Chapter 1

Introduction
Craniofacial malformations are serious congenital diseases in humans

Craniofacial malformations are very common in humans. Around 75% of patients with congenital birth defects have craniofacial deformities (Chai and Maxson, 2006).

Oral clefts are the most common craniofacial defects in humans (Jiang et al., 2006), which consist of two etiologically different classes: cleft lip with or without cleft palate (CLP), and isolated cleft palate (CP) (Jiang et al., 2006). Oral clefts appear with a spectrum of severities (Appendix 1.1A-D), and its estimated prevalence is 1 in 300 to 2500 births for CLP, and about 1 in 1500 births for CP, which varies across different geographical locations, races, and socio-economic conditions (Murray 2002; Cobourne, 2004; Stanier and Moore, 2004). Oral clefts usually impair patients’ ability of taking food, speaking and hearing, and affect their psychological development (Stanier and Moore, 2004). Current treatments are costly, and involve multiple rounds of plastic surgeries together with other therapies (Stanier and Moore, 2004), which many patient families can not afford.

Oral clefts can further be categorized into two distinct forms, non-syndromic or syndromic. In non-syndromic oral clefts, an oral cleft is the only defect found in affected patients, while the patients with syndromic oral clefts have other recognizable structural anomalies (Murray, 2002; Cobourne, 2004). While oral clefts have been associated with more than 300 Mendelian syndromes (Jiang et al., 2006), the majority of oral clefts (around 70% in CLP, and 50% in CP) are non-syndromic (Murray, 2002). The etiology of oral clefts is very complex. Both genetic and environmental factors are involved (Murray, 2002). Maternal smoking, maternal alcohol consumption and poor nutritional status are among the possible risk factors for human oral clefts (Murray, 2002). Moreover, gene-environment interactions are implicated in oral clefts (Murray, 2002).
Congenital teeth defects, including abnormal teeth number, defective teeth shape or size and defective teeth structures, are also common craniofacial birth defects (Thesleff, 2006; Fleischmannova et al., 2008). Abnormality in the teeth number, in particular, affects about 20% of the human population (Appendix 1.1E, F), and appears as two major classes: teeth agenesis and supernumerary teeth (Thesleff, 2006). Teeth agenesis, a tooth defect involving missing one or more teeth, could further be subdivided into several distinct forms according to the number of missing teeth: hypodontia, in which less than 6 teeth are missing in the patients; oligodontia, in which 6 or more teeth are lost in patients; and anodontia, in which the patients have no teeth at all (http://en.wikipedia.org/wiki/). In contrast to the teeth agenesis, the supernumerary teeth is a tooth defect with extra teeth (Fleischmannova et al., 2008).

Similar to oral clefts, congenital teeth defects appear either as one of syndromic features or as an isolated defect (Appendix 1.1E, F) (Thesleff, 2006; Fleischmannova et al., 2008). Abnormality in teeth number has been found in numerous syndromic disorders, such as ectrodactyly, ectodermal dysplasia, orofacial cleft (EEC) syndrome and hypohidrotic ectodermal dysplasia (HED) syndrome (Thesleff, 2006).

Craniofacial malformations, including oral clefts and teeth defects, arise from the disruption of various steps of the craniofacial developmental process; therefore understanding the morphogenetic and molecular mechanisms of craniofacial development will contribute significantly to developing better ways of diagnosis, prevention and treatment of craniofacial defects.
**Craniofacial development**

Craniofacial development is a very complex process. Cells derived from all three embryonic germ layers, i.e., ectoderm, endoderm and mesoderm, are involved in the formation of craniofacial structures (Chai and Maxson, 2006). Ectoderm-derived neural crest cells, in particular, contribute significantly to the mesenchymal structures of the head and neck such as palatal mesenchyme and tooth mesenchyme, and play extremely important roles in regulating craniofacial development (Chai and Maxson, 2006). Neural crest cells are a transient population of multipotent cells formed at the border between dorsal neural and epidermal ectoderm (Meulemans and Bronner-Fraser, 2004). After their induction, neural crest cells undergo epithelial-mesenchymal transformation and delaminate from the dorsal neural tube, and then migrate along several specific routes to multiple destinations, where they give rise to a variety of cell types (Meulemans and Bronner-Fraser, 2004).

During facial development, neural crest cells derived from the dorsal aspect of the developing brain, termed as cranial neural crest cells (CNC), migrate ventrolaterally to populate the facial primordia. More specifically, CNC derived from the forebrain and rostral midbrain contribute to the frontonasal process, while CNC from the caudal midbrain and the first two rhombomeres of the hindbrain populate the first pair of branchial arches (Osumi-Yamashita et al. 1994; Kontges and Lumsden 1996; Trainor, 2005), which later form the mandibular processes and the maxillary processes. Once migrated into their destinations, cranial neural crest cells proliferate and differentiate into a variety of craniofacial elements (Helms and Schneider 2003; Santagati and Rijli 2003).

The initial facial primordia are composed of 5 prominences: one frontonasal process, a pair of mandibular processes, and a pair of maxillary processes, which are located around the primitive oral cavity by 26 days of gestation in humans (E9.5 in
mice) (Appendix 1.2A; Appendix 1.3A). At around 32 days of gestation in humans (E10.0 in mice) (Appendix 1.2B; Appendix 1.3B), nasal placodes appear bilaterally on the ventrolateral part of the frontonasal prominence, which subsequently grows rapidly around the nasal placodes and develops into lateral nasal process and medial nasal process (reviewed in Jiang et al., 2006). Rapid outgrowth of the facial primordia, largely driven by the proliferation of CNC-derived mesenchymal cells, brings distal medial nasal process and lateral nasal process into direct contact, and fusion between them is initiated by about 35 days of gestation in humans (Appendix 1.2C; Appendix 1.3C), which may involve filopodia extension and cell adhesion (Cox, 2004; Jiang et al., 2006). While medial nasal process and lateral nasal process are still undergoing active fusion, fusion between maxillary processes and medial nasal processes is initiated (Appendix 1.2D; Appendix 1.3D). Fusion first forms a bilayered epithelial seam, which must be removed to form an intact lip. The mechanism of seam disintegration is under hot debate. While some argue for epithelial mesenchymal transformation, others support programmed cell death of epithelial cells (Jiang et al., 2006). Later on, further growth of the maxillary processes pushes two medial nasal processes medially. The groove between them gradually disappears with the growth and contribution of the mesenchymal cells of the medial nasal processes and the maxillary processes. While the maxillary processes fuse with the medial nasal processes to form the lateral lips, the paired medial nasal processes merge to form the intermaxillary segment, which give rise to a central lip and a primary palate. The primary palate later will fuse with the secondary palate. By the 48 days of gestation in humans (E12.5 in mice), the development of the upper lip is complete (reviewed in Jiang et al., 2006).

Like upper lip formation, developmental processes of secondary palate are similar in humans and mice (reviewed in Murray and Schutte, 2004; Chai and
Maxson, 2006; Gritli-Linde, 2007). It is initiated bilaterally as an internal outgrowth of the maxillary processes at around the 6th week of gestation in humans (E11.5-12 in mice; Appendix 1.4A). After initiation, the palatal shelves undergo vertical growth along the lateral sides of the developing tongue (E12.5-E13.5 in mice; Appendix 1.4B-D), and then elevate rapidly into the horizontal position when the tongue descends in the oral cavity (E14 in mice; Appendix 1.4E). Elevated palatal shelves grow continually, and eventually make direct contact in the midline (E14.5 in mice; Appendix 1.4F) to form a midline epithelial seam (MES), a transient epithelial structure. The MES must be removed to form an intact secondary palate. The exact mechanism for removing MES is still controversial. The proposed mechanisms include cell apoptosis, cell migration and epithelial-mesenchymal transformation. After removal of MES, two palatal shelves are fused to form mesenchymal continuity (E16.5 in mice; Appendix 1.4H).

Development of the upper lip and the secondary palate is largely driven by rapid proliferation of CNC-derived mesenchymal cells, and is regulated by the interaction between two tissues: epithelium and underlying CNC-derived mesenchyme. Similarly, tooth development in mammals involves a series of sequential and reciprocal signaling interactions between the adjacent epithelium and mesenchyme (Thesleff et al., 1995). Tooth development in mice is first seen morphologically as a thickening of the oral epithelium at around embryonic day 11 (E11) (Appendix 1.5). The presumptive dental epithelium proliferates and buds into the underlying neural crest-derived mesenchyme at specific sites and induces the mesenchyme to condense around the epithelial buds from E12 to E13. At E13.5, cells at the tip of the molar tooth buds stop dividing and form the primary enamel knot, which functions as a signaling center to stimulate the proliferation of the surrounding dental epithelium. Subsequently, the dental epithelium folds and invaginates farther
into the mesenchyme, surrounding the condensing mesenchyme to form “cap” (at E14) and then “bell”-shaped tooth germs (at about E16). As development proceeds, the epithelial cells in contact with the dental mesenchyme differentiate into the enamel-producing ameloblasts and their adjacent mesenchymal cells differentiate into the dentin-producing odontoblasts (Thesleff and Hurmerinta, 1981). The ameloblasts and odontoblasts deposit enamel and dentin matrices, respectively. The shape of the resultant tooth becomes effectively fossilized by subsequent mineralization of these matrices.

During tooth development, odontogenic potential, the capability to induce tooth formation, experiences a shift from tooth epithelium to tooth mesenchyme. Tooth inductive signals arise initially in the presumptive dental epithelium. As development proceeds, the dental epithelium rapidly loses tooth inductive potential and, at the same time, the underlying dental mesenchyme acquires the capability to instruct tooth morphogenesis from non-dental epithelia (Mina and Kollar, 1987; Lumsden, 1988).

Molecular genetic basis of craniofacial development and malformations in humans

Tremendous efforts have been made to understand molecular mechanisms of the craniofacial development and the molecular genetic basis of craniofacial malformations in humans mainly through linkage analysis and associated study.

In particular, some progress has been made in identifying causative genes for syndromic CLP and CP (Cobourne, 2004; Cox, 2004). Syndromic CLP accounts for around 30% of all CLP cases. Some syndromes with CLP as one of the characteristics are caused by mutations in single genes, which include $IRF6$ (interferon regulatory factor) for van der Woude syndrome (VWS) (Kondo et al.,
PVRL1 (Poliovirus receptor related) for CLP-ectodermal dysplasia (CLPED-1) syndrome (Suzuki et al., 2000); P63 (a homologue of p53) for ectrodactyly, ectodermal dysplasia, orofacial cleft (EEC) syndrome (Ianakiev et al., 2000); MID1 (encoding a microtubule-associated protein) for Opitz syndrome (OS) (Quaderi et al., 1997); WNT3 for Tetra-amelia syndrome (Niemann et al., 2004); FGFR1 (fibroblast growth factor receptor) for Kallmann syndrome (Dode et al., 2003; Kim et al., 2005); and PTCH1 (a receptor for SHH) for Gorlin syndrome (Evans, et al., 1993). Around half of all CP cases are syndromic. Mutations causing some of syndromic CP are found in several genes, including TCOF1 for Treacher Collins syndrome (TCS) (Edwards et al., 1997), SHH (Sonic hedgehog) for Holoprosencephaly (HPE) syndrome (Roessler et al., 1996), and TBX22 (a member of T-box family transcriptional factor) for X-linked CP (CPX) syndrome (Braybrook et al., 2001).

The etiology of non-syndromic oral clefts is much more complicated. Linkage analysis has found some potential chromosomal loci, which may harbor causative or susceptibility genes of oral clefts (Cobourne, 2004). On the other hand, candidate gene-based association studies have implicated several genes including TGFα and TGFβ3 in non-syndromic CLP (Feng et al., 1994; Field et al., 1994; Lidral et al., 1998). Moreover, PVRL1, a gene responsible for the above-mentioned syndromic CLP, has recently been shown to associate with non-syndromic CLP, suggesting that syndromic genes could contribute to non-syndromic CLP (Sozen et al., 2001). However, the progress to identify genes responsible for non-syndromic oral clefts is far from satisfactory. Linkage analysis and association study often produce inconsistent results, reflecting the complex and multifactorial nature of non-syndromic oral clefts (Cobourne, 2004).

Some advances were also achieved in searching for candidate genes responsible for both isolated and syndromic teeth abnormalities in humans.
Mutations in *Pax9* and *Msx1*, two homeobox genes, are found to be the cause of non-syndromic forms of teeth agenesis in some patients (Stockton et al., 2000; Lidral and Reising, 2002). Since *Pax9* and *Msx1* are tooth mesenchymal factors, these data indicate the importance of tooth mesenchymal cells in tooth development. Teeth agenesis is found in more than 49 syndromes (Li et al., 2008). Genes mutated in some of these syndromes have been discovered, including *p63* in ECC syndrome (Ianakiev et al., 2000); *PITX2* in Rieger syndrome (Semina et al., 1996); *IRF6* in van der Woude syndrome (Kondo et al., 2002); and *Eda* (a signal molecule of tumor necrosis factor pathway) in hypohidrotic ectodermal dysplasia (HED) syndrome (Kere et al., 1996); In addition, mutations in *RUNX2* are linked to cleidocranial dysplasia, a syndrome with extra teeth as one of its features (Mundlos et al., 1997).

One interesting finding in humans is that mutations in some genes such as *IRF6* and *Msx1* are associated with CLP, CP and dental defects (Van den Boogaard et al., 2000). Therefore, understating morphogenetic and molecular mechanisms of one of them will contribute to providing insights to other defects.

**Mouse as a model for investigating molecular mechanisms of craniofacial development and malformations**

Mice and humans share strikingly similar genome and processes of craniofacial development. Therefore mice are excellent models to study etiology and pathogenesis of craniofacial defects, including oral clefts and dental abnormalities. Moreover, genes identified in mice provide good candidates for association studies in humans.

Spontaneous mutant mice with craniofacial defects are valuable for identifying genes important for craniofacial development. While mice with CP are
quite common, mice with CLP are relatively rare. Several spontaneous mutant mice are reported to exhibit CLP in homozygous mutants, such as Dancer mice (Bush et al., 2004). Bush et al. have positionally-cloned the causative gene for the Dancer mutation, and showed that CLP in the Dancer mutant mice resulted from ubiquitous misexpression of Tbx10, a T-box transcriptional factor (Bush et al., 2004).

In the last few years, gene-targeting method along with gene expression analysis has been widely employed to study candidate genes for craniofacial defects such as oral clefts and teeth defects in mice. A growing number of genes are found to play critical roles in craniofacial development (Jiang et al., 2006; Chai and Maxson, 2006; Gritli-Linde, 2007). Most of them encode components of signal transduction pathways, including signal molecules, receptors, and transcriptional factors (Jiang et al., 2006; Chai and Maxson, 2006; Gritli-Linde, 2007). These findings highlight the importance of tissue-tissue interactions during craniofacial development.

One of the most exciting discoveries in developmental biology in recent decades is that only a handful of signaling pathways are employed over and over again during embryogenesis (Gregorian et al., 2008). This is also true for craniofacial development (Jiang et al., 2006; Chai and Maxson, 2006; Gritli-Linde, 2007). Several major signaling pathways, including Shh, Tgfβ/Bmp and Fgf, play important roles in different stages of craniofacial development, and are utilized in development of different craniofacial organs, such as secondary palate, upper lip, and teeth (Jiang et al., 2006; Chai and Maxson, 2006; Gritli-Linde, 2007).

As described above, CNC cells contribute significantly to craniofacial development. Therefore signaling regulating early aspects of neural crest development, including its induction, emigration and migration, plays important roles in craniofacial development and malformations (Chai and Maxson, 2006; Gritli-Linde, 2007). Moreover, signaling pathways play important roles in craniofacial
development by regulating epithelial-mesenchymal interactions after CNC cells have migrated into these regions. Here, I summarize recent understanding of roles of these signaling pathways in the latter aspect of craniofacial development, mainly focusing on development of upper lip, secondary palate and teeth.

1) Role of Sonic hedgehog (Shh) signaling in upper lip, secondary palate and early tooth development

Hedgehog (Hh) belongs to a family of secreted signal proteins, which includes 3 members in mammals, Desert hedgehog (Dhh), Indian hedgehog (Ihh) and Sonic hedgehog (Shh) (Ingham and McMahon, 2001). Hedgehog signaling participates in diverse developmental events by regulating a variety of cellular processes such as cell proliferation, cell fate decision and cell survival (Ingham and McMahon, 2001). Transduction of Hh signaling involves two transmembrane proteins: Smoothened (Smo) and the receptor Patched (Ptc1 and Ptc2 in mice). In the absence of Hh signals, Ptc interacts with and suppresses the activity of Smo. When Hh binds to Ptc, the suppression of Smo is relieved, which activates the expression of target genes through Gli family zinc finger transcription factors (Gli1, Gli2, and Gli3 in mouse) (Ingham and McMahon, 2001; Lan and Jiang, 2009)

While Ihh and Dhh are critical for skeletal differentiation and spermatogenesis, respectively (Clark et al., 2000; Rodda and McMahon, 2006), Shh signaling plays important roles in craniofacial development (Jiang et al., 2006). Mice lacking Shh had multiple developmental abnormalities including severe cranial defects (Chiang et al., 1996). During midfacial development, the expression of Shh is restricted to the epithelium of facial primordia, while Ptc1 and Smo are expressed in both the epithelium and the underlying mesenchyme (Jeong et al., 2004). Mice with conditionally deleted Smo in neural crest cells displayed defective facial patterning,
increased cell apoptosis at E9.5 and E10.5, and reduced cell proliferation at E11.5 (Jeong et al., 2004). These results are consistent with studies in chick. In chick, Shh signaling is required for both cell proliferation and cell survival of facial primordia (Ahlgren and Bronner-Fraser, 1999; Hu and Helms, 1999). Moreover, inhibiting Shh signaling in chick by implanting beads coated with anti-Shh antibody into mesenchymal cells caused cleft lip (Hu and Helms, 1999). All these results support important roles of Shh signaling in upper lip formation. However, severe craniofacial phenotypes of Shh mutant mice prevent direct investigation of the role of Shh signaling in upper lip formation.

In developing secondary palate, Shh is localized to the palatal epithelium (Rice et al., 2004, Lan and Jiang, 2009), while Ptc1 and Gli1, two target genes of Shh signaling, are expressed in both palatal epithelium and mesenchyme (Rice et al., 2004, Lan and Jiang, 2009). Deletion of Shh in the oral epithelium using K14-Cre resulted in incompletely penetrant cleft palate (Rice et al., 2004; Gritli-Linde, 2007; Lan and Jiang, 2009), while oral epithelium-specific abrogation of Smo, an essential autonomous component of Shh signaling, did not generate cleft palate (Rice et al., 2004), indicating that the epithelial Shh signals to the mesenchymal cells, and Shh signaling functions mainly in the palatal mesenchyme. Indeed, mice lacking Smo specifically in CNC-derived palatal mesenchymal cells exhibited cleft palate due in part to decreased cell proliferation (Lan and Jiang, 2009). It is now clear that Shh signaling in the palatal mesenchyme, not in the epithelium, plays an essential role in regulating palatal outgrowth (Lan and Jiang, 2009). While Shh signaling is essential for palatogenesis, its activity needs to be tightly controlled in the secondary palate. Transgenic overexpression of Shh driven by K14 promoter in oral epithelium impaired palatogenesis and led to cleft palate (Cobourne et al., 2009).

