Published in final edited form as:
Dev Biol. 2011 January 15; 349(2): 451–461. doi:10.1016/j.ydbio.2010.10.023.

BmprIa is required in mesenchymal tissue and has limited redundant function with *BmprIb* in tooth and palate development

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Abstract

The BMP signaling plays a pivotal role in the development of craniofacial organs, including the tooth and palate. *BmprIa* and *BmprIb* encode two type I BMP receptors that are primarily responsible for BMP signaling transduction. We investigated mesenchymal tissue-specific requirement of *BmprIa* and its functional redundancy with *BmprIb* during the development of mouse tooth and palate. *BmprIa* and *BmprIb* exhibit partially overlapping and distinct expression patterns in the developing tooth and palatal shelf. Neural crest specific inactivation of *BmprIa* leads to formation of an unusual type of anterior clefting of the secondary palate, an arrest of tooth development at the bud/early cap stages, and severe hypoplasia of the mandible. Defective tooth and palate development is accompanied by the down-regulation of BMP responsive genes and reduced cell proliferation levels in the palatal and dental mesenchyme. To determine if *BmprIb* could substitute for *BmprIa* during tooth and palate development, we expressed a constitutively active form of *BmprIb* (*caBmprIb*) in the neural crest cells in which *BmprIa* was simultaneously inactivated. We found that substitution of *BmprIa* by *caBmprIb* in neural rest cells rescues the development of molars and maxillary incisor, but the rescued teeth exhibit a delayed odontoblast and ameloblast differentiation. In contrast, *caBmprIb* fails to rescue the palatal and mandibular defects including the lack of lower incisors. Our results demonstrate an essential role for *BmprIa* in the mesenchymal component and a limited functional redundancy between *BmprIa* and *BmprIb* in a tissue specific manner during tooth and palate development.

Keywords

BMP signaling; *BmprIA*; *BmprIB*; tooth development; palatogenesis

Introduction

The family of bone morphogenetic proteins (BMPs) comprises over 20 multi-functional cytokines that belong to the TFG-β superfamily. BMPs play many important roles in embryonic development, postnatal growth, and regeneration. BMP signaling is transduced

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into cell via heteromeric receptor complexes of type I and type II transmembrane serine-threonine kinase receptors. Binding of BMP ligands to a heteromeric receptor complex induces phosphorylation of the type I receptor in the GS domain by the type II receptor. The activated type I receptor further phosphorylates in the cytoplasm the receptor-regulated Smads, primarily Smad-1, −5, and −8, which bind to common Smad (Smad4) and enter the nucleus where the Smad complex interacts with other transcription factors to regulate gene expression (Sieber et al., 2009). Besides this canonical BMP signaling pathway, BMPs can also activate Smad-independent mitogen-activated protein kinase (MAPK) signaling pathways. In addition to the two originally identified type I BMP receptors (BMPR-IA and BMPR-IB), Activin receptor type IA (ActRIa or Alk2) also binds to BMP ligands and transduces BMP signaling (Kawabata et al., 1998; Nohe et al., 2004). While mice deficient for *BmprIb* are viable with appendicular skeleton defects (Baur et al., 2000; Yi et al., 2000), mutations in either *BmprIa* or *Alk2* lead to embryonic lethality during early gestation stage (Mishina et al., 1995; 1999; Gu et al., 1999), suggesting distinct and potentially redundant roles between these receptors during embryonic development.