Shh signaling is also involved in early tooth development. Shh is expressed
in the tooth epithelial thickenings during tooth initiation (Hardcastle et al., 1998). Implanting beads containing Shh proteins into the oral epithelium adjacent to a molar tooth germ led to ectopic epithelial invaginations (Hardcastle et al., 1998). These data suggest a role of Shh in the initiation of tooth development (Hardcastle et al., 1998). However, whether Shh signaling plays an essential role in tooth initiation in vivo is still not clear. When the tooth epithelial thickenings are developing into tooth buds, Shh is expressed in the epithelial cells at the tip of tooth germs (Hardcastle et al., 1998). Blocking Shh signaling using anti-Shh monoclonal antibody at this stage resulted in tooth developmental arrest at epithelial thickenings (Cobourne et al., 2001). Moreover, tooth epithelium-specific deletion of Shh using K14-Cre resulted in smaller teeth (Dassule et al., 2000). These data clearly indicate that Shh acts as a mitogen to control tooth growth. When the tooth germs transition from bud to cap stage, the expression of Shh is increased dramatically in the presumptive primary enamel knots, implicating a role of Shh in tooth morphogenesis. Indeed mice lacking Shh in the tooth epithelium showed abnormal teeth shape (Dassule et al., 2000). The same study also demonstrated that Shh is not essential for tooth epithelial differentiation (Dassule et al., 2000).

2) Role of Tgfβ/Bmp signaling in upper lip and secondary palate development

The transforming growth factor-β (Tgfβ) superfamily of secreted signaling molecules, including TGFβs, bone morphogenetic proteins (BMPs) and activins, plays essential roles throughout animal development (Shi and Massague, 2003; Nohe et al., 2004). These molecules initiate cellular signaling by binding to and bringing together two types of receptor serine and threonine kinases. Upon ligand binding, the type-II receptor phosphorylates and activates the type-I receptor kinase, which in turn phosphorylates and activates a set of transcriptional coactivators called Smad proteins.
and leads to their nuclear translocation and transcriptional activation of downstream target genes (reviewed in Shi and Massague, 2003; Nohe et al., 2004).

Tgfβ signaling, particularly Bmp signaling, plays essential roles in upper lip development. In mice, the expression of Bmp4 is restricted to the distal epithelial cells of facial primordia just before and during lip fusion, whereas Msx1 and Msx2 are expressed in the underlying mesenchymal cells (Gong et al., 2003). Conditional knockout of either Bmp4 or Bmpr1a using Nestin-cre caused isolated cleft lip and bilateral CLP, respectively (Liu et al., 2005). In Bmp1a deficient mice, cleft lip phenotype was correlated with prematurely elevated apoptosis of nasal epithelium and subjacent mesenchyme and decreased cell proliferation in maxillary mesenchyme (Liu et al., 2005). In Chick, however, inhibiting Bmp signaling promoted cell survival (Ashique et al., 2002). Msx1 and Msx2 are downstream genes of Bmp signaling in mice. Although single knockout of either Msx1 or Msx2 did not cause cleft lip (Satokata and Maas, 1994; Satokata et al., 2000), double knockout mutants did display CLP (Ishii et al., 2005). Moreover, Bmp signaling is tightly regulated (Balemans et al., 2002). In Chick, either inhibiting or enhancing Bmp signaling led to defective lip formation (Ashique et al., 2002). Bambi (Bmp and activin membrane bound inhibitor), a putative negative regulator of Bmp signaling, is found to exhibit tight co-expression with Bmp4 in upper lip regions during murine upper lip development (Onichtchouk et al., 1999; Grotewold et al., 2000), suggesting a role of Bambi in upper lip formation by regulating Bmp signaling. To address this question, I have generated Bambi deficient mice using gene-targeting strategy (refer to Appendix 2). However, mice lacking Bambi did not show cleft lip phenotype. Since numerous inhibitors could regulate Bmp signaling at multiple levels (Balemans et al., 2002), functional redundancy of these inhibitors may obscure their importance in upper lip development.
Bmp signaling is also essential for secondary palate development. Inactivation of *Bmpr1a* in both epithelium and subjacent mesenchyme of maxillary processes caused cleft palate due in part to decreased cell proliferation of maxillary mesenchyme and defective anterior-posterior patterning of secondary palate (Liu et al., 2005). In contrast, ablation of *Bmpr1a* in oral epithelium using *K14-Cre* did not result in cleft palate (Kobielak et al., 2003; Andl et al., 2004), indicating that the function of Bmp signaling lies mainly in palatal mesenchyme. In line with this, palatal mesenchyme-specific deletion of *Bmpr1a* using *Osr2-IresCre* led to clefting of anterior palate (personal communication). Moreover, mice lacking *Msx1*, which is expressed in palatal mesenchyme and is a target gene of Bmp signaling, exhibited cleft palate due to defective cell proliferation (Satokata and Maas, 1994; Zhang et al., 2002). Further experiments have established the regulatory hierarchy: Early epithelial *Bmp4* induces expression of mesenchymal *Msx1*, which in turn regulates the expression of *Bmp4* and *Bmp2* in the palatal mesenchyme and *Shh* in the medial edge epithelium. Consistent with this hypothesis, transgenic expression of *Bmp4* in *Msx1* domains rescued cleft palate phenotype of *Msx1* mutants, which was correlated with restoration of cell proliferation (Zhang et al., 2002). However, secondary palate development appeared normal in mice with conditional disruption of *Bmp4* in either palatal epithelial or palatal mesenchymal cells (Liu et al., 2005; personal communication). Possible explanation for this result is insufficient cre activity or functional redundancy of *Bmp4* with other *Bmps* such as *Bmp2*. Further studies are necessary to answer this question. Along with Bmp signaling, Tgfβ signaling is also important for palatogenesis. It participates in regulating palatal growth and fusion (Taya et al., 1999; Ito et al., 2003; Dudas et al., 2006). Function of Tgfβ signaling in palatal fusion is mediated primarily through Tgfβ3 (Taya et al., 1999; Dudas et al., 2004).
3) Role of Fgf signaling in secondary palate development

Fibroblast growth factor (Fgf) is a large and complex family of signal molecules, which includes 22 members in vertebrates (Itoh and Ornitz, 2004; Pauws and Stanier, 2007). Fgf signaling plays important roles in developmental processes (Itoh and Ornitz, 2004; Pauws and Stanier, 2007). Fgf signaling is initiated when Fgfs bind to appropriate Fgf receptors that contain tyrosine kinase activity, which dimerize and transactivate in the cytoplasm, leading to activation of cascade of Ras/extracellular signal-regulated kinase (ERK) pathway to regulate expression of target genes in the nucleus (Itoh and Ornitz, 2004; Pauws and Stanier, 2007).

While the role of Fgf signaling in upper lip formation is still not clear, the important roles of Fgf signaling in mediating palatal epithelial-mesenchymal interactions during early secondary palate development were well illustrated by analysis of mice deficient for either Fgf10 or Fgfr2b. Fgfr2b is expressed in palatal epithelium while Fgf10 is expressed in subjacent palatal mesenchyme (Rice et al., 2004; Alappat et al., 2005). Disruption of Fgf signaling by targeted deletion of either Fgf10 or its epithelial receptor Fgfr2b resulted in cleft palate (Rice et al., 2004; Alappat et al., 2005). Further analysis of these mice revealed an important epithelial-mesenchymal signaling cascade: mesenchymal Fgf10 acts on epithelial cells via its receptor Fgfr2b to regulate their survival and proliferation and induce the expression of Shh in palatal epithelium, which signals back to mesenchymal cells to promote their proliferation (Rice et al., 2004; Alappat et al., 2005).

4) Role of Bmp and Fgf signaling in early tooth development

Members of Bmp and Fgf families have been shown to be critical tooth inductive signals (reviewed Jernvall and Thesleff, 2000; Pispa and Thesleff, 2003). Bmp2, Bmp4, Fgf8 and Fgf9 are expressed in the presumptive dental epithelium at
the beginning of tooth development (Wozney et al., 1988; Lyons et al., 1989; Vainio et al., 1993; Turecková et al., 1995; Neubuser et al., 1997; Dassule and McMahon, 1998). Bmp and Fgf signaling are necessary for activation of expression of the Msx1 and Pax9 transcription factors, respectively, in the presumptive tooth mesenchyme (Vainio et al., 1993; Neubuser et al., 1997; Tucker et al., 1998; Mandler and Neubuser, 2001). Mice lacking either Msx1 or Pax9 function exhibited tooth developmental arrest at the early bud stage (Satokata and Maas, 1994; Peters et al., 1998). Tissue recombination experiments demonstrated that both Msx1 and Pax9 are required for activation of odontogenic potential in the tooth mesenchyme (Peters et al., 1998; Bei et al., 2000). Interestingly, expression of Bmp4, which shifts from the presumptive dental epithelium to the developing tooth mesenchyme at about E12 during normal mouse tooth initiation, was significantly reduced in the developing tooth mesenchyme in either Msx1-/- or Pax9-/- mutant mice (Chen et al., 1996; Peters et al., 1998). Addition of recombinant Bmp4 protein rescued development of Msx1-/- mutant tooth germs to the late bell stage in explant cultures (Chen et al., 1996; Bei et al., 2000). In addition, Fgf8 induced Fgf3 expression in the dental mesenchyme in an Msx1-dependent manner (Bei and Maas, 1998). Although teeth developed normally in Fgf3-/- mutant mice (Mansour et al., 1994), mice homozygous for null mutations in both Fgf3 and Fgf10, which are both expressed in the developing tooth mesenchyme, had tooth developmental arrest at the bud stage (Wang et al., 2007). Moreover, mice with either tissue-specific inactivation in the oral epithelium of Bmpr1a, which encodes a type-1 receptor for Bmp signaling, or a deletion of Fgfr2b, which encodes the epithelial isoform of the type-2 Fgf receptor, exhibited tooth developmental arrest at the early bud stage (De Moerlooze et al., 2000; Andl et al., 2004; Liu et al., 2005). Thus, both Bmp and Fgf signaling are critical for the reciprocal interactions between the epithelium and mesenchyme during tooth initiation.
5) Canonical Wnt signaling in upper lip, secondary palate and early tooth development

Wnt signaling is another evolutionally conserved signaling pathway. The Wnt family of secreted proteins consists of 19 members in mammals and plays important roles in embryogenesis by regulating various cellular processes, such as cell proliferation, migration and cell fate determination (Logan and Nusse, 2004; Grigoryan et al., 2008). Wnt signaling could be mediated through either β-catenin-dependent canonical pathway or β-Catenin-independent non-canonical Wnt Signaling (Veeman et al., 2003; Logan and Nusse, 2004). The canonical Wnt signaling pathway is most studied, and its activation involves stabilization and nuclear accumulation of β-catenin (Logan and Nusse, 2004). In the absence of Wnt ligands, cytoplasmic β-catenin proteins are phosphorylated by a destructive complex containing a serine/threonine kinase glycogen synthase kinase-3β (GSK3β), and scaffolding proteins Axin and Adenomatous Polyposis Coli (APC). After phosphorylation, β-catenin proteins are targeted for degradation by the ubiquitination-proteosome pathway. Binding of Wnt to a Frizzled (Fz) receptor and a LRP5/6 co-receptors activates canonical Wnt signaling, and inhibits β-catenin phosphorylation, leading to stabilization of β-catenin proteins and their accumulation in the cellular nuclei where they interact with Tcf/Lef family DNA-binding proteins to activate transcription of downstream target genes (Logan and Nusse, 2004; Grigoryan et al., 2008).

In recent years, roles of the canonical Wnt signaling in craniofacial development start to be gradually discovered. Using TOPGAL transgenic mice, in which a LacZ reporter gene is driven by Tcf/Lef binding sites and a minimal promoter (DasGupta and Fuchs, 1999), Lan et al. showed that the canonical Wnt signaling is specifically activated in upper lip epithelium and the adjacent mesenchyme just prior
to upper lip fusion (Lan et al., 2006). Interestingly, Wnt9b and Wnt3 were found to be expressed in lip epithelium during upper lip formation (Lan et al., 2006). Moreover, knockout of Wnt9b in mice resulted in incompletely penetrant CLP (Carroll et al., 2005; unpublished data), suggesting that the canonical Wnt signaling in upper lip is at least partially triggered by Wnt9b. In humans, cleft lip phenotype was found in some of patients with WNT3 mutations (Niemann et al., 2004). Taken together, these results suggested that Wnt9b and Wnt3 may activate the canonical Wnt signaling during upper lip formation.

Several Wnt ligands appear to be expressed in the secondary palate (Mouse genome informatics, Jackson Laboratory). Wnt5a is expressed in palatal mesenchymal cells, and exhibits a gradient along anterior-posterior axis of palatal shelves (He et al., 2008). Knockout of Wnt5a led to a complete cleft secondary palate due to abnormal cell proliferation and defects of directional cell migration (He et al., 2008). However, Wnt5a is believed to activate Ror2-dependent non-canonical Wnt pathway (He et al., 2008). Whether the canonical Wnt signaling plays a role in secondary palate development is not clear. Moreover, two recent studies suggest that the canonical Wnt signaling need to be tightly regulated during secondary palate development. In mice, disruption of GSK3β, which phosphorylates β-catenin proteins targeted for degradation, led to cleft palate (Liu et al., 2007). In humans, mutations of Axin2 gene, an important inhibitor of the canonical Wnt signaling, were found to be associated with incomplete cleft palate (Letra et al, 2009). However, no study has focused on investigating requirement of inactivation/inhibition of the canonical Wnt signaling in the secondary palate development. The first part of my thesis is aimed to address these questions.

Wnt/β-catenin signaling also plays essential roles in early tooth development. Epithelium-specific inactivation of β-catenin or epithelial expression of
Dkk1, an inhibitor of the canonical Wnt signaling, caused tooth developmental arrest at the early bud stage (Andl et al., 2002; Liu et al., 2008). In addition, Mice lacking Lef1, an important nuclear mediator of canonical Wnt signaling, exhibited the similar phenotype (Kratochwil et al., 2002). Lef1 has been shown to directly regulate transcription of Fgf4 (Kratochwil et al., 2002), and be a critical survival factor of dental epithelial cells (Sasaki et al., 2005). On the other hand, tooth epithelium-specific stabilization of β-catenin, thus mimicking the constitutive activation of canonical Wnt signaling in tooth epithelium, led to extra budding from the normal tooth and ectopic epithelial invaginations (Jarvinen et al., 2006; Liu et al., 2008). These ectopic epithelial invaginations can develop further into teeth in the kidney capsules (Jarvinen et al., 2006). Taken together, these data established the essential role of intraepithelial Wnt signaling/β-catenin in the early tooth development. In addition, since Wnts are diffusible signal molecules, Wnts expressed in tooth epithelium could signal to tooth mesenchymal cells. In line with this, nuclear-localized β-catenin was observed in some of tooth mesenchymal cells in a time when tooth mesenchyme is acquiring odontogenic potential from tooth epithelium (Liu et al., 2008). However, the study directly addressing the role of Wnt/β-catenin in tooth mesenchymal cells is still lacking. The second part of my thesis is aimed to address this question.

**Goal of this thesis**

Craniofacial malformations, particularly oral clefts and dental defects, cause the devastating effect on affected patients, and occur in high frequency. In recent years, some progress has been made in identifying causative genes for oral clefts and dental defects in both humans and mice. However, morphogenetic and molecular mechanisms of oral clefts and dental defects are still poorly understood.
The goal for my thesis is to further understand the molecular genetic basis of oral clefts and dental defects, more specifically the roles of the canonical Wnt signaling in secondary palate and early tooth development using mice as models. In the second chapter, I investigated the role of the canonical Wnt signaling in secondary palate development. In the third chapter, I investigated the role of tooth mesenchymal-specific canonical Wnt signaling in early tooth development.

Since the roles of mesenchyme-specific canonical Wnt signaling in secondary palate and early tooth development were unknown, my study adds significant new insights into the molecular mechanisms of oral clefts and dental defects.
Chapter 2
Investigating the role of Wnt/β-catenin signaling in palatogenesis
Summary

Cleft palate (CP) is a serious congenital disease, and arises from the disruption of normal palatogenesis. However, the molecular mechanism of secondary palate development and the etiology of CP are poorly understood. Several major signaling pathways, including Shh, Tgfβ/Bmp and Fgf pathways, have been shown to be extremely important in secondary palate development. However, very little is known about the role of canonical Wnt signaling, if any, in palatogenesis. Here I report the findings of my investigation of the role of canonical Wnt signaling in secondary palate development. I first analyzed the expression of BAT-gal, a LacZ reporter responsive to the canonical Wnt signaling, and found that BAT-gal expression in the developing palate is highly restricted to the anterior region. I found that while several Wnt ligands are expressed, several Wnt antagonists are also expressed in the developing palate. To test whether inactivation/inhibition of the canonical Wnt signaling is required for normal palatogenesis, I utilized the Cre/Lox system to conditionally stabilize β-catenin, an obligatory transducer of the canonical Wnt signaling, in palatal mesenchymal cells. Gross morphological examination revealed that persistent activation of the canonical Wnt signaling in palatal mesenchymal cells led to cleft palate. Detailed histological analysis of embryos from various developmental stages indicated that elevation of palatal shelves did not occur in the β-catenin gain-of-function mutants. Analysis of cell proliferation by BrdU labeling indicated that cell proliferation was reduced in both palatal epithelium and mesenchyme in mutants compared to control littermates at E13.5. However, examination of cell death by TUNEL assay did not detect abnormal cell death in mutants. Further analysis of the expression of the genes important for secondary palate development by in-situ hybridization revealed that the expression of Lef1, Bmp4, Bmp2 and Osr2 was increased, while the expression of Shox2 was totally
abolished in palatal mesenchyme of mutant embryos at E13.5. In conclusion, our data indicate that inactivation or inhibition of the canonical Wnt signaling in palatal mesenchymal cells is required for normal palatogenesis. In addition, I also analyzed mice deficient for β-catenin in palatal mesenchymal cells, and revealed a Wnt-independent function of β-catenin in secondary palate development.
Introduction

Secondary palate development is a very complex process, involving initiation, rapid growth, elevation and fusion of the bilateral palatal shelves at the midline (Chai and Maxson, 2006; Gritli-Linde, 2007; Murray and Schutte, 2004). Disturbance in any of these steps could cause cleft palate, a common birth defect with a prevalence of 1 in 1500 births (Murray 2002; Stanier and Moore, 2004).

Development of secondary palate involves two tissues: palatal epithelium and palatal mesenchyme. Previous lineage tracing experiments have showed that the majority of palatal mesenchymal cells are derived from cranial neural crest (CNC) cells (Chai et al., 2000; Ito et al., 2003), which are migratory multipotent cells induced at the border between the dorsal neural and epidermal ectoderm during early embryogenesis (Chai and Maxson, 2006). After their induction, CNC cells delaminate and migrate along defined routes to the frontonasal process and branchial arches, thus contributing significantly to the mesenchymal structures of head and neck (Chai and Maxson, 2006). Therefore, it is not surprising that many mutant mice deficient in genes important for various steps of CNC development, including induction, cell expansion, survival, migration and cell differentiation, exhibited cleft palate phenotype (reviewed in Chai and Maxson, 2006; Gritli-Linde, 2007).

Palatogenesis is regulated by reciprocal and sequential interactions between palatal mesenchyme and epithelium. Several major signaling pathways, including Shh, Tgfl3/Bmp and Fgf signaling, have been shown to be important for mediating palatal epithelium-mesenchyme interactions (reviewed in Chai and Maxson, 2006; Gritli-Linde, 2007). The canonical Wnt signaling is known to control early aspects of neural crest development, including its induction, cell expansion, survival, and cell fate determination (reviewed in Grigoryan et al., 2008). However, whether the canonical Wnt signaling also plays any role in regulating proliferation and
differentiation of palatal mesenchymal cells or in mediating palatal mesenchymal-epithelial interactions is not clear. Specific deletion of β-catenin, an essential effector of the canonical Wnt signaling, in early migratory neural crest cells led to almost total loss of CNC-derived craniofacial structures prior to secondary palate development (Brault et al., 2001), which precluded direct investigation of role of the canonical Wnt signaling in palatogenesis.

While the canonical Wnt signaling is required for early neural crest development, several lines of evidence suggest that the canonical Wnt signaling needs to be turned down/off at certain stages during secondary palate development. In mice, disruption of GSK3β, which phosphorylates β-catenin targeted for degradation, led to cleft palate (Liu et al., 2007). Recently, mutations of *Axin2* gene, an important inhibitor of the canonical Wnt signaling, were found to be associated with incomplete cleft palate in humans (Letra et al, 2009).

Here, I test the requirement for the canonical Wnt signaling to be kept inactive in CNC-derived palatal mesenchymal cells during secondary palate development using the Cre/Lox system. Our data revealed the importance of stage-specific inactivation or inhibition of the canonical Wnt signaling in these cells for normal palatogenesis. Moreover, our data also revealed a Wnt-independent role of β-catenin in secondary palate development.
Results

Activity of canonical Wnt signaling is highly restricted during palate development

As a first step to investigate the role of canonical Wnt signaling in secondary palate development, I examined expression of a LacZ transgene driven by seven repeats of Lef/Tcf sequences as a reporter of the canonical Wnt/β-catenin activation in the developing palate (Maretto et al., 2003). X-gal staining of E13.5 BAT-gal transgenic embryos indicated that β-galactosidase activity was restricted to the anterior tips of the palatal shelves (Figure 2.1A, D), while the palatal mesenchymal cells in other parts of palatal shelves did not show any β-galactosidase activity (Figure 2.1A, B, C), indicating that the canonical Wnt signaling is not activated or is suppressed in the majority of palatal mesenchymal cells.

Since β-catenin is an essential nuclear mediator of the canonical Wnt signaling, I analyzed its expression in the secondary palate by immunofluorescence. I found that β-catenin protein was ubiquitously expressed in both palatal epithelial and mesenchymal cells throughout the entire secondary palate during palatal development (see Figure 2.10A, C). While β-catenin exhibited the strongest expression at cell membrane, it appeared that β-catenin didn’t accumulate in the nucleus, further indicating that β-catenin-mediated canonical Wnt signaling is not activated in those cells.

By searching Mouse Genome Informatics gene expression database (http://www.informatics.jax.org/), I found that several Wnt ligands and inhibitors are expressed in the secondary palate. To confirm their expression in the secondary palate, I performed RT-PCR assays of total RNA isolated from E13.5 palatal shelves, and detected strong expression of Wnt signaling inhibitors including Frzb, sFRP4, and Wif1 along with several Wnt ligands including Wnt10a and Wnt6 (Figure 2.1E).
Collectively, these data indicate that although several Wnt ligands are expressed in the secondary palate, the canonical Wnt signaling is not activated in the palatal mesenchymal cells except those at the anterior tips of palatal shelves. Our data further suggest that the Wnt inhibitors including *Frzb* sFRP4 and *Wif1* may antagonize canonical Wnt signaling activity in the developing palate.

**Stabilization of β-catenin in CNC-derived palatal mesenchymal cells resulted in cleft palate**

To test whether inhibition of the canonical Wnt signaling in palatal mesenchyme is critical for the development of the secondary palate, I used the Cre/Lox system to specifically stabilize β-catenin in the developing palatal mesenchymal cells in mice. The *Osr2-CreKI* mice express Cre highly specifically in the palatal mesenchymal cells, but not in the palatal epithelial cells (personal communications). The *Catnb<sup>lox(ex3)/+</sup>* mice carry the targeted β-catenin allele in which Exon3, encoding the GSK3β phosphorylation sites, is flanked by two direct repeats of *LoxP* sequences (Harada et al., 1999). To confirm the palatal mesenchyme-specific stabilization of β-catenin, I analyzed β-catenin protein expression in both mutant and control littermates by immunofluorescence. I found that the expression of β-catenin protein was strongly elevated in palatal mesenchymal cells, but not in palatal epithelial cells of *Catnb<sup>lox(ex3)/+</sup>;Osr2-CreKI* embryos at E12.5 and E13.5 in comparison with the control littermates (Figure 2.2 A-D).

The *Catnb<sup>lox(ex3)/+</sup>;Osr2-CreKI* mice died at birth and displayed open eyelids and cleft secondary palate (Figure 2.3A-D). I then performed detailed histological analysis of both control and *Catnb<sup>lox(ex3)/+</sup>;Osr2-CreKI* littermates at various developmental stages (Figure 2.4A-H). At E12.5, the palatal shelves in the mutants were morphologically similar to those of control littermates (compare Figure
2.4B to A). At E13.5, the palatal shelves of mutant embryos appeared shorter and wider, as compared to those of control embryos (Figure 2.4C, D). Grooves on the ventrolateral sides of the palatal shelves (arrowheads in Figure 2.4C, D) were not as deep as those of control embryos. At E14.5, the palatal shelves had elevated above the tongue and initiated fusion at the midline (black arrow in Figure 2.4E). In contrast, the palatal shelves in Catnb<sub>lox(ex3)/+</sub>;Osr2-CreKI mutants did not elevate (Figure 2.4F). By E16.5, the palatal shelves in control embryos had completed fusion (Figure 2.4G). In Catnb<sub>lox(ex3)/+</sub>;Osr2-CreKI mutants, however, the palatal shelves were still vertically oriented (Figure 2.4H). In addition, I also observed that the palatal mesenchyme in Catnb<sub>lox(ex3)/+</sub>;Osr2-CreKI mutants grew more and more condensed from E13.5 onward.

Catnb<sub>lox(ex3)/+</sub>;Osr2-CreKI embryos exhibited reduced cell proliferation in the palatal mesenchyme at E13.5

Failure of palatal elevation is often associated with impaired palatal growth (Lan et al., 2004; Liu et al., 2008). To further investigate the cellular mechanism of cleft palate in the Catnb<sub>lox(ex3)/+</sub>;Osr2-CreKI mutants, I analyzed cell proliferation and cell death in Catnb<sub>lox(ex3)/+</sub>;Osr2-CreKI and their littermate controls at E12.5 and E13.5, two critical stages for palatal outgrowth. At E12.5, no significant change in cell proliferation was detected in either palatal epithelium or palatal mesenchyme in Catnb<sub>lox(ex3)/+</sub>;Osr2-CreKI mutant embryos, compared to the control littermates (Figure 2.5A, B). At E13.5, BrdU analysis indicated that Catnb<sub>lox(ex3)/+</sub>;Osr2-CreKI embryos exhibited significant reduction of cell proliferation in both palatal mesenchymal cells and epithelial cells (Figure 2.5C, D), compared to their littermate controls (P<0.05). I further analyzed cell proliferation in palatal shelves of both control and mutant embryos using immunostaining of the Ki67 protein, a marker for
proliferating cells. Ki67 is expressed in G1, S, G2 and M phases, but not in G0 phase of cell cycles (Scholzen and Gerdes, 2000). As shown in Figure 2.6, Ki67 staining was comparable between control and mutant embryos at E12.5 (Figure 2.6A, B). However, Ki67 staining in both palatal epithelial and mesenchymal cells of mutant embryos was greatly reduced at E13.5, in comparison with their control littermates (Figure 2.6C, D). Since activation of the canonical Wnt signaling occurred only in the palatal mesenchyme but not in the palatal epithelium of \textit{Catnb}^{lox(ex3)/+};\textit{Osr2-CreKI} embryos, the reduction of cell proliferation in palatal epithelium in mutants suggests that activation of the canonical Wnt signaling represses the expression of signal molecules required for regulating proliferation of palatal epithelial cell. However, examination of cell death activity in the palatal shelves did not detect significant difference between control and \textit{Catnb}^{lox(ex3)/+};\textit{Osr2-CreKI} mutant embryos at E13.5 (Figure 2.7).

**Stabilization of β-catenin in the palatal mesenchyme activated Bmp signaling in both palatal epithelial and mesenchymal cells**

Bmp signaling has been shown to function downstream of Wnt/β-catenin signaling in some developmental processes (Huelsken, 2001; Kim et al., 2002). Previous studies have established the important function of Bmp signaling in secondary palate development (Zhang et al., 2002; Liu et al., 2005). Deletion of type I Bmp receptor (\textit{Bmpr1a}) in the palatal primordia caused altered cell proliferation, resulting in cleft secondary palate (Liu et al., 2005). I therefore examined the expression of \textit{Bmp2} and \textit{Bmp4} by in-situ hybridization, and activity of Bmp signaling by immunostaining of phosphorylated Smads 1, 5,8 (pSmad 1,5,8) in the palatal shelves of both control and \textit{Catnb}^{lox(ex3)/+};\textit{Osr2-CreKI} mutant embryos at E13.5. In-situ hybridization revealed that expression of \textit{Bmp4} and \textit{Bmp2} was ectopically
activated in palatal mesenchymal cells in \( \text{Catnb}^{\text{lox(ex3)/+}};\text{Osr2-CreKI} \) mutant embryos at E13.5 (Figure 2.8 A-D). Analysis of pSmad 1,5,8 indicated that Bmp signaling was activated in both the palatal epithelial and mesenchymal cells of mutant embryos at E13.5 (Figure 2.8E-F). Bmp signaling is tightly regulated at multiple levels (Balemans et al., 2002). Either too much or too little Bmp signaling could cause developmental defects (Smith 1999; Balemans et al., 2002; Ashique et al., 2002; Liu et al., 2005). The ectopic activation of Bmp signaling in the palatal epithelial and mesenchymal cells may contribute to cleft palate phenotype in \( \text{Catnb}^{\text{lox(ex3)/+}};\text{Osr2-CreKI} \) embryos.

**Forced stabilization of β-catenin in the palatal mesenchymal cells activated expression of Lef1 and Osr2, and repressed expression of Shox2**

Along with Bmp2 and Bmp4, the transcription factors Lef1, Osr2 and Shox2 were also found to be misexpressed in \( \text{Catnb}^{\text{lox(ex3)/+}};\text{Osr2-CreKI} \) mutant embryos at E13.5. Lef1 has been shown to be a transcriptional target of Wnt3a/β-catenin signaling (Filali et al., 2002). A short Wnt-responsive element (WRE) is located in Lef1 promoter (Filali et al., 2002). Therefore, Lef1 is a useful marker for activity of the canonical Wnt signaling. Indeed, I found that Lef1 was ectopically expressed in the palatal mesenchyme of \( \text{Catnb}^{\text{lox(ex3)/+}};\text{Osr2-CreKI} \) embryos at E13.5 (Figure 2.9A-B), indicating that stabilization of β-catenin has activated the canonical Wnt signaling in palatal mesenchymal cells. Previous studies have demonstrated that Lef1 is involved in the palatal fusion by promoting epithelial mesenchymal transformation (EMT) (Nawshad and Hay 2003). However, Lef1 is not required for the palatal growth, since it is not expressed in the palatal shelves at this stage (Figure 2.9A), and Lef1 knockout mice have normal secondary palate development (Van Genderen et al., 1994).
Odd-skipped related 2 (Osr2) is homologous to the odd-skipped zinc finger transcription factor in *Drosophila* (Lan et al., 2004). Consistent with the previous report (Lan et al., 2004), I found that Osr2 is expressed robustly in the palatal mesenchyme with higher expression levels in lateral regions than medial regions in E13.5 control embryos (Fig 2.9 C). In Catnb<sup>lax(ex3)/+</sup>;Osr2-CreKI embryos, one copy of Osr2 gene has been deleted, and replaced by the Cre cDNA. Therefore, the expression level of Osr2 gene in mutants was expected to be less than that of littermate controls. However, I found that Osr2 mRNA level in Catnb<sup>lax(ex3)/+</sup>;Osr2-CreKI embryos was higher than that in control embryos (Compare Figure 2.9D to C), and the lateral-medial expression gradient was disrupted (Figure 2.9D). Previous study has demonstrated that Osr2 is an important regulator of palatal development (Lan et al., 2004). Misexpression of Osr2 in Catnb<sup>lax(ex3)/+</sup>;Osr2-CreKI mutants, therefore, may partially account for the CP phenotype. Recently, research in human fibroblasts indicated that Wnt3a could induce Osr2 and Osr1 expression (Klapholz-Brown et al., 2007), suggesting that Osr2 is a downstream target of the canonical Wnt signaling in certain microenvironments.

*Shox2*, a short stature homeobox gene, is specifically expressed in the anterior portion of the secondary palate (Yu et al., 2005). Knockout of Shox2 led to clefting of anterior palate without disrupting the formation of posterior secondary palate (Yu et al., 2005). In Shox2 mutants, the palatal shelves exhibited reduced cell proliferation and increased cell apoptosis (Yu et al., 2005). I found that the expression of Shox2 was repressed in most palatal mesenchymal cells in Catnb<sup>lax(ex3)/+</sup>;Osr2-CreKI embryos at E13.5 (Figure 2.9E-F). One interesting observation is that the expression domain of residual Shox2 is complementary to that of ectopic Bmp2 in Catnb<sup>lax(ex3)/+</sup>;Osr2-CreKI mutant embryos (Figure 2.9), suggesting ectopic Bmp2 may be responsible for repressing Shox2 expression in
palatal mesenchymal cells or vice versa. Taken together, our data indicate that constitutive activation of the canonical Wnt signaling disturbs normal molecular program of secondary palate development, which may account for the cleft palate phenotype.

**Palatal mesenchyme-specific deletion of β-catenin also led to cleft secondary palate**

While I did not detect the canonical Wnt signaling activity using the BAT-gal reporter mice, the β-catenin protein was indeed expressed in the palatal mesenchymal cells. To investigate whether palatal mesenchymal expression of β-catenin plays a role in the palatogenesis, I crossed *Osr2-IresCre* mice to mice carrying floxed alleles of β-catenin (*Catnb*), which contain two *LoxP* sites flanking Exon2-Exon6 and are inactivated upon cre-mediated deletion of *LoxP*-flanked region (Brault et al., 2001).

I confirmed the palatal mesenchyme-specific ablation of β-catenin by immunostaining. As shown in Figure 2.10, β-catenin protein was significantly reduced in the palatal mesenchymal cells of *Catnb*;*Osr2-IresCre* mutant embryos at E12.5 and E13.5 (asterisks), while its expression in palatal epithelial cells (marked by arrows in Figure 2.10) was similar in control and mutant embryos. In contrast, some vessel-like structures in the palatal mesenchyme of mutant embryos still exhibited strong β-catenin protein staining at E12.5 and E13.5 (indicated by green arrowheads in Figure 2.10 B, D), which is consistent with the fact that cre activity of *Osr2-IresCre* mice is not detected in these structures. Previous lineage tracing indicated that some of palatal mesenchymal cells are not derived from CNC (Ito et al., 2003). These *Osr2*-negative cells may come from the origins other than CNC. More importantly, β-catenin staining in other mesenchymal cells (indicated by white
arrowheads in Figure 2.10A-D) and the palatal epithelial cells (marked by white arrows) that do not express cre recombinase was similar in both control and mutant embryos at E12.5 and E13.5.

\textit{Catnbf}^{0f};\textit{Osr2-IresCre} pups died after birth, and exhibited open-eyelids, cleft cheeks, craniofacial skeletal defects, and cleft palate phenotypes (Figure 2.11, 2.12). I then collected embryos from various developmental stages to perform histological analysis. H&E staining revealed that the palatal defects appeared as early as E12.5 (Figure 2.13A, B), and became more severe at E13.5 (Figure 2.13C, D). At E13.5, the palatal shelves of \textit{Catnbf}^{0f};\textit{Osr2-IresCre} mutant embryos appeared wider and shorter, compared to those of control embryos. Bending of the palatal shelves on the vetrolateral sides (indicated by black arrows) was impaired in mutant embryos. At E14.5, the palatal shelves in control embryos had elevated above the tongue and formed an epithelial seam at the midline (Figure 2.13E, F). In \textit{Catnbf}^{0f};\textit{Osr2-IresCre} mutants, the anterior and middle portions of the palatal shelves had elevated to the horizontal position but were too short to make a direct contact at the midline, whereas the posterior portion of the palatal shelves did not elevate (data not shown). By E16.5, the palatal shelves in controls had completed fusion (Figure 2.13G). In the mutants, the posterior portion of the palatal shelves was still vertically oriented (data not shown), while the anterior and middle portions of the bilateral palatal shelves had elevated, but failed to reach the midline (Figure 2.13H).

\textit{Catnbf}^{0f};\textit{Osr2-IresCre} mutant embryos exhibited palatal growth defects

\textit{Catnbf}^{0f};\textit{Osr2-IresCre} embryos exhibited the tissue loss of the maxillary-mandible junction (white arrow in Figure 2.11B) and other craniofacial skeletal defects (Figure 2.12), which could contribute to the abnormal shape of the palate shelves. In this case, cleft palate phenotype may be a secondary effect of other
craniofacial defects. However, I found that the palatal shelves in $Catnb^{ff}; Osr2$-IresCre embryos appear smaller than those of control littermates at E13.5 (Figure 2.13C, D). To investigate whether β-catenin plays a primary role in the palatal growth, I performed TUNNEL and BrdU assays to analyze cell death and cell proliferation in the palatal shelves. As shown in Fig. 2.14, the mutant mice and their control littermates exhibited comparable level of cell death in the palatal shelves at E12.5 and E13.5, excluding cell apoptosis as a cause of the smaller palatal shelves in the mutants.

I then compared the percentage of BrdU positive cells in the palate mesenchyme in control and mutant embryos at E12.5 and E13.5, and found that the percentage of BrdU positive cells was significantly reduced in $Catnb^{ff}; Osr2$-IresCre mutant embryos (P<0.05) at both E12.5 (20.5% reduction) and E13.5 (15% reduction) (Fig. 2.15A-E). These data suggest that β-catenin plays a primary role in the palatal growth.

### Analysis of expression of marker genes in $Catnb^{ff}; Osr2$-IresCre mutant embryos at E13.5

To investigate the molecular basis of cleft palate in $Catnb^{ff}; Osr2$-IresCre embryos, I further performed in-situ hybridization analysis to examine expression of several genes known to be important for secondary palate development. I first examined the expression of markers exhibiting anterior or posterior-specific expression. Shox2 was specifically expressed in the anterior portion of secondary palate (Yu et al., 2005). Ablation of Shox2 led to incomplete cleft palate (Yu et al., 2005). In Shox2 mutants, the palatal shelves exhibited reduced cell proliferation and increased cell apoptosis (Yu et al., 2005). Similar to its expression in control embryos (Figure 2.16A, C), Shox2 was strongly expressed in the mesenchymal cells of anterior
palate (Figure 2.16B), but absent in the posterior palatal shelves (Figure 2.16D) of E13.5 $\text{Catnbf}^{ff}$; $\text{Osr2-IresCre}$ embryos. $\text{Meox2}$ is commonly used as a marker for the posterior palatal shelves, and is absent in the anterior portion of secondary palate (Ding and Jin et al, 2006). Disruption of $\text{Meox2}$ in mice resulted in incompletely penetrant posterior cleft palate (Ding and Jin et al, 2006). I found that $\text{Meox2}$ was expressed at similar level in posterior palatal shelves in both control and mutant embryos at E13.5 (Figure 2.16E, F). Collectively, these data indicate that the anterior-posterior patterning of the palatal shelves is not affected in $\text{Catnbf}^{ff}$; $\text{Osr2-IresCre}$ mutant embryos.