The development of mammalian tooth and palate is governed by interactions between pharyngeal ectoderm and cranial neural crest-derived mesenchyme. Among many regulators, BMP signaling plays a pivotal role in mediating the epithelial-mesenchymal interaction during the development of these craniofacial organs (Nie et al., 2006). During palatogenesis, several *Bmp* genes, including *Bmp2*, *Bmp3*, *Bmp4*, *Bmp5*, and *Bmp7*, exhibit dynamic and differential expression patterns along the anterior-posterior (A-P) axis of the developing palatal shelves (Lu et al., 2000; Zhang et al., 2002; Hilliard et al., 2005; Nie, 2005; Levi et al., 2006). In the anterior portion of developing palatal shelves, *Bmp4*, *Msx1*, *Shh*, and *Bmp2* form a genetic hierarchy to regulate cell proliferation (Zhang et al., 2002); and BMP signaling is also required for the expression of *Shox2* whose inactivation causes formation of a rare type of anterior clefting of the secondary palate in mice (Yu et al., 2005; Gu et al., 2008). In the posterior palate, a balanced BMP activity is essential for the maintenance of palatal epithelial integrity (Xiong et al., 2009; He et al., 2010). Numerous studies have implicated BMP signaling in many aspects of tooth development, from determination of tooth forming sites and tooth types (Neubüser et al., 1997; Tucker et al., 1998), progression from the bud stage to the cap stage and formation of the enamel knot (Chen et al., 1996; Jernvall et al., 1998; Zhang et al., 2000; Zhao et al., 2000), to tooth root formation and tooth eruption (Yamashiro et al., 2003; Hosoya et al., 2008, Huang et al., 2010, Yao et al., 2010). Among those *Bmp* genes that are expressed in developing tooth, *Bmp4* was suggested to play a central role as a morphogen during early tooth morphogenesis (Vainio et al., 1993; Thesleff and Mikkola, 2002).

The crucial role of BMP signaling in tooth and palate development was further revealed by studies that used mice carrying conditionally inactivated type I BMP receptors. In contrast to the lack of any visible craniofacial defect in *BmprIb* null mice (Baur et al., 2000; Yi et al., 2000), tissue-specific inactivation of *BmprIa* in the palatal and dental epithelium results in a cleft palate formation and an arrest of molar development at the bud/cap stages, and causes various incisor phenotypes depending on different *Cre* transgenic mouse lines that were used (Andl et al., 2004; Liu et al., 2005). Furthermore, tissue specific deletion of *Alk2* in the neural crest lineage leads to multiple craniofacial defects including cleft palate and hypoplastic mandible; however, a tooth phenotype was not reported in this study (Dudas et al., 2004). Despite that these receptors are highly homologous and can activate both Smad and Smad-independent pathways, and that they may function redundantly to certain extent, each of them mediates specific and non-redundant signaling during embryogenesis (Sieber et al., 2009).

Despite the essential role for *BmprIa* in the epithelial component for tooth and palate development, the requirement of *BmprIa* in the mesenchymal component remains unknown. This is likely due to the unavailability of a dental and palatal mesnechyme specific Cre deletor mouse line and the fact that mice bearing deletion of *BmprIa* in the neural crest cell by *Wnt1-Cre* die around E12.5 when tooth and palate development just starts (Stottmann et al., 2004). It was recently demonstrated that embryonic lethality in mice lacking *BmprIa* in the neural crest lineage is due to norepinephrine depletion instead of cardiac defects (Morikawa et al., 2009). Administration of the β-adrenergic receptor agonist isoproterenol prevents embryonic lethality, and *Wnt1Cre;BmprIa*$^{F/-}$ embryos survive to term, making it possible to examine the role of *BmprIa* throughout development of neural crest-derived tissues. Taking this advantage, we investigated the role of *BmprIa* in the mesenchymal tissue and further tested if BMPR-IB mediated signaling is able to substitute for the loss of *BmprIa* in tooth and palate development.