Along with $\text{Shox2}$ and $\text{Meox2}$, other transcriptional factors such as $\text{Satb2}$, $\text{Osr2}$ and $\text{Pax9}$ play the important roles in palatogenesis (Gritli-Linde, 2007). $\text{Satb2}$, a gene encoding a nuclear matrix protein, is detected in palatal mesenchymal cells. In humans, mutations in $\text{SATB2}$ have been found to be associated with cleft palate (FitzPatrick et al., 2003; Leoyklang et al., 2007). In line with its role in humans, knockout of $\text{Satb2}$ led to craniofacial and skeletal defects, including cleft palate (Dobreva et al., 2006). I therefore examined its expression in $\text{Catnbf}^{ff}$; $\text{Osr2-IresCre}$ and control embryos at E13.5. However, I found that its expression was similar in $\text{Catnbf}^{ff}$; $\text{Osr2-IresCre}$ and control embryos at this stage (Figure 2.17A, B). Odd-skipped related 2 ($\text{Osr2}$) is expressed in palatal mesenchymal cells during secondary palate development (Lan et al., 2004). Mice deficient for $\text{Osr2}$ showed cleft palate (Lan et al., 2004). I therefore compared its expression between $\text{Catnbf}^{ff}$; $\text{Osr2-IresCre}$ and control embryos at E13.5. However, I did not detect any significant difference between them (Figure 2.17C, D). $\text{Pax9}$, a paired box gene, is expressed strongly in mesenchymal cells of the developing palate shelves. Mice lacking $\text{Pax9}$ exhibited cleft palate and other developmental defects (Peters et al., 1998). Both $\text{Pax9}$ and $\text{Catnbf}^{ff}$; $\text{Osr2-IresCre}$ mutant embryos showed impairment of ventrolateral
indentation of palatal shelves (Peters et al., 1998). However, the expression of Pax9 in the palatal shelves of E13.5 Catnb<sup>ff</sup>; Osr2-IresCre mutant embryos is comparable with that in control littermates (Figure 2.17E, F).

Fgf signaling is important for mediating palatal epithelial-mesenchymal interactions: palatal mesenchymal Fgf10 acts on palatal epithelial cells via its receptor Fgfr2b to regulate their survival and proliferation and induce the expression of Shh in the palatal epithelium, which signals back to palatal mesenchymal cells to promote their proliferation (Rice et al., 2004; Alappat et al., 2005). I therefore compared the expression of Fgf10, Fgfr2 and Shh in the palatal shelves between Catnb<sup>ff</sup>; Osr2-IresCre and control embryos at E13.5. I found that the expression of Fgf10, Fgfr2 and Shh appeared normal in mutant embryos compared with their expression in control littermate (Figure 2.18).

In summary, using Cre/Lox system, I specifically stabilized β-catenin in palatal mesenchymal cells, thus mimicking the activation of the canonical Wnt signaling. Stabilization of β-catenin in palatal mesenchymal cells led to complete cleft palate, at least partially resulting from failure of palatal shelves to elevate and reduced cell proliferation in the palatal mesenchymal cells. Further marker gene analysis revealed that activation of the canonical Wnt signaling in the palatal mesenchyme disturbed the normal molecular program of palatogenesis. Collectively, these data indicate a role of inactivation or inhibition of the canonical Wnt signaling in secondary palate development during the palatal growth. Moreover, by specifically deleting β-catenin in palatal mesenchymal cells, I demonstrated that β-catenin might also play a primary role in normal palatogenesis by regulating cell proliferation via a Wnt-independent mechanism.
Discussion

Secondary palate development involves initiation, rapid growth, elevation, and fusion of the bilateral palatal shelves, and is regulated by sequential and reciprocal interactions between palatal epithelium and mesenchyme (Gritli-Linde, 2007). Previous studies have revealed critical roles of several major signaling pathways, including Shh, Tgfβ/Bmp and Fgf pathways, in secondary palate development (Gritli-Linde, 2007). Recently, Wnt5a has been shown to regulate the proliferation of palatal mesenchymal cells and the anterior-posterior patterning of the palatal shelves through Ror2-mediated non-canonical Wnt signaling (He et al., 2008). However, while the canonical Wnt/β-catenin signaling pathway has been shown to play critical roles in numerous developmental processes (Grigoryan et al., 2008), whether it is required for secondary palate development is not known. Mice deficient for β-catenin, an obligatory mediator of the canonical Wnt signaling, exhibited early embryonic lethality (Haegel et al., 1995). Specific deletion of β-catenin in early migratory neural crest cells also led to severe craniofacial defects prior to palatal development (Brault et al., 2001). In this thesis, I report the first direct investigation of β-catenin-mediated canonical Wnt signaling in secondary palate development.

Inhibition of canonical Wnt signaling and normal palatogenesis

My study indicates that the canonical Wnt signaling activity is restricted to mesenchymal cells at the anterior tips of the developing palatal shelves. While the exact role of the canonical Wnt signaling in these cells is unknown, I suspect that it may be involved in fusion of secondary palate with primary palate. Previous studies showed that the canonical Wnt signaling was activated in the upper lip primordia just prior to their fusion (Lan et al., 2006). The canonical Wnt signaling may play a similar role in the fusion of primary palate with secondary palate. However,
Osr2-IresCre mice exhibited cre activity in the palatal mesenchymal cells throughout the entire palatal shelves (Lan et al., 2007), and Catnb\textsuperscript{+/--} Osr2-IresCre mutant mice exhibited complete cleft palate phenotype, which could mask the fusion defect. Further studies will be necessary to define the exact role of the canonical Wnt signaling activity in the anterior secondary palate.

While the canonical Wnt signaling may play a role in fusion between primary palate and secondary palate, its activity is not detected in most regions of developing palatal shelves. Moreover, several canonical Wnt signaling antagonists are expressed in the palatal shelves. These data suggest that the canonical Wnt signaling is actively inhibited in the palatal shelves. To test whether the inhibition of the canonical Wnt signaling is critical for normal palatogenesis, I investigated palatogenesis in mice with tissue-specific stabilization of β-catenin in the developing palatal mesenchyme.

**Roles of the canonical Wnt signaling in proliferation and differentiation of palatal mesenchymal cells**

Forced stabilization of β-catenin significantly inhibited proliferation of palatal mesenchymal cells. Some of these palatal mesenchymal cells had exited the cell cycle, and appeared to undergo differentiation, suggesting that activation of Wnt/β-catenin signaling may change the fate of palatal mesenchymal cells. It will be interesting to determine what kind of cell type the palatal mesenchyme cells in $Catnb^{lox(\alpha3)/+};Osr2-CreKI$ mutants have differentiated into. One possibility is that these cells had become cartilage or bone cells, since they condensed in a way similar to mesenchymal cells differentiating into cartilage or bone. It will also be interesting to determine whether the canonical Wnt signaling plays a role in later stages of secondary palate development, such as regulating formation of palatine bones. Further studies are needed to answer these questions.
Wnt-independent β-catenin function in palatogenesis

β-catenin has dual functions (Perez-Moreno and Fuchs, 2006). It is both an essential component of the canonical Wnt signaling pathway and a component of cell-cell adheren junctions. Although I did not detect activity of canonical Wnt signaling in palatal mesenchymal cells using the BAT-gal reporter, or nuclear localization of β-catenin in palatal mesenchymal cells by using immunostaining, the Catnb−/−;Osr2-IresCre mice exhibit complete cleft of the secondary palate. While the cleft palate could result from secondary effects of other craniofacial defects, such as the cleft cheek, the fact that cell proliferation is significantly reduced in the palatal mesenchyme in these mutant mice suggests that β-catenin plays a primary role in normal development of the palatal mesenchyme. It is possible that low levels of canonical Wnt signaling activity, un-detected by BAT-gal reporter assay and immunostaining, exists and plays essential roles in the developing palatal mesenchymal cells. Alternatively, since β-catenin is involved in cell-cell adhesion, β-catenin may play a role in maintaining the integrity of cell-cell interactions of palatal mesenchymal cells.
Methods

Mouse lines, breeding, and genotyping

Generation and detailed analysis of Cre activity during secondary palate development in Osr2-IresCre mice has been previously reported (Lan et al., 2007). Characterization of Osr2-CreKI mice will be described elsewhere. Catnb^{ff} (Brault et al., 2001) mice and R26R mice (Soriano, 1999) were purchased from the Jackson Laboratory (Bar Harbor, ME). Catnb^{lox(ex3)/+} (Harada et al., 1999) mice were gifts from Dr. Di Chen (University of Rochester, NY). Catnb^{ff}; Osr2-IresCre mice, in which β-catenin is conditionally deleted in the palatal mesenchymal cells, were obtained by crossing Catnb^{ff+}; Osr2-IresCre mice to Catnb^{ff} mice. For palatal mesenchymal-specific activation of β-catenin, Catnb^{lox(ex3)/lox(ex3)} mice were crossed to Osr2-CreKI mice. Genotyping information for above mouse strains has been previously reported (Soriano, 1999; Brault et al., 2001).

Histology and Immunostaining

For histological analysis, embryos from various developmental stages were collected, fixed either in Bouin’s fixative or in 4% PFA, and dehydrated through ethanol series, and embedded in paraffin for section. Slides were stained with hematoxylin and eosin (H&E) method.

For immunostaining, frontal sections from 4% PFA fixed embryos were treated by 3% hydrogen peroxide, boiled in pressure cooker containing trilogy solution for antigen retrieval, and then incubated with primary antibodies against phospho-Smad1/5/8 (polyclonal rabbit serum, Cell Signaling Technology; 1:200), and β-catenin (mouse monoclonal clone 15B8, Sigma; 1:500). M.O.M kit (Vector Laboratories), combined with streptavidin-conjugated Texas-Red (Vector
Laboratories, 1:100) was used for detection of β-catenin. Histostain Plus Rabbit Primary (DAB) kit (Zymed Laboratories) was used for detection of phospho-Smad 1/5/8.

**X-Gal Staining**

To detect β-galactosidase activity, embryos were fixed in 0.25% glutaraldehyde, passed through sucrose series, embedded in OCT freezing medium, and sectioned at 14 um thickness using a cryostat microtome. X-gal staining of sections was performed as described previously (Hogan et al., 1994). Slides were counterstained with eosin.

**BrdU labeling and TUNEL assays**

BrdU assay was performed for detection of cell proliferation using bromodeoxyuridine (BrdU) labeling and Detection Kit (Roche Diagnostics Corporation, Indianapolis) following manufacturer’s instructions. Percentage of BrdU positive cells was calculated from data collected from bilateral palatal shelves of five consecutive sections. Students’ $t$-test was performed to evaluate the significance of difference. $P$-value less than 0.05 is considered statistically significant. TUNEL assay was performed for detection of cell death using DeadEnd™ Fluorometric TUNEL System (Promega, Madison) following manufacturer’s instructions.

**Section in situ hybridization**

4% PFA-fixed embryos were dehydrated through ethanol series and embedded in paraffin for section. Frontal sections were hybridized with in-vitro transcribed digoxigenin–labeled antisense RNA probes as described elsewhere (Zhang et al. 1999).
Figure 2.1 Activity of the canonical Wnt signaling is highly restricted during secondary palate development. (A) Whole-mount view of X-gal stained BAT-gal embryos at E13.5. Cells with BAT-gal activity appeared blue. At E13.5, BAT-gal activity was restricted to anterior tips of secondary palate (marked by white arrowheads). (B-D) X-gal stained frozen sections from posterior (B) middle (C), and anterior (D) portions of the palatal shelves of E13.5 BAT-gal embryos. The BAT-gal activity was only detected in palatal mesenchymal cells of anterior tips of palatal shelves at E13.5 (D). White arrows point to first molar tooth buds. (E) RT-PCR analysis of total RNA isolated from E13.5 palatal shelves. Expression of sFRP4, Frzb, Wif1, Wnt6 and Wnt10a was detected. P, palatal shelf of the secondary palate; T, tongue.
Figure 2.2 *Osr2-CreKI*-mediated stabilization of β-catenin in CNC-derived palatal mesenchymal cells. Frontal sections from both E12.5 (A, B) and E13.5 (C, D) control (A, C) and *Catnb*<sup>lox(ex3)/+</sup>;*Osr2-CreKI* mutant (B, D) embryos were subjected to immunofluorescence with anti-β-catenin antibody (red), and counterstained with DAPI (blue). At both E12.5 and E13.5, the expression of β-catenin protein was strongly elevated in palatal mesenchymal cells of mutant embryos (B, D), compared to its expression in palatal mesenchymal cells of control embryos (A, C). In contrast, palatal epithelial β-catenin staining was similar in control and mutant embryos at E125 and E13.5. P, palatal shelves of secondary palate; T, tongue.
Figure 2.3 *Catnb* \(^{lox(ex3)/+}\);*Osr2-CreKI* mutants exhibited open eyelids and cleft secondary palate. (A, B) Lateral view of E17.5 control (A) and *Catnb* \(^{lox(ex3)/+}\);*Osr2-CreKI* mutant (B) embryos. Mutant embryos exhibited open eyelids (B, white arrowhead), while the control littermates showed closed eyelids at E17.5 (A, white arrowhead). (C, D) Oral view of E17.5 control (C) and *Catnb* \(^{lox(ex3)/+}\);*Osr2-CreKI* mutant (D) embryos. At E17.5, the palatal shelves of control embryos had completed fusion (C), while the palatal shelves in mutant embryos were still widely open. In contrast, the primary palate was formed normally in both control and mutant embryos. PP, primary palate; P, palatal shelf of secondary palate.
Figure 2.4 Histological analysis of control and \( \text{Catnb}^{\text{lox(ex3)I/}};\text{Osr2-CreKI} \) mutant embryos during secondary palate development. Frontal sections from middle palate region (the palate region corresponding to the first molar tooth) of control and mutant embryos were stained by H&E method. (A, B) At E12.5, the morphology of the palatal shelves was similar in control (A) and \( \text{Catnb}^{\text{lox(ex3)I/}};\text{Osr2-CreKI} \) mutant (B) embryos. (C, D) At E13.5, the palatal shelves in the \( \text{Catnb}^{\text{lox(ex3)I/}};\text{Osr2-CreKI} \) mutant embryos (D) appeared wider and shorter, compared to those in control embryos. Bending of palatal shelves at ventrolateral sides of first molar tooth (arrowheads in C and D) was impaired in mutant embryos. (E, F) At E14.5, the palatal shelves in control embryos had elevated to the horizontal position and made a direct contact at the midline (E, indicated by black arrow). By comparison, the palatal shelves of \( \text{Catnb}^{\text{lox(ex3)I/}};\text{Osr2-CreKI} \) mutant embryo failed to elevate (F). (G, H) At E16.5, the palatal shelves had completed fusion in the control embryos (G), while the palatal shelves in \( \text{Catnb}^{\text{lox(ex3)I/}};\text{Osr2-CreKI} \) mutant embryos were still in the vertical position. Note that the palatal mesenchyme in mutant embryos grew more and more condensed from E13.5 onward in comparison with that in control embryos (compare D, F, H with C, E, G). P, palatal shelf; T, tongue.
Figure 2.5 *Catnb*<sup>lox(ex3)/+</sup>*;Osr2-CreKI* mutant embryos exhibited reduced cell proliferation in palatal shelves at E13.5. (A-D) BrdU assay of frontal sections from middle regions of palatal shelves (corresponding to first molar tooth) of control (A, C) and *Catnb*<sup>lox(ex3)/+</sup>*;Osr2-CreKI* (B, D) at E12.5 (A, B) and E13.5 (C, D) (E-F) Quantitative analysis of the percentage of BrdU-positive cells in palatal mesenchyme (E) and epithelium (F) of control and *Catnb*<sup>lox(ex3)/+</sup>*;Osr2-CreKI* embryos at E12.5 and E13.5. Standard deviations were used for error bars. Asterisks in E and F marked significant differences between control and mutant embryos (P<0.05). P, palatal shelf.
Figure 2.5

A B C D

Control Catnb^{lox(ex3)\mu} Osr2-Cre Kl

E

Mesenchyme

Percentage of Bad positive cells

F

Epithelium

Percentage of Bad positive cells

E12.5 E13.5 E12.5 E13.5

Control GOF mutant Control GOF mutant

*
Figure 2.6 *Catnb\textsuperscript{lox(ex3)/+;Osr2-CreKI} mutant embryos had less Ki67 positive cells in both palatal epithelium and mesenchyme at E13.5.* Frontal sections from middle regions of palatal shelves (corresponding to first molar tooth) of control (A, C) and *Catnb\textsuperscript{lox(ex3)/+;Osr2-CreKI} (B,D) at E12.5 (A, B) and E13.5 (C,D) were stained with anti-Ki67 antibody. Ki67 positive cells appeared brown. (A, B) At E12.5, control (A) and mutant (B) embryos exhibited comparable Ki67 level in palatal shelves. (C, D) At E13.5, Ki67 positive cells were greatly reduced in both palatal epithelium (marked by black arrowheads) and mesenchyme (indicated by black arrows) in *Catnb\textsuperscript{lox(ex3)/+;Osr2-CreKI} mutant embryos (D), in comparison with their control littermates (C). P, palatal shelf; T, tongue.
Figure 2.7 Cell death was not altered in the palatal shelves of Catnb\textsuperscript{lox(ex3)/+}\textsubscript{Os2-CreKI} embryos. Frontal sections from posterior and anterior portions of the palate shelves of E13.5 control (A, C) and Catnb\textsuperscript{lox(ex3)/+}\textsubscript{Os2-CreKI} mutant (B, D) embryos were subjected to TUNEL assays. The dead cells appeared green. The nuclei were counterstained with DAPI (blue). At E13.5, cell death activity in the palatal shelves was comparable between control and Catnb\textsuperscript{lox(ex3)/+}\textsubscript{Os2-CreKI} mutant embryos. P, palatal shelf; T, tongue.
Figure 2.8 Bmp signaling was activated in both palatal epithelial and mesenchymal cells of Catnb<sup>loxp(ex3)/+;Osr2-CreKI</sup> mutant embryos at E13.5. (A, B) At E13.5, no Bmp4 was detected in mesenchymal cells of middle palatal regions in control embryos (A), however, its expression was activated in the palatal mesenchymal cells but not palatal epithelial cells of Catnb<sup>loxp(ex3)/+;Osr2-CreKI</sup> mutant embryos (B). (C, D) The expression of Bmp2 was ectopically activated in the palatal mesenchymal cells of Catnb<sup>loxp(ex3)/+;Osr2-CreKI</sup> mutant embryos at E13.5, in comparison with their control littermates. (E, F) At E13.5, the majority of cells in the palatal epithelium and mesenchyme of Catnb<sup>loxp(ex3)/+;Osr2-CreKI</sup> mutant embryos exhibited robust expression of pSmad1, 5,8 protein. By contrast, only a few cells from both palatal mesenchyme and epithelium of control embryos expressed pSmad1, 5,8. Arrowheads and arrows point to palatal epithelium and mesenchyme, respectively. T, tongue.
Figure 2.8

Control

Catnb$^{lox(\text{ex3})/+}$; Osr2-CreKI

A

B

T

T

Bmp4

Bmp4

C

D

T

T

Bmp2

Bmp2

E

F

T

T

pSmad1.5,8

pSmad1.5,8
Figure 2.9 Forced stabilization of β-catenin in CNC-derived palatal mesenchymal cells activated expression of Lef1 and Osr2, and repressed expression of Shox2. Frontal sections from E13.5 control (A, C, E) and Catnb<sup>lox(ex3)/+</sup>;Osr2-CreKI mutant (B, D, F) embryos were subjected to in-situ hybridization with digoxygenin-labeled probe for Lef1 (A, B), Osr2(C, D), and Shox2 (E, F). (A, B) At E13.5, Catnb<sup>lox(ex3)/+</sup>;Osr2-CreKI mutants showed strong Lef1 expression in palatal mesenchymal cells (B). In contrast, control littermates did not exhibit Lef1 expression in these cells (C, D) The expression of Osr2 was greatly increased in palatal mesenchymal cells of Catnb<sup>lox(ex3)/+</sup>;Osr2-CreKI mutant embryos (D) in comparison with its expression in control littermates (C). The lateral-medial gradient shown in the controls (C) was disrupted in Catnb<sup>lox(ex3)/+</sup>;Osr2-CreKI mutants (D). (E, F) Shox2 was strongly expressed in mesenchymal cells of anterior palate in controls (E), while its expression was abolished in most palatal mesenchymal cells of Catnb<sup>lox(ex3)/+</sup>;Osr2-CreKI mutant embryos (F).
Figure 2.9

Control

A

E13.5

Lef1

B

E13.5

Lef1

Catnb\textsuperscript{lox(ex3)/+; Osr2-CreKI}

C

E13.5

Osr2

D

E13.5

Osr2

E

E13.5

Shox2

F

E13.5

Shox2
Figure 2.10 β-catenin was deleted specifically in palatal mesenchymal cells in Catnb<sup>ff</sup>;Osr2-IresCre mutant embryos. Frontal sections from both E12.5 (A, B) and E13.5 (C, D) control (A, C) and Catnb<sup>ff</sup>;Osr2-IresCre mutant (B, D) embryos were subjected to immunofluorescence with anti-β-catenin antibody (red). At both E12.5 and E13.5, the expression of β-catenin protein was almost abolished in most palatal mesenchymal cells of mutant embryos (B, D; asterisks), except some vessel-like structures that still showed strong β-catenin staining (pointed to by green arrowheads), while palatal mesenchymal cells of control embryos exhibited ubiquitous expression of β-catenin protein (A, C; asterisks). In contrast, β-catenin staining in other mesenchymal cells (indicated by white arrowheads in A-D) and palatal epithelial cells (marked by white arrows in A-D) that did not express Cre recombinase was similar in control and mutant embryos at E12.5 and E13.5. T, tongue.
Figure 2.11 *Catnb*^{ff};*Osr2-IresCre* mutants exhibited open eyelids, wide-open mouth, curved limbs, and cleft secondary palate. (A, B) Lateral view of E16.5 control (A) and *Catnb*^{ff};*Osr2-IresCre* mutant (B) embryos. Mutant embryos exhibited open eyelids (B, white arrowhead) and cleft cheeks (B, white arrow) while the control littermates showed closed eyelids (A, white arrowhead) and intact cheeks (A, white arrow at E16.5. (C, D) Ventral view of E16.5 control (C) and *Catnb*^{ff};*Osr2-IresCre* mutant (D) embryos. *Catnb*^{ff};*Osr2-IresCre* mutant embryos showed curved forelimbs and hindlimbs. (E, F) Oral view of E16.5 control (E) and *Catnb*^{ff};*Osr2-IresCre* mutant (F) embryos. At E16.5, palatal shelves of control embryos had completed fusion (E), while the palatal shelves in mutant embryos were still widely open. Oral roof in *Catnb*^{ff};*Osr2-IresCre* mutant embryos appeared wider than that in control littermates (indicated by double arrows). In contrast, the primary palate was formed normally in both control and mutant embryos. PP, primary palate; P, palatal shelf of secondary palate
Figure 2.12 *Catnb1F;Osr2-IresCre* mutants exhibited craniofacial skeletal defects.

Skeletal preparations were compared between control (A, C, E) and (B, D, F) *Catnb1F;Osr2-IresCre* mutant littermates. (A, B) Lateral view of stained skeletal preparations of control (A) and *Catnb1F;Osr2-IresCre* mutant (B) embryos at E16.5. *Catnb1F;Osr2-IresCre* mutant embryos showed overall reduction of mineralization (red) in skull, in comparison with their control littermates. (C, D) Ventral view of skulls of control (C) and *Catnb1F;Osr2-IresCre* (D) mutant embryos at E16.5. Mutant embryos had cartilages (blue, arrowheads) at the place where bone normally formed in controls (red, arrowheads). (E, F) Top view of Meckel's cartilage and the mandible bone in control and *Catnb1F;Osr2-IresCre* mutant embryos at E16.5. *Catnb1F;Osr2-IresCre* embryos had much thicker Meckel's cartilages, while the mandibles appeared normal in mutants. f; frontal bone, p; parietal bone, ip; interparietal bone, sq; squamous bone, mn; mandible bone; m, Meckel's cartilage.
Figure 2.12

Control

Catnb\textsuperscript{ld}; Osr2-IresCre

A p f sq ip

B p f sq ip

C

D

E M → Mn

F M → Mn
Figure 2.13 Histological analysis of control and *Catnb*/*Osr2-IresCre* mutant embryos during secondary palate development. Frontal sections from middle palate region (the palate region corresponding to the first molar tooth) of control and *Catnb*/*Osr2-IresCre* mutant embryos were stained by H&E method. (A, B) At E12.5, the palatal shelves in *Catnb*/*Osr2-IresCre* embryos (B) appeared slightly smaller than those in control littermates (A). (C, D) At E13.5, the palatal shelves in *Catnb*/*Osr2-IresCre* mutant embryos (D) appeared wider and shorter, compared to those in control littermates. Bending of palatal shelves at ventrolateral side of first molar tooth (indicated by black arrows in C and D) was impaired in *Catnb*/*Osr2-IresCre* mutant embryos. (E, F) At E14.5, the palatal shelves in both control (E) and *Catnb*/*Osr2-IresCre* mutant (F) embryos had elevated to the horizontal position. However, the palatal shelves in *Catnb*/*Osr2-IresCre* mutant embryos were too short to make a direct contact at the midline (E, F). (G, H) At E16.5, the palatal shelves had completed fusion in the control embryos (G), while the palatal shelves in *Catnb*/*Osr2-IresCre* mutant embryos were still widely spaced (H). P, palatal shelf; T, tongue.
Figure 2.13

Control

Catnb^{fr} /; Osr2-IresCre
Figure 2.14 Cell death was not altered in palatal shelves of *Catnb<sup>0/0</sup>;Osr2-IresCre* embryos at E12.5 and E13.5. Frontal sections from middle palate region (the palate region corresponding to the first molar tooth) of control (A, C) and *Catnb<sup>0/0</sup>;Osr2-IresCre* mutant (B, D) embryos were subjected to TUNEL assays. The dead cells appeared green. The nuclei were counterstained with DAPI (blue). At both E12.5 (A, B) and E13.5 (C, D), the palatal shelves in *Catnb<sup>0/0</sup>;Osr2-IresCre* embryos showed similar level of cell death activity, compared with their control littermates. P, palatal shelf.
Figure 2.15 **Catnb\textsuperscript{ff};Osr2-IresCre** mutant embryos exhibited palatal growth defects at E12.5 and E13.5. (A-D) BrdU assay of frontal sections from middle regions of palatal shelves (corresponding to first molar tooth) of control (A, C) and **Catnb\textsuperscript{ff};Osr2-IresCre** (B, D) at E12.5 (A, B) and E13.5 (C, D) (E) Quantitative analysis of the percentage of BrdU-positive cells in palatal mesenchyme at E12.5 and E13.5. Standard deviations were used for error bars. Asterisks marked significant differences between control and **Catnb\textsuperscript{ff};Osr2-IresCre** mutant embryos (P<0.05). P, palatal shelf; T, tongue.
Figure 2.15

A  Control

B  Catnb<sup>fr</sup>; Osr2-IresCre

C

D

E

Percentage of BrdU positive cells

<table>
<thead>
<tr>
<th></th>
<th>E12.5</th>
<th>E13.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Mutant</td>
<td>15</td>
<td>10</td>
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* denotes statistical significance.
Figure 2.16 Expression of anterior and posterior markers of secondary palate was not altered in \textit{Catnb}^{ff}; \textit{Osr2-IresCre} mutant embryos at E13.5. Frontal sections from anterior (A, B) and posterior (C-F) portions of secondary palate of control (A, C, E) and \textit{Catnb}^{ff}; \textit{Osr2-IresCre} mutant (B, D, F) embryos were subjected to in-situ hybridization with probes for anterior palate marker \textit{Shox2} (A-D) and posterior palate marker \textit{Meox2} (E, F). (A, B) \textit{Shox2} was expressed at similar level in anterior palatal shelves in E13.5 control (A) and \textit{Catnb}^{ff};\textit{Osr2-IresCre} mutant (B) embryos. (C, D) \textit{Shox2} was absent in posterior palatal shelves of E13.5 control (C) and mutant (D) embryos. (E, F) The expression of \textit{Meox2} in posterior palatal shelves was comparable between E13.5 control (E) and \textit{Catnb}^{ff};\textit{Osr2-IresCre} mutant (F) embryos.
Figure 2.16

Control | Catnb""; Osr2-IresCre

A  
T  P
Shox2

B  
T
Shox2

C  
T  P
Shox2

D  
T  P
Shox2

E  
T  P
Meox2

F  
T  P
Meox2
Figure 2.17 Marker gene analysis of Catnb\textsuperscript{f/f}; Osr2-IresCre mutants (I). Frontal sections from E13.5 littermate controls (A, C, E) and Catnb\textsuperscript{f/f}; Osr2-IresCre mutants (B, D, F) were subjected to in-situ hybridization with digoxygenin-labeled probes for Satb2 (A, B), Osr2(C, D), and Pax9 (E, F). (A, B) At E13.5, the expression of Satb2 was not affected in Catnb\textsuperscript{f/f}; Osr2-IresCre mutants. (C, D) The expression of Osr2 in palatal shelves was similar in control (C) and Catnb\textsuperscript{f/f};Osr2-IresCre mutant (D) embryos. (E, F) The expression of Pax9 in palatal shelves was indistinguishable between control and Catnb\textsuperscript{f/f};Osr2-IresCre mutant embryos at E13.5.
Figure 2.17

Control  Catnb<sup>br</sup>; Osr2-IresCre

A  B

Satb2  Satb2

C  D

Osr2  Osr2

E  F

Pax9  Pax9
Figure 2.18 Marker gene analysis of Catnb$^{ff}$; Osr2-IresCre mutants (II). Frontal sections from E13.5 littermate controls (A, C, E) and Catnb$^{ff}$; Osr2-IresCre mutants (B, D, F) were subjected to in-situ hybridization with digoxigenin-labeled probes for Fgf10 (A, B), Fgfr2(C, D), and Shh (E, F). (A, B) At E13.5, the expression of Fgf10 in the palatal mesenchyme was similar in control and Catnb$^{ff}$; Osr2-IresCre mutant embryos. (C, D) The expression of Fgfr2 in the palatal epithelium was indistinguishable between control and mutant embryos at E13.5. (E, F) The expression of Shh in the medial epithelium edge (marked by arrowheads) and cells that would form palatal rugaes (indicated by arrows) was not altered in Catnb$^{ff}$; Osr2-IresCre mutants, in comparison with their control littermates. P, palatal shelf; T, tongue.
Chapter 3
Investigating the role of Wnt/β-catenin signaling in early tooth development
Summary

The developing tooth has been used as a model system for elucidating the molecular mechanisms regulating epithelial-mesenchymal interactions during organogenesis. Classical tissue recombination studies demonstrated that initiation of tooth development depends on activation of odontogenic potential in the developing tooth mesenchyme by signals from the presumptive dental epithelium. Several signaling molecules, including members of the Bmp, Fgf and Wnt families are expressed in the presumptive dental epithelium at the beginning of tooth initiation in mice. Both Bmp and Fgf signaling play essential roles in the induction of odontogenic potential in the early tooth mesenchyme. Wnt signaling is required within the developing tooth epithelium for tooth morphogenesis. However, whether Wnt signaling is directly involved in the activation of the odontogenic mesenchyme is not known. In this chapter, I utilized the Cre/Lox system to show that tissue-specific inactivation of β-catenin, a central component of the canonical Wnt signaling pathway, in the early developing tooth mesenchyme caused tooth developmental arrest at the bud stage in mice. I showed that mesenchymal β-catenin function is required for the expression of Lef1 and Fgf3 in the developing tooth mesenchyme and for the induction of primary enamel knot in the developing tooth epithelium. Moreover, I found that constitutive stabilization of β-catenin in the developing palatal mesenchyme induced ectopic tooth initiation from the palatal epithelium. Together, these results reveal that Wnt ligands expressed in the presumptive dental epithelium signal directly to the developing tooth mesenchyme to activate mesenchymal odontogenic program during tooth initiation.
Introduction

The developing mouse tooth has long been used as a model system for studying the molecular mechanisms regulating organ development (reviewed by Maas and Bei, 1997; Pispa and Thesleff, 2003). Like development of many other organ systems, development of the tooth, from its initiation through morphogenesis to cytodifferentiation, involves a series of sequential and reciprocal signaling interactions between the adjacent epithelium and mesenchyme (Thesleff et al., 1995; Pispa and Thesleff, 2003). In mice, tooth development begins as a thickening of the oral epithelium at around the 11th day of embryonic development (E11). The presumptive dental epithelium proliferates and buds into the underlying neural crest-derived mesenchyme at specific sites and induces the mesenchyme to condense around the epithelial buds from E12 to E13. At E13.5, cells at the tip of the epithelial tooth buds form the primary enamel knot, which secrete a number of growth factors to stimulate the proliferation of the surrounding dental epithelium. Subsequently, the dental epithelium folds and invaginates farther into the mesenchyme, surrounding the condensing mesenchyme to form “cap” and then “bell”-shaped tooth germs. As development proceeds, the epithelial cells in contact with the dental mesenchyme differentiate into the enamel-producing ameloblasts and their adjacent mesenchymal cells differentiate into the dentin-producing odontoblasts (Thesleff and Hurmerinta, 1981). The ameloblasts and odontoblasts deposit enamel and dentin matrices, respectively. The shape of the resultant tooth becomes effectively fossilized by subsequent mineralization of these matrices (Lumsden, 1988).

Tissue recombination experiments have demonstrated that tooth inductive signals arise initially in the presumptive dental epithelium. The E9-E11 mouse oral epithelium induced tooth formation when recombined with non-dental mandibular or second branchial arch mesenchyme (Mina and Kollar, 1987; Lumsden, 1988;
Ohazama et al., 2005). As development proceeds, the dental epithelium rapidly loses tooth inductive potential and, at the same time, the underlying dental mesenchyme acquires the capability to instruct tooth morphogenesis from non-dental epithelia (Mina and Kollar, 1987; Lumsden, 1988). It was demonstrated that the E13.5 dental mesenchyme induced tooth formation from various non-dental epithelia, including limb epithelium, causing enamel organ morphogenesis and amelogenesis (Ruch et al., 1973; Ruch, 1984; Kollar and Fisher, 1980; Mina and Kollar, 1987; Lumsden, 1988). Thus, whereas normal tooth initiation depends on a site-specific epithelium, the principal outcome of the epithelial-mesenchymal interactions during the tooth initiation stage is the acquisition by the cranial neural crest-derived mesenchyme of the ability to instruct the overlying epithelium to undergo tooth morphogenesis (Lumsden, 1988).

Members of the bone morphogenetic protein (Bmp) and fibroblast growth factor (Fgf) families have been shown to be critical tooth inductive signals (refer to Chapter 1). The Wnt signaling pathway also plays essential roles in early tooth development. The Wnt family of secreted proteins consists of 19 members in mammals and can trigger various cellular responses through several distinct pathways (Logan and Nusse, 2004). The canonical Wnt signaling pathway involves stabilization and nuclear accumulation of β-catenin. In the absence of Wnt signaling, cytoplasmic β-catenin is phosphorylated by the serine/threonin kinase GSK3β through interactions with the scaffolding proteins Axin and APC and targeted for degradation by the ubiquitination-proteosome pathway. Activation of Wnt signaling inhibits β-catenin phosphorylation, leading to stabilization of β-catenin and its accumulation in the cellular nuclei where it interacts with and converts the Tcf/Lef family DNA-binding proteins from transcriptional repressors to activators. Several Wnt genes, including Wnt4, Wnt6, Wnt10a and Wnt10b, as well as Lef1, are strongly
expressed in the presumptive dental epithelium at the tooth initiation stage in mice (Kratochwil et al., 1996; Dassule and McMahon, 1998; Sarkar and Sharpe, 1999). As the dental placode invaginated to form the tooth bud at E12, Lef1 mRNA expression shifted to the underlying presumptive dental mesenchyme (Kratochwil et al., 1996). At the same developmental stage, nuclear β-catenin was observed in both the dental epithelium and underlying mesenchyme (Liu et al., 2008). Application of Wnt-expressing cells specifically induced Lef1 mRNA expression in the mesenchyme of E11 mouse mandibular explants (Dassule and McMahon, 1998). These data indicate that the Wnt/β-catenin signaling pathway is activated in the dental mesenchyme during tooth initiation. However, whereas mice lacking Lef1 exhibited tooth developmental arrest at the bud stage (Kratochwil et al., 1996), tissue recombination experiments suggested that Lef1 function is required in the dental epithelium, but not in the dental mesenchyme, for tooth morphogenesis (Kratochwil et al., 1996; 2003). Consistent with an essential role of active Wnt/β-catenin signaling in the dental epithelium for early tooth development, epithelium-specific inactivation of β-catenin or epithelial expression of Dkk1, an inhibitor of the canonical Wnt signaling, caused tooth developmental arrest at the early bud stage (Andl et al., 2002; Liu et al., 2008). However, whether Wnt/β-catenin signaling in the developing dental mesenchyme is required for early tooth morphogenesis is not known. Here I found that β-catenin function is required in the developing dental mesenchyme for the transition of tooth morphogenesis from the bud to cap stage. In addition, I showed that ectopic stabilization of β-catenin in developing palatal mesenchyme induced de novo formation of ectopic tooth buds from the palate epithelium. Together, these data indicate that Wnt/β-catenin signaling plays critical roles in the activation of the mesenchymal odontogenic potential during early tooth development.
Results

Deletion of β-catentin in early tooth mesenchyme caused tooth developmental arrest at the bud stage

Since the tooth mesenchyme is derived from the cranial neural crest, the most straightforward approach for investigating the roles of the canonical Wnt signaling in the developing tooth mesenchyme would be to specifically inactivate the β-catentin gene in the early neural crest cells. However, Cre/loxP-mediated tissue-specific inactivation of the β-catentin gene in the early neural crest cells in mice using the Wnt1-Cre transgenic mouse strain resulted in severe disruption of formation of the facial primordia prior to initiation of tooth development (Braut et al., 2001). Our lab recently generated the Osr2-IresCre knockin mice, in which the IresCre bicistronic expression cassette was inserted in the 3’ region of the Osr2 gene, and showed that these mice exhibited Cre activity in the mesenchyme of the developing secondary palate and molar tooth germs (Lan et al., 2007). In contrast to the Wnt1-Cre transgenic mice, which express Cre in the premigratory neural crest cells (Danielian et al., 1998; Chai et al., 2000), Cre activity was not detected in the craniofacial tissues in the Osr2-IresCre mice until after neural crest migration to the facial primordia was complete (Lan et al., 2007). To analyze the spatial and temporal pattern of Cre activity during tooth development in Osr2-IresCre mice, I crossed Osr2-IresCre mice to the R26R reporter mice, in which LacZ expression is only activated by Cre-mediated recombination (Soriano, 1999). At E12.5, as the molar tooth epithelium invaginated into the mesenchyme, β-galactosidase activity was detected in mesenchymal cells lingual to the molar tooth buds, while mesenchymal cells buccal to the molar tooth buds showed very little β-galactosidase activity (Figure 3.1A, B). The lingual bias of Cre activity in the developing tooth mesenchyme corresponds to preferential expression of the Osr2 gene in the lingual side of the
developing tooth mesenchyme (Gao et al., 2009; Zhang et al., 2009). At E13.5, 
β-galactosidase activity in the upper molar mesenchyme still exhibited lingual bias 
(Figure 3.1C), while it was detected in the mesenchymal cells in both lingual and 
buccal sides of lower molar tooth buds (Figure 3.1D). By E15.5, β-galactosidase was 
expressed robustly in the dental papilla in both upper molar and lower molar tooth 
germs (Figure 3.1E, F). Importantly, no β-galactosidase expression was detected in 
the tooth epithelial cells throughout these stages.