Materials and methods

Animals and embryo collection

The generation and genotyping of transgenic and gene-targeted animals, including *Wnt1Cre*, *BmprIa*$^{+/-}$, *BmprIa*$^{F/F}$, have been described previously (Mishina et al., 1995; Danielian et al., 1998). The *pMes-caBmprIb* conditional transgenic line contains a constitutively active form (with Gln203 to Asp change) of BMPR-IB (named *caBmprIb*), which is linked to the 5' end of the *IRES-Egfp* sequence and to the 3' end of the *LoxP* flanked *STOP* cassette, under the control of the chick β-actin promoter, as described previously (He et al., 2010). Embryos containing inactivated *BmprIa* in their neural crest cells (*Wnt1Cre;BmprIa*$^{F/-}$) were obtained by crossing *Wnt1Cre;BmprIa*$^{+/-}$ mice with *BmprIa*$^{F/F}$ line. To obtain embryos carrying *Wnt1Cre;BmprIa*$^{F/-}$alleles and a *pMes-caBmprIb* transgenic allele, *Wnt1Cre;BmprIa*$^{+/-}$ mice were crossed with *BmprIa*$^{F/+}$;*pMes-caBmprIb* mice. Mice containing such compounded alleles are referred as *Wnt1Cre;BmprIa*$^{F/-}$;*caIb*.

Embryos with *BmprIa* deficiency in their neural crest cells (*Wnt1Cre;BmprIa*$^{F/-}$) die at E12.5 (Stottmann et al., 2004), due to norepinephrine depletion (Morikawa et al., 2009). Administration of the β-adrenergic receptor agonist isoproterenol prevents embryonic lethality, allowing *Wnt1Cre;BmprIa*$^{F/-}$ embryos to survive to term (Morikawa et al., 2009). This was done by supplementing the drinking water of dams with 200μg/ml isoproterenol and 2.5 mg/ml ascorbic acid from 7.5 post-coitum (dpc), as described previously (Morikawa and Cserjesi, 2008). To be consistent, all embryos used throughout this study, including the wild type controls, *Wnt1Cre;BmprIa*$^{F/-}$, and *Wnt1Cre;BmprIa*$^{F/-}$;*caIb*, were obtained from dams fed with isoproterenol and ascorbic acid.

Embryos were collected from timed-mate pregnant females in ice-cold PBS. Embryonic head samples were dissected and fixed individually in 4% paraformaldehyde (PFA) overnight at 4°C, and processed for paraffin section for histological and in situ hybridization analyses or for frozen section for immunostaining. A tail sample form each embryo was used for PCR-based genotyping (primers information available upon request).

Mouse kidney capsule grafting

For subrenal culture in mice, E13.5 embryos were harvested from mating of *Wnt1Cre;BmprIa*$^{+/-}$ mice with *BmprIa*$^{F/+}$;*pMes-caBmprIb* mice, and placed in PBS on ice. Embryos carrying *Wnt1Cre;BmprIa*$^{F/-}$ or *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* compounded alleles exhibited craniofacial abnormalities, and could be easily distinguished from embryos with other genotypes. *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* embryo could be further distinguished from *Wnt1Cre;BmprIa*$^{F/-}$ mice by the expression of *Egfp* in craniofacial region. Tail samples

from targeted embryos were subjected to genotyping. E13.5 embryos collected from crosses of wild type mice were used as positive control. Mandibular molar germs were isolated from *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* embryos and wild type controls, and were subjected to subrenal culture. Adult CD-1 male mice were used as hosts for subrenal culture. Mice were anesthetized by intraperitoneal injection of Newbutal sodium solution at a dose of 0.01 mg/g of body weight. Kidney capsule grafting was performed following the procedure described in details previously (Zhang et al., 2003). Samples were harvested 2 weeks after subrenal culture.

Histology, in situ hybridization, and immunostaining

For histological and in situ hybridization analyses, paraffin sections were made at 10-μm and subjected for standard Hematoxylin/Eosin staining and non-radioactive in situ hybridization, as described previously (St. Amand et al., 2000). Frozen sections, made at 10-μm, were applied for immunohistochemical staining, as described previously (Xiong et al., 2009). Polyclonal antibodies against p-Smad1/5/8 were purchased from Cell Signaling (cat. #: 9511) and used at the concentration of 1:200. Green fluorescent-conjugated secondary antibodies were obtained from Invitrogen.