To investigate whether β-catenin function in the developing tooth 
mesenchyme is required for tooth morphogenesis, I crossed Osr2-IresCre mice to 
mice carrying a targeted conditional β-catenin allele, Catnb\textsuperscript{f/f} (Braut et al., 2001). 
Catnb\textsuperscript{f/f};Osr2-IresCre pups were born with cleft palate and died shortly after birth. To 
examine tooth development in Catnb\textsuperscript{f/f};Osr2-IresCre mice, I carried out histological 
analyses of embryos harvested at E12.5 to E16.5. At E12.5, molar tooth development 
had initiated and tooth epithelium invaginated into the underlying mesenchyme in 
both control and mutant embryos (data not shown). At E13.5, molar tooth germs had 
reached the bud stage in both control and mutant embryos (Figure 3.2A, B). By E14.5, 
tooth development in the control embryos had reached the cap stage (Figure 3.2C), 
while molar tooth germs in the Catnb\textsuperscript{f/f}; Osr2-IresCre mutants remained at the bud 
stage (Figure 3.2D). At E16.5, molar tooth germs in the control embryos continued to 
develop toward the bell stage (Figure 3.2E), but the molar tooth germs in the Catnb\textsuperscript{f/f}; 
Osr2-IresCre mutants remained arrested at the bud stage (Figure 3.2F). Similarly, 
development of the lower incisors were also arrested at the late bud stage in the 
Catnb\textsuperscript{f/f}; Osr2-IresCre mutant mice (Figure 3.3). These data indicate that β-catenin 
function is required in the developing dental mesenchyme for tooth morphogenesis 
beyond the bud stage.
\( \beta \)-catenin function is required in the developing tooth mesenchyme for induction of primary enamel knot.

As the developing tooth germ transitions from the bud to the cap stage, mesenchymal signals induce the cells at the tip of the epithelial tooth bud to form the primary enamel knot, an epithelial signaling center that expresses multiple signaling molecules and transcriptional factors. The enamel knot is an important regulator of tooth shape, and its induction is a prerequisite for the tooth germ to develop into the cap stage (reviewed by Jernvall and Thesleff, 2000). To investigate whether induction of enamel knot had taken place in \( \text{Catnb}^{\beta} \); \( \text{Osr2-IresCre} \) mutant embryos, I analyzed expression of \( \text{Fgf4} \) and \( \text{Shh} \), two well established molecular markers of the primary enamel knot, in the developing tooth germs. At E14.5, the first molar tooth germs in control embryos exhibited strong localized expression of \( \text{Fgf4} \) and \( \text{Shh} \) mRNAs at the center of the cap-shaped tooth epithelium (Figure 3.4A, C). In contrast, examination of serial sections throughout the developing first molar tooth germs in the \( \text{Catnb}^{\beta} \); \( \text{Osr2-IresCre} \) mutant littermates failed to detect any \( \text{Fgf4} \) expression in the developing tooth epithelium while only a few cells at the tip of the maxillary, but not mandibular, first molar tooth buds showed weak \( \text{Shh} \) expression (Figure 3.4B, D). Similarly, in the developing lower incisor tooth germs at E14.5, only a few cells at the tip of the \( \text{Catnb}^{\beta} \); \( \text{Osr2-IresCre} \) mutant tooth buds showed weak \( \text{Shh} \) expression, in contrast to the robust \( \text{Shh} \) expression in the distal incisor epithelium in the control littermates (Figure 3.4E, F).

While the primary enamel knot acts as a signaling center for stimulating tooth morphogenesis from the bud to the cap stage, the enamel knot cells themselves undergo programmed cell death (reviewed by Jernvall and Thesleff, 2000). Consistent with the \( \text{Shh} \) expression data shown above, I found activated Caspase3 activity in a few cells in the center of the maxillary first molar tooth buds (Figure 3.5B), but not in
the mandibular first molar tooth buds (Figure 3.5D), in the E14.5 Catnb conditional; Osr2-IresCre mutant embryos, compared with strong and localized activated Caspase3 activity in the enamel knot cells in both the maxillary and mandibular first molar tooth germs in the control littermates (Figure 3.5A, C). Importantly, no increase in activation of Caspase3 activity was detected in the developing tooth epithelium and mesenchyme in the mutant embryos from E12.5 through E14.5, compared with their control littermates (Figure 3.5A-H). These data indicate that the tooth developmental arrest in the Catnb conditional; Osr2-IresCre mutant embryos was not associated with increased cell death.

The subtle differences between the progression of maxillary and mandibular first molar development may be due to the differences in the domains of Cre-mediated β-catenin inactivation in the developing tooth mesenchyme. I compared the expression of the β-catenin protein in the developing tooth germs in the control and Catnb conditional; Osr2-IresCre embryos by immunohistochemical staining. Similar to previous reports, I detected strong expression of β-catenin protein, with nuclear localization in a subset of cells, in the developing tooth mesenchyme directly underlying the developing epithelial tooth buds from E12.5 to E14.5 in control embryos (Figure 3.7A, and data not shown). By comparison, β-catenin protein levels are dramatically reduced throughout the developing tooth mesenchyme surrounding the mandibular first molar tooth buds by E13.5 in the Catnb conditional; Osr2-IresCre embryos (Figure 3.7B). Consistent with the lingual bias of Cre activity, in particular around the developing maxillary molar tooth germs, β-catenin was dramatically reduced in the developing tooth mesenchyme lingual and distal to, but not in the buccal side of, the maxillary first molar tooth buds in the E13.5 Catnb conditional; Osr2-IresCre embryos (Figure 3.7B). Importantly, the level of β-catenin protein in the developing tooth epithelium was not altered in the Catnb conditional; Osr2-IresCre embryos, in comparison with the control
littermates (Figure 3.7A, B). Thus, whereas previous studies showed that β-catenin function is required in the developing tooth epithelium for early tooth morphogenesis (Liu et al., 2008), these data indicate that β-catenin function is also required in the developing tooth mesenchyme for the induction of primary enamel knot formation and for tooth developmental transition from the bud to the cap stage.

**Cell autonomous requirement of β-catenin for maintenance of expression of Lef1 and Fgf3 in the developing tooth mesenchyme**

Induction of the primary enamel knot and transition of tooth development from the bud to the cap stage depends on adequate Bmp4 expression in the developing tooth mesenchyme (Chen et al., 1996; Bei et al., 2000; Zhao et al., 2000). Mice lacking either Msx1 or Pax9, two transcription factors expressed in the early developing tooth mesenchyme, exhibited tooth developmental arrest at the bud stage with loss of mesenchymal Bmp4 expression (Chen et al., 1996; Peters et al., 1998). Addition of exogenous Bmp4 to tooth germ explant cultures or transgenic expression of Bmp4 in the tooth mesenchyme rescued Msx1−/− mutant molar tooth development to the cap stage (Bei et al., 2000; Zhao et al., 2000). I thus compared the expression of Bmp4, Msx1, and Pax9 during tooth development in the Catnbflo/flo; Osr2-IresCre mutant and control embryos. I found that expression of these genes in the developing tooth mesenchyme was similar in the Catnbflo/flo; Osr2-IresCre mutant and control littermates (Figure 3.6A-F), although Bmp4 expression was detected in the enamel knot in the E14.5 control molar tooth germs (arrowheads in Figure 3.6A) but not in the mutant molar tooth germs (Figure 3.6B).

Mice lacking the transcription factor Lef1 exhibited tooth developmental arrest at late bud stage without disrupting mesenchymal Bmp4 expression (Kratochwil et al., 1996), similar to the Catnbflo/flo; Osr2-IresCre mutant mice. I compared the
expression of *Lef1* mRNA during tooth development in the *Catnb<sup>β<sup>−</sup></sup>; *Osr2-IresCre* mutant and control littermates. At E13.5, *Lef1* mRNA was highly expressed in the distal tooth bud epithelium and mesenchyme in control embryos (Figure 3.7C). In comparison, *Lef1* mRNA expression was dramatically reduced in the developing tooth mesenchyme in the *Catnb<sup>β<sup>−</sup></sup>; *Osr2-IresCre* mutant littermates (Figure 3.7D). In particular, the domains of *Lef1* downregulation correlated well with the domains of loss of β-catenin in the tooth mesenchyme such that *Lef1* mRNA expression was reduced throughout the mandibular molar tooth mesenchyme but persisted on the buccal side of the maxillary molar tooth mesenchyme (compare Figure 3.7D with Figure 3.7B). By E14.5, *Lef1* mRNA was strongly expressed in the primary enamel knot and the underlying dental mesenchyme in control embryos (Figure 3.7E). In contrast, little *Lef1* mRNA expression was detected in the developing tooth mesenchyme while strong *Lef1* mRNA expression was present in the distal tooth epithelium in *Catnb<sup>β<sup>−</sup></sup>; *Osr2-IresCre* mutant littermates (Figure 3.7F). These data indicate that β-catenin function is required cell-autonomously in the developing tooth mesenchyme for maintenance of *Lef1* mRNA expression.

*Leff1<sup>−/−</sup>* mutant embryos lacked *Fgf3* and *Fgf4* expression in the developing tooth mesenchyme and epithelium, respectively (Kratochwil et al., 2002). I found that *Fgf4* expression was absent in the developing tooth epithelium of *Catnb<sup>β<sup>−</sup></sup>; *Osr2-IresCre* mutant embryos although strong *Lef1* expression persisted in the mutant tooth epithelium (Fig. 3.4B and Figure 3.7F). I further examined expression of *Fgf3* expression during tooth development in the *Catnb<sup>β<sup>−</sup></sup>; *Osr2-IresCre* mutant embryos. As shown in Figure 3.7G, H, expression of *Fgf3* was significantly reduced in the developing tooth mesenchyme in *Catnb<sup>β<sup>−</sup></sup>; *Osr2-IresCre* mutant embryos compared with the control littermates.
Constitutive stabilization of β-catenin in the palatal mesenchyme activated ectopic tooth initiation

To further investigate the role of β-catenin in the activation of mesenchymal odontogenic potential, I crossed the \( \text{Catnb}^{\text{lox(ex3)}} \) mice, in which Exon-3 of the β-catenin gene was flanked by \( \text{loxP} \) sequences (Harada et al., 1999), to \( \text{Osr2-CreKI} \) mice. Deletion of Exon-3 from the β-catenin gene results in generation of a constitutively more stable β-catenin protein product due to lack of phosphorylation sites for GSK3β, thus mimicking activation of canonical Wnt signaling (Harada et al., 1999; Jarvinen et al., 2006; Liu et al., 2008). Similar to \( \text{Osr2-IresCre} \) mice, \( \text{Osr2-CreKI} \) mice exhibit highly specific Cre expression in the \( \text{Osr2} \)-expressing cells, including the developing palatal and tooth mesenchyme. Whereas a small percentage of \( \text{Osr2-IresCre} \) mice exhibited ectopic Cre activity (Lan et al., 2007), none of over 200 \( \text{Osr2-CreKI} \) mouse embryos displayed ectopic Cre activity outside of \( \text{Osr2} \)-expressing tissues (Lan et al., unpublished data).

\( \text{Catnb}^{\text{lox(ex3)}}/+;\text{Osr2-CreKI} \) mice died shortly after birth and displayed cleft secondary palate. Histological analyses revealed that development of molar teeth at their normal locations was not significantly affected in \( \text{Catnb}^{\text{lox(ex3)}}/+;\text{Osr2-CreKI} \) mutant embryos (Figure 3.8B, E, H), compared with their control littermates (Figure 3.8A, D, G). However, multiple epithelial invaginations were detected at the nasal sides of the developing palatal shelves by E16.5 in the \( \text{Catnb}^{\text{lox(ex3)}}/+;\text{Osr2-CreKI} \) mutants (Figure 3.8F). From E17.5 to P0, these ectopic epithelial invaginations formed morphologically distinct epithelial buds (Figure 3.8I).

To ascertain that the palatal epithelial invaginations resulted from stabilization of β-catenin in the palatal mesenchyme, I examined β-catenin protein expression in the control and \( \text{Catnb}^{\text{lox(ex3)}}/+;\text{Osr2-CreKI} \) mutant embryos. At E13.5, there was little β-catenin protein detected in the palatal mesenchyme in the control
embryos (Figure 3.9A). In contrast, strong β-catenin staining was detected throughout the palatal mesenchyme in the Catnb\textsuperscript{lox(ex3)/+;Osr2-CreKI} mutant littermates (Figure 3.9B) while β-catenin staining in the palatal epithelium was comparable in the control and mutant embryos (Figure 3.9A, B). Interestingly, following the formation of the palatal epithelial invaginations in the Catnb\textsuperscript{lox(ex3)/+;Osr2-CreKI} mutant embryos, intense β-catenin staining localized to the mesenchyme cells immediately underlying the invaginated palatal epithelium (Figure 3.9C). Consistent with the changes in the pattern of β-catenin protein distribution, Lef1 mRNA expression was ectopically activated throughout the developing palatal mesenchyme in the Catnb\textsuperscript{lox(ex3)/+;Osr2-CreKI} mutant embryos at E13.5 (Figure 3.9E), while no Lef1 expression was detected in the palatal mesenchyme in the control littermates (Figure 3.9D). In the Catnb\textsuperscript{lox(ex3)/+;Osr2-CreKI} mutants at P0, strong Lef1 mRNA expression was localized to the palatal mesenchyme immediately adjacent to the invaginated palatal epithelium (Figure 3.9F). These data suggest, following the initial overall accumulation of stabilized β-catenin and activation of the canonical Wnt signaling pathway resulting from Cre-mediated deletion of Exon-3 of the β-catenin gene in the palatal mesenchyme in the Catnb\textsuperscript{lox(ex3)/+;Osr2-CreKI} mutant embryos, that subsequent β-catenin expression and accumulation in the palatal mesenchyme underlying the invaginated palatal epithelium was regulated by epithelial-mesenchymal interactions.

To investigate the identity of the invaginated palatal epithelial structures in the Catnb\textsuperscript{lox(ex3)/+;Osr2-CreKI} mutants, I performed section in situ hybridization with probes for genes important for tooth development. Pitx2 is a marker for developing teeth, and is not expressed in other embryonic epithelial appendages (Mucchielli et al., 1997; Liu et al., 2008). Pitx2 expression was detected throughout the invaginated palatal epithelium in the Catnb\textsuperscript{lox(ex3)/+;Osr2-CreKI} mutants (Figure 3.9G), suggesting
that the abnormal epithelial structures represent ectopic tooth initiation. *Shh* is normally expressed in the enamel knot cells in the developing tooth germs (Dassule et al., 2000). *Shh* expression was observed specifically at the tip of some of the palatal epithelial buds in the *Catnblox(ex3)/+;Osr2-CreKI* mutants (Figure 3.9H). Similarly, *Bmp4* expression was detected at the tip of some of the palatal epithelial buds and in the underlying mesenchymal cells in the *Catnblox(ex3)/+;Osr2-CreKI* mutants (Figure 3.9I), which closely resembles *Bmp4* expression in the primary enamel knot and mesenchyme of the late bud stage tooth germs. These data indicate that stabilization of β-catenin in the palatal mesenchyme induces ectopic tooth initiation.

Taken together, our data indicate that β-catenin function is required in the developing tooth mesenchyme for the complete activation of mesenchymal odontogenic potential and that activation of β-catenin-mediated canonical Wnt signaling in the palatal mesenchyme causes ectopic activation of mesenchymal odontogenic potential.
Discussion

At the beginning of tooth development, the presumptive dental epithelium provides the tooth initiation signals to activate odontogenic potential in the developing tooth mesenchyme (Mina and Kollar, 1987; Lumsden, 1988; Ohazama et al., 2005). Several signaling molecules, including Bmp2, Bmp4, Fgf8, Fgf9, Shh, Wnt4, Wnt6, Wnt10a and Wnt10b, are expressed in the presumptive dental epithelium at the beginning of tooth development (Wozney et al., 1988; Lyons et al., 1989; Neubuser et al., 1997; Dassule and McMahon, 1998; Sarkar and Sharpe, 1999). Previous studies demonstrated that both Bmp and Fgf signaling play essential roles in the induction of odontogenic potential in the developing tooth mesenchyme by activating expression of the Msx1 and Pax9 transcription factors, respectively (Neubuser et al., 1997; Sarkar et al., 1998; Mandler and Neubuser, 2001). In contrast, Shh signaling appears not directly involved in the activation of odontogenic potential in the developing tooth mesenchyme because mice lacking Smoothened, the obligate transducer of Shh signaling, throughout cranial neural crest derived tissues, including the developing tooth mesenchyme, developed molars and upper incisors (Jeong et al., 2004). In this thesis, I provide the first experimental evidence that β-catenin mediated canonical Wnt signaling is also required for the activation of odontogenic potential in the developing tooth mesenchyme for tooth development to transition from the bud to cap stage.

Wnt/β-catenin signaling plays a direct and essential role in activation of odontogenic signals in the developing tooth mesenchyme

β-catenin is a central component of both the cadherin-mediated cell adhesion complex and the canonical Wnt signaling pathway. I found that inactivation of β-catenin in the developing tooth mesenchyme caused tooth developmental arrest
at the bud stage. Because tooth development from the bud to cap stage is accompanied by condensation of the developing tooth mesenchyme to form the dental papilla, it is possible that the tooth developmental arrest in the \textit{Osr2-IresCre;Catnb^{f/f}} mutant mice may be due in part to defects in cadherin-mediated cell adhesion during tooth mesenchyme condensation. However, the failure of induction of enamel knot markers in these mutants indicates that β-catenin is required for activation of mesenchymal odontogenic activity. Consistent with hypothesis, I found that \textit{Fgf3} expression was significantly reduced in the developing tooth mesenchyme in the \textit{Osr2-IresCre;Catnb^{f/f}} mutant mice. Moreover, the cell-autonomous reduction in \textit{Lef1} expression in the tooth mesenchyme cells lacking β-catenin in the \textit{Osr2-IresCre;Catnb^{f/f}} mutant mice indicates that β-catenin mediates the canonical Wnt signaling in the developing tooth mesenchyme.

Previous studies demonstrated that both \textit{Bmp4} and \textit{Wnt10b} were capable of inducing \textit{Lef1} expression in the E11 mouse mandibular mesenchyme explants (Kratochwil et al., 1996; Dassule and McMahon, 1998). I found that \textit{Lef1} expression was specifically reduced but \textit{Bmp4} expression was unaltered in the developing tooth mesenchyme in the \textit{Osr2-IresCre;Catnb^{f/f}} mutant embryos, in comparison with control littermates. Thus, in the absence of β-catenin mediated canonical Wnt signaling, Bmp4 was insufficient to maintain \textit{Lef1} expression in the developing tooth mesenchyme.

The tooth developmental arrest phenotype of the \textit{Osr2-IresCre;Catnb^{f/f}} mutant mice is remarkably similar to that of the \textit{Lef1^{-/-}} mutant mice. Both mutant strains exhibited tooth developmental arrest at the late bud stage with losses of \textit{Fgf4} and \textit{Shh} expression from the tooth epithelium and of \textit{Fgf3} expression in the tooth mesenchyme (Kratochwil et al., 1996). However, tissue recombination experiments indicated that \textit{Lef1} function was required in the tooth epithelium, but not in the tooth
mesenchyme, for tooth morphogenesis in explant cultures (Kratochwil et al., 1996). Kratochwil et al. (2002) further demonstrated that exogenous Fgf4 rescued development of the \textit{Lef1}\textsuperscript{−/−} mutant tooth germs and that Wnt signaling was able to directly activate reporter gene expression driven by the \textit{Fgf4} gene promoter. These studies led to the hypothesis that Lef1-mediated canonical Wnt signaling acts as a relay mechanism within the developing tooth epithelium through regulation of Fgf4 expression, which in turn signals to the mesenchyme to activate Fgf3 and other mesenchymal odontogenic factors to induce tooth development from the bud to the cap stage (Kratochwil et al., 2002). In the \textit{Osr2-IresCre;Catnbf/f} mutant mice, however, β-catenin was inactivated in the developing tooth mesenchyme but not in the tooth epithelium. Lef1 expression was specifically down-regulated in the developing tooth mesenchyme but not in the tooth epithelium in these mutants. The lack of \textit{Fgf4} expression in the developing tooth epithelium in \textit{Osr2-IresCre;Catnbf/f} mutant embryos indicates intraepithelial Wnt/Lef1 signaling is insufficient to activate Fgf4 expression in the developing tooth epithelium in the absence of signals downstream of Wnt/β-catenin signaling in the developing tooth mesenchyme. The difference between our finding that Wnt/β-catenin signaling in the developing tooth mesenchyme is required for tooth development in vivo and the finding of Kratochwil et al. (1996) that Lef1 function was not required in the developing tooth mesenchyme for tooth morphogenesis in recombinant explants is most likely due to functional redundancy between Lef1 and other Tcf family members. Indeed, \textit{Tcf1} is coexpressed with \textit{Lef1} in many embryonic tissues, including the developing tooth mesenchyme (Oosterwegel et al., 1993; Galceran et al., 1999). Moreover, Lef1 appears to function partially redundantly with Tcf1 and Tcf4, respectively, during limb and midfacial development (Galceran et al., 1999; Brugmann et al., 2007). Thus, by specifically inactivating β-catenin in the developing tooth mesenchyme, we have revealed a direct
role for Wnt/β-catenin signaling in the activation of odontogenic signals in the developing tooth mesenchyme.