Cell proliferation and TUNEL assays

BrdU labeling was performed to determine cell proliferation rate and TUNEL assay was applied to detect apoptotic cells, as described previously (Zhang et al., 2002; Alappat et al., 2005). These were done by using BrdU Labeling and Detection Kit and In Situ Cell Death Detection Kit, both from Roche Diagnostics Corporation. Cell proliferation rates were measured by counting BrdU-positive cells and total cells in defined arbitrary areas in the palatal and dental mesenchyme, respectively. The outcome was presented as percentage of labeled cells among total cells in the defined arbitrary areas. Three continuous sections from each of three individual samples of wild type and mutants were counted, respectively. The sums from both genotypes were subjected to Student's *t*-test to determine the significance of difference. Three independent BrdU labeling experiment with 6 samples of each genotype and four independent TUNEL assay with 4 samples of each genotype were performed.

Results

Expression of BmprIa and BmprIb in the developing tooth and palate

Numerous previous studies have implicated BMP signaling in tooth and palate development. However, detailed expression patterns of *BmprIa* and *BmprIb* during tooth and palate development have not been documented. We therefore began with examination of *BmprIa* expression in the developing tooth at several critical developmental stages, including the bud, the cap, and the bell stages, and in the developing secondary palatal shelves. *BmprIb* expression was also examined in parallel. In the developing tooth at the E13.5 bud stage, both *BmprIa* and *BmprIb* exhibit overlapped but distinct expression patterns (Fig. 1A-F). In the upper (maxillary) incisors, *BmprIa* is expressed in both dental epithelium and mesenchyme (Fig. 1A), but in the lower (mandibular) incisors, *BmprIa* expression is restricted in the dental epithelium (Fig. 1C). *BmprIb* is only expressed in the dental epithelium of both upper and lower incisor germs at this stage (Fig. 1B, D). In the molars, a relatively lower level of *BmprIa* expression was observed in the dental epithelium and mesenchyme, with a scattered pattern in the mesenchyme (Fig. 1E). *BmprIb* is also expressed in the epithelium and mesenchyme, with a much stronger level in the upper molar mesenchyme as compared to its expression in the lower molar (Fig. 1F). At the E14.5 cap stage, *BmprIa* remains its expression in both epithelial and mesenchymal compartments of upper incisors and molars, and in the epithelium of lower incisor (Fig. 1G, H, and Supplemental Figure 1). In contrast, *BmprIb* is restrictedly expressed in the dental

epithelium of all types of tooth germ, with a high level in the future inner enamel organ of molars (Fig. 1I, J, and Supplemental Figure 1). At the E16.5 bell stage, *BmprIa* expression is mainly restricted in the epithelial component of upper and lower incisors, but an above background expression was also observed in the dental papilla of upper incisors. We also detected *BmprIa* expression in the inner enamel epithelium as well as odontoblasts in molars (Supplemental Figure 1). At this stage, *BmprIb* expression becomes weaker and is completely restricted in the dental epithelium of incisors and molars (Supplemental Figure 1).

In the developing palatal shelves at E12.5 and E13.5, *BmprIa* is expressed in the epithelium and mesenchyme of the anterior palate, while in the posterior palate, *BmprIa* expression is mainly restricted in the palatal epithelium, with an above background level in the mesenchyme (Fig. 1K, M, and Supplemental Figure 1). At the same stages, *BmprIb* is only expressed in the anterior portion of the palatal shelf in both the epithelium and mesenchyme, but its expression is completely absent in the posterior palatal mesenchyme (Fig. 1L, N, and Supplemental Figure 1).