**Wnt/β-catenin signaling and ectopic tooth initiation**

Our finding that constitutive stabilization of β-catenin in the developing palatal mesenchyme caused ectopic tooth initiation from the palatal epithelium is very intriguing. Two laboratories recently independently demonstrated that constitutively stabilizing β-catenin through epithelium-specific deletion of Exon-3 of the β-catenin gene using the K14-Cre transgenic mice resulted in continuous sequential tooth production from the embryonic molar tooth germs (Jarvinen et al., 2006; Liu et al., 2008). In addition, K14-Cre mediated epithelium-specific deletion of the Apc gene resulted in extra tooth formation next to the molar tooth germs in the K14-Cre;Apc^{cko/cko} mutant mice (Kuraguchi et al., 2006). These results indicate that constitutive activation of Wnt/β-catenin signaling in the oral epithelium can trigger ectopic initiation of tooth development. Interestingly, although many epithelial invaginations formed in the oral epithelium in the Catnb^{ex3K14/+} mice, extra teeth only developed from the dental epithelium (Jarvinen et al., 2006; Tummers and Thesleff, 2009), indicating that ectopic intraepithelial Wnt/β-catenin signaling was insufficient to activate the mesenchymal odontogenic program outside of the tooth developmental field. In contrast, ectopic initiation of tooth bud-like structures in the Catnb^{lox(ex3)/+;Osr2-CreKI} mutant mouse resulted from direct reprogramming of the developing palatal mesenchyme by stabilized β-catenin. Further investigation of how Wnt/β-catenin signaling interacts with other signaling pathways to activate the mesenchymal odontogenic potential will guide research efforts in biological tooth regeneration for replacement therapy.
Methods

Mouse strains, breeding and genotyping

Generation and characterization of *Osr2-IresCre* mice have been reported previously (Lan et al., 2007). Detailed analysis of *Osr2-CreKI* mice, in which a Cre cDNA replaced the coding region of the *Osr2* gene, will be described elsewhere. The *Catnb^{ff}* mice, homozygous for a β-catenin allele with the DNA sequences from Exon-2 to Exon-6 flanked by two repeated loxP sites (Brault et al., 2001), and the *R26R* mice (Soriano, 1999) were purchased from the Jackson Laboratory (Bar Harbor, ME). *Catnb^{lox(ex3)}* mice (Harada et al., 1999) were generously provided by Dr. Di Chen (University of Rochester).

For analysis of Cre activity in *Osr2-IresCre* mice, *Osr2-IresCre* mice were crossed to *R26R* mice and the embryos processed for X-gal staining. Mice lacking β-catenin in the dental mesenchymal cells was obtained by crossing *Osr2-IresCre/+;Catnb^{fp}+* mice with *Catnb^{ff}+* mice. For ectopic mesenchyme-specific activation of β-catenin, *Catnb^{lox(ex3)}* mice were crossed to *Osr2-CreKI* mice. Timed mating was set up in the late afternoon and examined for the presence of vaginal plugs the next morning. The noon of plug day is assumed as 0.5 day of gestation (E0.5). Embryos were collected at various developmental stages. For genotyping, genomic DNA from either yolk sac or tail tissues was extracted. Primers for genotyping *Catnb* and *R26R* alleles have been described previously (Brault et al., 2001; Soriano, 1999). Other primers used for genotyping are: 1733, AGGGTACCTGAAGCTCAGCG and 1734, CAGTGGCTGACAGCAGCTTT for the *Catnb^{lox(ex3)}* allele; and Cre83, GTCCAATTACTGACCAGCTACACC and Cre85, GTTATTCCGATCATCAGCTACACC for both *Osr2-IresCre* and *Osr2-CreKI* alleles.
X-Gal Staining

To detect β-galactosidase activity, embryos were fixed in 0.25% glutaraldehyde, passed through sucrose series, embedded in OCT freezing medium, and sectioned at 14 μm thickness using a cryostat microtome. X-gal staining of sections was performed as described previously (Hogan et al., 1994). Slides were counterstained with eosin.

Histology and immunohistochemical staining

For histological analysis, staged embryos were collected, fixed either in Bouin’s fixative or in 4% paraformaldehyde (PFA), dehydrated through ethanol series, embedded in paraffin, and sectioned serially at 7 μm. Slides were stained with hematoxylin and eosin, and mounted with Permount.

For immunostaining, frontal sections from 4% PFA-fixed embryos were treated by 3% hydrogen peroxide, boiled in a pressure cooker containing trilogy solution, and incubated with primary antibodies against phospho-Smad1/5/8 (polyclonal rabbit serum, Cell Signaling Technology; 1:200), β-catenin (mouse monoclonal clone 15B8, Sigma; 1:500), and active-caspase-3 (rabbit monoclonal antibody, BD Biosciences, 1:250). M.O.M kit (Vector Laboratories), in combination with streptavidin-conjugated Texas-Red (Vector Laboratories, 1:100) was used for detection of β-catenin. Histostain Plus Rabbit Primary (DAB) kit (Zymed Laboratories) was used for detection of phospho-Smad 1/5/8 and active-caspase-3, following manufacturer’s instructions.

Section in situ hybridization

Embryos were fixed in 4% PFA, dehydrated through ethanol series, embedded in paraffin, and sectioned at 7 μm thickness. Frontal sections were
hybridized with digoxigenin–labeled antisense RNA probes that are generated by in vitro transcription of linearized cDNA templates. Slides were subsequently incubated with an alkaline phosphatase-conjugated anti-DIG antibody (Roche). Hybridization signal was detected by BM purple substrate (Roche) as described previously (Zhang et al., 1999).
Figure 3.1 Analysis of cre activity during molar tooth development in Osr2-IresCre; R26R double heterozygous embryos. Frontal sections from Osr2-IresCre; R26R embryos at E12.5 (A, B), E13.5 (C, D), and E15.5 (E, F) were assayed by X-gal staining. Cells expressing cre recombinase appeared blue. A, C, E: upper molars; B, D, F: lower molars. (A, B) At E12.5, β-galactosidase activity was detected in mesenchymal cells lingual to the molar tooth buds, while mesenchymal cells buccal to the molar tooth buds showed very little β-galactosidase activity.. (C, D) At E13.5, β-galactosidase activity in the upper molar mesenchyme still exhibited lingual bias, while it was detected in the mesenchymal cells in both lingual and buccal side of lower molar tooth buds. (E, F) By E15.5, β-galactosidase was expressed robustly in the dental papilla in both upper molar and lower molar tooth germs. Black arrows point to mesenchymal cells in buccal side of molar tooth buds. Note that cre activity was only detected in dental mesenchymal cells, not in dental epithelia. tb, tooth bud; de, dental epithelium; dm, dental mesenchyme.
Figure 3.1

Upper Molar | Lower Molar
---|---
A | B
E12.5 | E12.5

C | D
E13.5 | E13.5

E | F
E15.5 | E15.5

tb | tb
dm | dm
de | de
Figure 3.2 Conditional ablation of β-catenin in early tooth mesenchyme caused molar tooth developmental arrest at the bud stage. Frontal sections from both controls (A, C, E) and Catnb<sup>f/f</sup>; Osr2-IresCre mutants (B, D, F) were subjected to hematoxylin and eosin (H&E) staining at E13.5 (A, B), E14.5 (C, D) and E15.5 (E, F). (A, B) At E13.5, molar tooth germs had reached the bud stage in both control and mutant embryos. (C, D) By E14.5, tooth development in the control embryos had reached the cap stage, while molar tooth germs in the Catnb<sup>f/f</sup>; Osr2-IresCre mutants remained at the bud stage. (E, F) At E16.5, molar tooth germs in the control embryos continued to develop toward the bell stage, but the molar tooth germs in the Catnb<sup>f/f</sup>; Osr2-IresCre mutants remained arrested at the bud stage. Black arrows point to molar tooth germs.
Figure 3.2

Control

Catnb<sup>fr</sup>; Osr2-IresCre
Figure 3.3 Conditional ablation of β-catenin in early tooth mesenchyme caused lower incisor developmental arrest at the bud stage. Frontal sections from both controls (A, C, E) and Catnb f/f; Osr2-IresCre mutants (B, D, F) were subjected to hematoxylin and eosin (H&E) staining at E13.5 (A, B), E14.5 (C, D) and E15.5 (E, F). Black arrows point to lower incisors. Note that development of lower incisor teeth was arrested at the bud stage.
Figure 3.4 β-catenin function is required in the developing tooth mesenchyme for induction of primary enamel knot. (A–D) Frontal sections in the molar teeth region from E13.5 littermate controls (A, C) and Catnb\textsuperscript{ff}; Osr2-IresCre mutants (B, D) were subjected to in-situ hybridization with digoxigenin-labeled probe for Fgf4 (A, B) and Shh (C, D). (E–F) Frontal sections in the incisor region from E13.5 littermate controls (E) and Catnb\textsuperscript{ff}; Osr2-IresCre mutants (F) were subjected to in-situ hybridization with the probes for Shh. Black arrows point to molar tooth buds (A–D) and lower incisors (E–F).
Figure 3.4

Control

Cat\textsuperscript{lo} \text sup\textsubscript{in}; Osr2-lresCre

\textbf{A} E14.5 \hspace{1cm} \textbf{B} E14.5

\textit{Fgf4}

\textbf{C} E14.5 \hspace{1cm} \textbf{D} E14.5

\textit{Shh}

\textbf{E} E14.5 \hspace{1cm} \textbf{F} E14.5

\textit{Shh}
Figure 3.5 Cell apoptosis was not altered in Catnb$^{ff}$; Osr2-IresCre mutant embryos. Frontal sections of littermate controls (A, C, E, G) and Catnb$^{ff}$; Osr2-IresCre mutant embryos (B, D, F, H) were subjected to immunostaining with anti-cleaved capsase-3 antibody. Cell apoptotic activity in dental mesenchyme was comparable between control and mutant embryos at E14.5 (A, B, C, D), E12.5 (E, F) and E13.5 (G, H). A cluster of cells were undergoing cell apoptosis in the primary enamel knots of upper molar (A) and lower molar (C) of E14.5 control embryos. Activated Caspase3 activity was also detected in a few cells in the center of the maxillary first molar tooth buds (B), but not in the mandibular first molar tooth buds (D).
Figure 3.5

Control | Catnb<sup>fl/fl</sup>; Osr2-IresCre

A E14.5 | B E14.5

Upper molar

C E14.5 | D E14.5

Lower molar

E E12.5 | F E12.5

G E13.5 | H E13.5
Figure 3.6 β-catenin is not required in the tooth mesenchyme for Bmp4 expression. Frontal sections from both control (A, C, E) and Catnb<sup>ff</sup>; Osr2-IresCre mutant embryos (B, D, F) at either E13.5 (E, F) or E14.5 (A-D) were subjected to in-situ hybridization with digoxygenin-labeled probes for Bmp4 (A, B), Msx1 (C, D), Pax9 (E, F). The expression of Bmp4, Msx1, and Pax9 in dental mesenchyme is similar in control and mutant embryos. The expression of Bmp4 is also detected in enamel knot of control embryos at E14.5 (marked by black arrowhead in A), while it is not expressed in tooth epithelium of mutant embryos (B).
Figure 3.6

Control  \hspace{1cm} Catnb^{fr}; Osr2-IresCre

A \hspace{1cm} E14.5  \hspace{1cm} B \hspace{1cm} E14.5

Bmp4

C \hspace{1cm} E14.5  \hspace{1cm} D \hspace{1cm} E14.5

Msx1

E \hspace{1cm} E13.5  \hspace{1cm} F \hspace{1cm} E13.5

Pax9
Figure 3.7 Cell autonomous requirement of β-catenin for maintenance of expression of *Lef1* and *Fgf3* in the developing tooth mesenchyme. Frontal sections from both control (A, C, E) and *Catnb*<sup>fl/fl*, Osr2-IresCre* mutant embryos (B, D, F) at either E13.5 (A, B, C, D) or E14.5 (E, F, G, H) were subjected to immunofluorescence with anti-β-catenin antibody (A, B) or in-situ hybridization with digoxygenin-labeled probes for *Lef1* (C, D, E, F), and *Fgf3* (G, H). Arrows point to dental mesenchymal cells of maxillary molar germs; arrowheads indicate dental mesenchymal cells of mandibular molar germs.
Figure 3.7
Figure 3.8 Constitutive stabilization of β-catenin in the palatal mesenchyme activated ectopic tooth-like epithelial buds. H&E staining of frontal section from control (A, D, G) and Catnb\textsuperscript{lox(ex3)/+};Osr2-CreKI embryos (B, C, E, F, H, I) were performed. Panels C, F, and I are higher magnification of secondary palate in panels B, E, and H, respectively. (A, B, D, E, G, H) Development of molar teeth at their normal locations was not significantly affected in Catnb\textsuperscript{lox(ex3)/+};Osr2-CreKI mutant embryos, compared with their control littermates. (C) At E15.5, epithelial thickening appeared at the nasal sides of the developing palatal shelves (arrowheads). (F) By E16.5, multiple epithelial invaginations were detected at the nasal sides of the developing palatal shelves in the Catnb\textsuperscript{lox(ex3)/+};Osr2-CreKI mutants (arrowheads). (I) At E17.5, the ectopic epithelial invaginations at the nasal sides of the palatal shelves in the Catnb\textsuperscript{lox(ex3)/+};Osr2-CreKI mutants formed morphologically distinct epithelial buds (arrowheads). um1, first upper molar; lm1, first lower molar.
Figure 3.8

<table>
<thead>
<tr>
<th>Control</th>
<th>Catnb&lt;sup&gt;lox/ox3&lt;/sup&gt;/&lt;sup&gt;+&lt;/sup&gt;, Osr2-CreKI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
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Figure 3.9 Constitutive stabilization of β-catenin in the palatal mesenchyme activated *Lef1*, and several tooth marker genes. Frontal sections from either E13.5 (A, B, D, E) or P0 (C, F, G, H, I) control (A, D) and Catnb^{lox(ex3)/+};Os2-CreKI mutant embryos (B, C, E, F, G, H, I) were subjected to immunofluorescence with anti-β-catenin antibody (A, B, C) or in-situ hybridization with digoxygenin-labeled probes for *Lef1* (D, E, F), *Pitx2* (G), *Shh* (H), and *Bmp4* (I).
References


He, F., Xiong, W., Yu, X., Espinoza-Lewis, R., Liu, C., Gu, S., Nishita, M.,


Klapholz-Brown, Z., Walmsley, G. G., Nusse, Y. M., Nusse, R., and Brown, P. O.


Appendix 1

Figures for Chapter 1
Appendix 1.1 Oral clefts and dental abnormalities are serious birth defects in humans. (A-D) Photos taken from the patients with various oral clefts. (A) Unilateral cleft lip without cleft palate (B) Unilateral cleft lip with cleft palate (C) Bilateral cleft lip with cleft palate (D) Isolated cleft palate (E-F) Two cases of dental abnormalities. (E) A patient with syndromic oligodontia (F) A patient with non-syndromic tooth agenesis. A red asterisk marked the position of a mandibular premolar, which was missing in this patient. (Tucker and Sharp, 2004; Stoll et al., 2004)
Appendix 1.1

(Tucker and Sharp, 2004; Stoll et al., 2004)
Appendix 1.2 Scanning electron micrographs of human embryos during midfacial development. (A) Frontal view of a Stage 13 (Carnegie Stage System of human embryos) human embryo. At this stage, the facial primordia consisting of frontonasal process, mandibular processes and maxillary processes are located around the primitive oral cavity. (B) Frontal view of a Stage 14 (30-32 days of gestation) human embryo. At this stage, the nasal placodes appear bilaterally on the ventrolateral part of the frontonasal prominence. (C) Frontal view of a Stage 15 (35 days of gestation) human embryo. At this stage, the frontonasal prominence is growing rapidly around nasal placodes and is developing into lateral nasal process and medial nasal process. (D) Side view of a Stage 16 (38 days of gestation) human embryo. By this stage, while the medial nasal process and the lateral nasal process are still undergoing active fusion, fusion between the medial nasal process and the maxillary process has initiated. FNP, frontonasal process; LNP, lateral nasal process; Mn, mandibular process; MNP, medial nasal process; Mx, maxillary process; NP, nasal placode; SA, secondary branchial arch. (Hinrischen, 1986; Bush, 2004; Jiang et al., 2006)
Appendix 1.2

(Hinrichsen, 1986)
Appendix 1.3 Scanning electron micrographs of mouse embryos during midfacial development. (A) Frontal view of an E9.5 mouse embryo. At this stage, the facial primordia consisting of frontonasal process, mandibular processes and maxillary processes are located around the primitive oral cavity. The nasal placodes start to appear bilaterally on the ventrolateral part of frontonasal prominence. (B) Frontal view of an E10.25 mouse embryo. The frontonasal prominence has developed into the lateral nasal process and the medial nasal process. (C) Frontal view of an E10.75 mouse embryo. The two mandibular processes have merged to form the mandible. Fusion between the medial nasal process and the lateral nasal process has been initiated. (D) Frontal view of an E11.5 mouse embryo. The groove between two medial nasal processes disappears with the medial growth of the medial nasal processes and the maxillary processes. The medial nasal process and the maxillary process are undergoing active fusion. FNP, frontonasal process; LNP, lateral nasal process; Mn, mandibular process; MNP, medial nasal process; Mx, maxillary process; NP, nasal placode; SA, secondary branchial arch. (Kaufman, 1998)
Appendix 1.3

(Kaufman, 1998)
Appendix 1.4 Development of the secondary palate in mice. (A) The maxillary processes bud into the oral cavity to form the primordia of palatal shelves. (B-D) The palatal shelves undergo the vertical growth along the lateral sides of the developing tongue (removed in B and D). (E) The palatal shelves then elevate rapidly into the horizontal position at E14. (F) Elevated palatal shelves grow continually, and eventually make direct contact in the midline at E14.5 to form a midline epithelial seam (indicated by an arrow). (G) The palatal shelves are undergoing active fusion at E15.5. (F) The formation of the secondary palate is completed at E16.5. p, palatal shelf of the secondary palate; pp, primary palate; t, tongue. (Kaufman, 1998)
Appendix 1.4

(Kaufman, 1998)
Appendix 1.5 Important stages of molar tooth development in mice. The tooth development is initiated as a thickening of oral epithelium at around mouse embryonic day 11.5 (E11.5), which then proliferates and invaginates into the underlying neural crest-derived mesenchyme to form a tooth bud at E13.5, a cap-shaped tooth germ at E14.5, and then a bell-shaped tooth germ at E16.5. From late E16, the teeth undergo cytodifferentiation. By E18.5, the epithelial cells in contact with the dental mesenchyme differentiate into the enamel-producing ameloblasts and their adjacent mesenchymal cells differentiate into the dentin-producing odontoblasts. The teeth erupt around 5 weeks after birth in mice.
Appendix 1.6 Diagram of the canonical Wnt signaling pathway. (A) In the absence of Wnt ligands, cytoplasmic β-catenin proteins are phosphorylated by a destructive complex containing a serine/threonine kinase glycogen synthase kinase-3β (GSK3β), and scaffolding proteins Axin and Adenomatous Polyposis Coli (APC). After the phosphorylation, β-catenin proteins are degraded by the ubiquitination-proteosome pathway. (B) Wnt ligands bind to a Frizzled receptor and a LRP5/6 co-receptor. These receptors consequently send a signal to Disheveled (Dsh) and Axin, which somehow inhibit β-catenin phosphorylation, and therefore lead to stabilization of β-catenin proteins and their accumulation in the cellular nuclei where they interact with Tcf/Lef family DNA-binding proteins to activate the transcription of downstream target genes.
Appendix 2

The TGFβ pseudoreceptor gene *Bambi* is dispensable for mouse embryonic development and postnatal survival

Work described in this chapter has been reported in:

Summary

The Bambi (Bmp and activin membrane bound inhibitor) gene encodes a transmembrane protein highly similar in amino acid sequence to transforming growth factor-beta (TGFβ) type-I receptors. Unlike the TGFβ receptors, however, the Bambi intracellular domain is short and lacks a serine/threonine-kinase domain that is essential for transducing TGFβ signaling. Previous biochemical assays showed that Bambi interacts directly with BMP receptors and antagonizes BMP signaling. Interestingly, the expression of Bambi largely overlaps, both temporally and spatially, with that of Bmp4 during early embryonic development in Xenopus, zebrafish, and mice, which led to the hypothesis that Bambi may function to regulate BMP signaling during embryogenesis. To directly analyze the roles of Bambi during embryonic development, we generated mice carrying a conditional allele of Bambi, Bambi\textsuperscript{flox}, with loxP sequences flanking the first exon that encodes the N-terminus and signal peptide region of the Bambi protein. Mice homozygous for this targeted conditional allele appear normal and fertile. We crossed the Bambi\textsuperscript{flox} mice to the EIIa-Cre transgenic mice and generated mice carrying deletion of the first exon of the Bambi gene. Surprisingly, mice homozygous for the deleted allele were viable, fertile and did not exhibit any discernible developmental defect. Our data exclude an essential role for Bambi in mouse embryonic development and postnatal survival.
Introduction

The transforming growth factor-beta (TGFβ) superfamily of secreted signaling molecules, including TGFβs, bone morphogenetic proteins (BMPs), and activins, plays essential roles throughout animal development. These molecules initiate cellular signaling by binding to and bringing together two types of receptor serine and threonine kinases. Upon ligand binding, the type-II receptor phosphorylates and activates the type-I receptor kinase, which in turn phosphorylates and activates a set of transcriptional coactivators called Smad proteins and leads to their nuclear translocation and transcriptional activation of downstream target genes (reviewed in Nohe et al., 2004; Shi and Massague, 2003). In an expression screen for components involved in BMP4 signaling in Xenopus embryos, Onichtchouk et al. (1999) identified a gene encoding a transmembrane protein highly related to type-I TGFβ receptors. They named the gene product BAMBI (BMP and activin membrane-bound inhibitor) because it lacked an intracellular kinase domain and its overexpression antagonized BMP and activin signaling in Xenopus embryos (Onichtchouk et al., 1999). The Bambi gene is evolutionarily conserved and its homologs have been identified in humans (Degen et al., 1996), Zebrafish (Tsang et al., 2000), mouse (Grotewold et al., 2001), and rat (Loveland et al., 2003).

Ectopic expression of Bambi mRNA in Xenopus and zebrafish embryos resulted in phenotypes resembling inhibition of BMP signaling (Onichtchouk et al., 1999; Tsang et al., 2000). In vitro biochemical assays showed that the Bambi protein associated with type-I and type-II BMP/TGFβ receptor complexes in the presence of ligands, suggesting that Bambi inhibits BMP/TGFβ signaling by interfering with receptor complex formation or phosphorylation of type-I receptors (Onichtchouk et al., 1999; Tsang et al., 2000). Interestingly, Bambi is coexpressed with Bmp4 in many developmental processes during embryogenesis in Xenopus (Onichtchouk et al.,
1999), zebrafish (Tsang et al., 2000), and mouse (Grotewold et al., 2001). The coexpression and the ability of Bambi to antagonize BMP/TGFβ signaling suggest that Bambi may play important roles in the regulation of BMP4 signaling during embryonic development.

BMP4 signaling is essential for many developmental processes. Mouse embryos lacking BMP4 function die at midgestation with severe defects in mesoderm formation (Winnier et al., 1995). BMP4+/− heterozygous mice exhibit variable defects in craniofacial, eye, kidney, and limb development (Dunn et al., 1997). Moreover, mice lacking chordin and noggin, endogenous inhibitors of BMP signaling, have multiple developmental defects (Bachiller et al., 2000, 2003; Brunet et al., 1998; McMahon et al., 1998), indicating that the levels of BMP signaling are highly regulated during normal embryonic development. Since Bambi is a unique inhibitor of Bmp signaling at the receptor level and since Bambi mRNA is coexpressed with Bmp4 during craniofacial, eye, kidney, and limb development (Grotewold et al., 2001), it may play important roles in these developmental processes by fine-tuning BMP signaling.
Results

To investigate the roles of Bambi in mammalian development, we generated mice carrying a conditional null allele of the *Bambi* gene, *Bambi*\(^{\text{flox}}\), through homologous recombination in mouse embryonic stem cells and subsequent production of chimeric mice (see Methods, and Appendix 2.1A). Both *Bambi*\(^{\text{flox}/+}\) and *Bambi*\(^{\text{flox}/\text{flox}}\) mice were viable, fertile and didn’t display any obvious phenotypic abnormality.

We crossed *Bambi*\(^{\text{flox}/+}\) mice to either *EIIa-cre* or *CMV-cre* transgenic mice, both of which could mediate deletion of *loxP*-flanked sequences in germ cells (Lakso et al., 1996; Dupe et al., 1997). *Bambi*\(^{\text{del}/+}\) mice were then intercrossed. The deletion of *loxP*-flanked sequence was confirmed by Southern hybridization analysis of *EcoRI*-digested genomic DNA using a probe corresponding to the deleted region (Appendix 2.1). A 7.7 Kb band representing the wildtype allele was detected in both wildtype and *Bambi*\(^{\text{del}/+}\) heterozygous mice, but absent in *Bambi*\(^{\text{del}/\text{del}}\) homozygotes (Appendix 2.1C). The presence of genomic DNA in all lanes was confirmed by hybridizing the same membrane to a probe corresponding to genomic DNA 5’ to the deletion (data not shown). The deletion was also verified by PCR genotyping. The wildtype allele gives a 215 bp PCR product using the primer pair F2/R2 and the deleted allele results in a 316 bp PCR product using the primers F2 and R4 (Appendix 2.1D). Furthermore, we performed RT-PCR analysis of total RNA from wildtype and *Bambi*\(^{\text{del}/\text{del}}\) mutant mouse embryos using primers RF1 and RR1. As expected, a 329 bp product was amplified from wildtype but not homozygous mutant littermate RNA samples (Appendix 2.2B). Similarly, a 586 bp product was amplified from wildtype but not homozygous mutant littermate RNA samples using primers flanking the exons 1 and 2 (Appendix 2.2C); indicating that the *loxP*-flanked sequence had been
successfully deleted by the Cre recombinase. Since the 1075 bp genomic deletion includes the Bambi gene promoter and the first exon encoding the signal peptide, the deleted allele should represent a null allele of Bambi.

Of 125 pups from heterozygous intercross genotyped at two weeks of age, 30 (24.0%) were of wildtype, 59 (47.2%) were heterozygous, and 36 (28.8%) were homozygous for the Bambi<sup>del</sup> allele. Chi-square analysis indicated that the distribution of these three genotypes was not significantly different from the expected Mendelian ratio. Morphological and histological examination of Bambi<sup>del/del</sup> homozygous mice didn’t detect any overt defects, indicating that the Bambi gene was not essential for normal mouse embryogenesis and postnatal survival.

To assess whether loss of Bambi function has any effect on postnatal growth, the body weights of homozygous mutant, heterozygous and wild type littermates were measured at regular internals up to two months after birth (Appendix 2.3). Independent student’s t-test indicated that the body weights of Bambi<sup>del/del</sup> female mice were not significantly different from those of wildtype and heterozygous littermates before weaning (pups were usually weaned at 21 – 22 days after birth). However, the Bambi<sup>del/del</sup> homozygous mutant female mice exhibited a small (9%-10%) but statistically significant (P<0.05) reduction in body weight, compared to their wildtype littermates. The Bambi<sup>del/+</sup> heterozygous female mice had body weights in between and not statistically significant from the wildtype and homozygous mutant littermates. For male mice, body weights of Bambi<sup>del/del</sup> homozygous mutants were not significantly different from those of wildtype and heterozygous littermates at all the stages examined.

I further investigated whether loss of Bambi function affected male and female fertility. Bambi<sup>del/del</sup> homozygous males and females were intercrossed and littersizes were monitored. Pups were born with normal litter sizes and were normally
nursed by their mothers, suggesting that deletion of *Bambi* has no significant effect on fertility. These data also exclude any maternal or paternal effect on the *Bambi* gene.

Since BMP signaling plays important roles in skeletal development, and genetic inactivation of other endogenous Bmp inhibitors resulted in skeletal defects (e.g., Balemans et al., 2002), we examined skeletal preparations of newborn pups. Compared with wildtype littermates, *Bambi*\textsuperscript{del/del} mutant mice didn’t exhibit any obvious defect in the skeleton (data not shown).

*Bambi* is co-expressed with Bmp4 during craniofacial and limb development (Grotewold et al., 2001). We carefully examined these structures in newborn mice by histological and skeletal preparations. *Bambi*\textsuperscript{del/del} mutant mice didn’t display any obvious craniofacial or limb defects (Appendix 2.4).

In conclusion, we have generated mice carrying a floxed conditional allele of *Bambi*. These mice were crossed to Cre-transgenic mice and mice carrying the deleted null *Bambi* allele were generated. Analysis of *Bambi*\textsuperscript{del/del} mutant mice indicated that Bambi function is not essential for embryonic development and postnatal survival although it has some minor effect on the growth of female mice after weaning.
Methods

Generation of \(\text{Bambi}^{\text{flox}}\) and \(\text{Bambi}^{\text{del}}\) mice

A BAC clone containing the \(\text{Bambi}\) genomic region was obtained by screening the RPCI-22 129/SvEvTac mouse BAC library (BACPAC Resources, Children’s Hospital of Oakland, Oakland, CA). An 7.7 Kb EcoRI fragment containing exons 1 and 2 as well as an 13.5 Kb \(\text{BamHI}\) fragment containing the entire gene were subcloned into pBluescript plasmid vectors for construction of the targeting vector. The targeting vector contained the following fragments in the 5’ to 3’ order: (1) a diphtheria toxin (DTA) expression cassette, (2) an 2.9 Kb EcoRI/PmlI fragment from 5’ to the \(\text{Bambi}\) gene promoter as the 5’ homologous arm, (3) a \(\text{loxP}\) sequence, (4) the 1.07 Kb PmlI fragment containing the Bambi gene promoter and first exon, (5) another \(\text{loxP}\) sequence followed by an FRT-flanked PGKneo expression cassette, and (6) the 3.6 Kb PmlI-Sacl fragment from Intron1 to exon3 region as the 3’ homology arm (Appendix 2.1A). The final targeting vector was linearized at a unique KpnI restriction site 3’ to the 3’ homology arm and electroporated into CJ7 mouse embryonic stem (ES) cells.

Following electroporation, ES cells were subjected to G418 selection as described previously (Swiatek and Gridley, 1993). G418-resistant ES clones were screened by Southern hybridization analysis of \(\text{BglII}\)-digested ES genomic DNA using a 5’ external probe. Three independent targeted ES clones were microinjected into C57BL/6J blastocysts that were subsequently transferred into the uterus of pseudopregnant C57BL/6J female mice to generate chimeric mice. Chimeric mice from two of the three ES clones gave germ-line transmission of the targeted allele and were crossed to C57BL/6J female mice to generate \(\text{Bambi}^{\text{flox/+}}\) heterozygous mice, which were than intercrossed to generate and test whether there is any obvious defect.
in the \( Bambi^{\text{flo}x/\text{flo}x} \) mice. To generate mice with loss of \( Bambi \) gene function, \( Bambi^{\text{flo}x/+} \) heterozygous mice were crossed to either \( E11a-Cre \) or \( CMV-Cre \) transgenic mice (purchased from The Jackson Laboratory, Bar Harbor, ME). Mice carrying the targeted allele and the \( Cre \) transgene were backcrossed to \( \text{C57BL/6J} \) mice to generate mice heterozygous for the deletion of the floxed first exon (\( Bambi^{\text{del}+/} \)) but without the \( Cre \) transgene. The \( Bambi^{\text{del}+/} \) heterozygous males and females were intercrossed for generation and analysis of \( Bambi^{\text{del}+/} \) homozygous mutant mice.

All animal protocols were approved by the University of Rochester Committee on Animal Resources in accordance with National Institute of Health guidelines.

**Genotyping**

For genotyping, DNA was isolated from either tail or embryonic yolk sac samples. To distinguish wildtype from the \( Bambi^{\text{flo}x} \) allele, sense primer F2 (\( \text{GGGATCCGGGTCTTGGAGAC} \)) and antisense primer R2 (\( \text{TCGGAGTTTGCTACGAGACC} \)) were used for PCR, amplifying a 215 bp product from the wildtype allele and a 336 bp product from the \( Bambi^{\text{flo}x} \) allele. The \( Bambi^{\text{del}} \) allele was detected by PCR using the sense primer F2 and antisense primer R4 (\( \text{TTCGGAATAGGAACTTCGTCG} \)), which yielded a 316 bp product. The program used for PCR genotyping reactions is as follows: 1 cycle of 94°C for 4 minutes; followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 62°C, and 40 seconds at 72°C. PCR products were resolved on 1.5% agarose gels.

**RT-PCR**

Total RNAs were extracted from E10.5 whole embryos using Trizol reagents (Invitrogen), treated with RNase-free DNase I (Promega) to remove any
residual genomic DNA, and purified further using the RNAeasy kit (QIAGEN). 3 μg of each purified total RNA sample was reverse-transcribed to generated first-strand cDNAs using the SuperScript II reverse transcription kit (Invitrogen). The following primers were used for RT-PCR analysis of Bambi: RF1, GTGTAGGCGTTGCTCTCTGT; RR1, CTTTGGTGAGCAGCACAGCC; and RR2, CGTCATGCAGTCTCGATAA. The following PCR program was used for the PCR reactions: 1 cycle of 94°C for 4 min; followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 40 seconds at 72°C.

Skeletal Preparation, Growth Curve and Statistical Analysis

Skeletal preparation was performed as described previously (Martin et al., 1995). For growth curve analysis, the body weights of mice were measured every 10 days for a period of 60 days starting from the 10th day after birth. Male and female growth curves were generated separately. For statistical analysis, independent Student’s t test was used, and a P value less than 0.05 was regarded as significant. Chi-square test was used for determining whether distribution of genotypes of progeny from Bambi del/+ heterozygous intercrosses reflected the expected Mendelian ratio, and P value less than 0.05 was considered significant.

Acknowledgements

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Appendix 2.1 Generation of $Bambi^{\text{flo}}$ and $Bambi^{\text{del}}$ mice. (A) Schematic representation of the $Bambi$ genomic locus, the gene targeting construct and the targeted ($Bambi^{\text{flo}}$) allele. $LoxP$ sequences are indicated by black triangles. D marks the deletion probe; DTA, diphtheria toxin A expression cassette; E1, exon 1; E2, exon 2; E3, exon 3; Neo, Frt-flanked PGK-Neo cassette. The restriction enzyme recognition sites are: B, Bgl I; E, EcoRI; P, PmlI; S, SacI. (P) marks PmlI site destroyed during subcloning. (B) Southern hybridization analysis of BglII-digested genomic DNA from representative G418-resistant ES clones using the 5' external probe. The upper band (11 Kb) represents the targeted allele and the lower band (9.6 Kb) represents the wildtype allele. (C) Southern hybridization analysis of EcoRI-digested tail genomic DNA from $Bambi^{+/+}$, $Bambi^{\text{del}+}$, and $Bambi^{\text{del}+/\text{del}}$ mice using the deletion probe. A 7.7 Kb band was only detected in the wildtype and heterozygous mouse DNA samples, but not in DNA from the homozygous $Bambi^{\text{del}+/\text{del}}$ mice. (D) PCR analysis of tail DNA of $Bambi^{+/+}$, $Bambi^{\text{del}+}$, and $Bambi^{\text{del}+/\text{del}}$ mice using primers F2, R2 and R4. The upper fragment (215 bp) represents the wildtype allele, and the lower band (316 bp) represents the $Bambi^{\text{del}}$ allele.
Appendix 2.1

A  wildtype Bamhi locus

![Diagram of wildtype Bamhi locus]

B

![Diagram showing genotypes and banding patterns]

C

![Image showing deletion probe results]

D

![Genotype analysis results]

11 kb 9.6 kb

5' external probe

deletion probe

F2/R2

F2/R4
Appendix 2.2 RT-PCR analysis of RNAs isolated from E10.5 wildtype and \textit{Bambi}^{del/de} embryos. (A) The approximate positions of primers used in (b) and (c) are indicated by arrows. E1, exon 1; E2, exon 2. (B) A 329 bp fragment corresponding to the entire exon 1 was only amplified from wildtype but not \textit{Bambi}^{del/de} embryo RNA samples. As internal control, RT-PCR product corresponding to the \textit{Hprt} mRNA was amplified from both wildtype and \textit{Bambi}^{del/de} embryo RNA samples. RT, with reverse transcription; -RT, without reverse transcription. (C) A 586 bp fragment containing Exon 1 and Exon 2 sequences was amplified from wildtype but not mutant RNA samples using primers RF1 and RR2 as indicated in (a). RT-PCR amplification of a fragment from \textit{Hprt} mRNA was used as internal control.
Appendix 2.3 Growth curves of wild type mice, \(Bambi^{del/+}\) heterozygous, and \(Bambi^{del/del}\) homozygous mice. \(A\) Growth curves of female wildtype (\(n=7\)), \(Bambi^{del/+}\) heterozygous (\(n=12\)), and \(Bambi^{del/del}\) homozygous (\(n=7\)) mice. From 30 days onwards, the body weights of the \(Bambi^{del/del}\) female mice were 9%-10% lower than those of wildtype mice (\(P<0.05\)). \(B\) Growth curve shows the average body weights of male wildtype (\(n=9\)), \(Bambi^{del/+}\) heterozygous (\(n=18\)), and \(Bambi^{del/del}\) homozygous (\(n=10\)) mice taken on the days indicated for a period of 60 days. At all the stages examined, body weights of \(Bambi^{del/del}\) male mice were not significantly different from those of wildtype and heterozygous male mice.
Appendix 2.3

A

Female

Body weight (g)

Age (days after birth)

B

Male

Body weight (g)

Age (days after birth)
Appendix 2.4 *Bambi* is not essential for limb and craniofacial development. Skeletal preparations of the forelimb (A, B), hindlimb (C, D), and skull (E, F) were compared between wildtype (A, C, E) and *Bambi*<sup>del/del</sup> homozygous (B, D, F) newborn littermates. No significant difference was found. (E) and (F) show the ventral view of the skulls.