Neural crest inactivation of BmprIa causes formation of an unusual type of cleft palate

To generate embryos that were deficient for *BmprIa* in the neural crest cells, we crossed *Wnt1Cre;BmprIa*$^{+/-}$ mice to *BmprIa*$^{F/F}$ mice. In order to obtain *Wnt1Cre;BmprIa*$^{F/-}$ embryos at term, dams were fed with isoproterenol and ascorbic acid in drinking water to prevent embryonic lethality at mid-gestation stage (Morikawa and Cserjesi, 2008; Morikawa et al., 2009). Wild type mice or mice with other genotypes except *Wnt1Cre;BmprIa*$^{F/-}$ mice were indistinguishable from with type mice from dams fed with normal drinking water. Gross examination of *Wnt1Cre;BmprIa*$^{F/-}$ mice identified dramatic craniofacial defects, including an extremely shortened mandible, hypoplastic maxillary prominence, and an unusual type of anterior clefting of the secondary palate (Fig. 2A-D). The shortened mandible with hypoplastic distal region exposed the tongue to external view (Fig. 2B). Histological analysis of E13.5 mutant embryo revealed shortened but horizontally positioned palatal shelves in the anterior region (Fig. 1F). However, the posterior palatal shelves in the mutant assumed a vertical position as the wild type control and appeared similar to the control in size and shape (Fig. 2G, H). At E14.5 when the palatal shelves in wild type control have elevated to the position above the tongue and met at the midline, the palatal shelves in the mutant were also positioned above the tongue (Fig. 2I-L). While the posterior palatal shelves in mutants made contact at the midline, the anterior palatal shelves appeared too short to make contact. This unique type of cleft palate defect was also observed in *Shox2* mutant mice (Yu et al., 2005; Gu et al., 2008). In addition, a deformed tongue was also seen in mutants. Thus deletion of *BmprIa* in the neural crest-derived palatal mesenchyme causes a defective growth in the anterior palatal shelves, and ultimately leads to the formation of anterior cleft of the secondary palate, consistent with a restricted expression of *BmprIa* in the anterior palatal mesenchyme. *BmprIa* is required not only in the epithelium (Andl et al., 2004; Liu et al., 2005), but also in the mesenchyme for normal palate development.

Deletion of BmprIa in neural crest-derived mesenchyme arrests tooth development

Histological examination failed to identify any definite tooth structure in *Wnt1Cre;BmprIa* newborn mice (data not shown), suggesting a defective tooth development at early phase. Analysis of mutant embryos at E13.5 revealed formation of molars at the bud stage (Fig. 3F), However, as compared to the wild type controls (Fig. 3E), the molar buds in the mutant appeared a little bit delay in development with less condensed dental mesenchymal cells surrounding the epithelial bud. The incisor phenotype in mutants appeared much dramatic (Fig. 3A-D). The upper incisors formed a single, medially located tooth bud that was arrested at the early bud stage (Fig. 3B), while the lower incisor buds never formed (Fig.

3D), likely due to a substantially shortened mandible (Fig. 2B). At the E16.5 bell stage, in mutants, we could not find any residual structure of upper incisors (Fig. 3H), but observe residual molar germs (Fig. 3L). Among 8 samples examined, all the lower molars were arrested at the bud stage, but 6 residual upper molars appeared to be arrested at the early cap stage (Fig. 3L). Taking together, these observations indicate an absolute requirement of *BmprIa* in the mesenchyme for tooth development beyond the bud stage or the early cap stage.

Reduced cell proliferation in the palatal and tooth mesenchyme of Wnt1Cre;BmprIa$^{F/-}$ embryos

To ensure that deletion of *BmprIa* in the neural crest cells disrupts BMP signaling in the palatal and dental mesenchyme, we examined the expression of phosphorylated Smad1/5/8 (pSmad1/5/8) by immunohistochemical staining. In wild type controls at E13.5, we detected abundant pSmad1/5/8 positive cells in the anterior palatal mesenchyme primarily on the future nasal side, and in the condensed dental mesenchyme as well as the dental epithelium (Fig. 4A, C). In contrast, as we expected, the number of pSmad1/5/8 positive cells was significantly reduced in the palatal and dental mesenchyme of *Wnt1Cre;BmprIa*$^{F/-}$ mutants, although the staining in the epithelial compartment remained unchanged (Fig. 4B, D).

To investigate cellular defects that may contribute to a cleft palate formation and to the arrest of tooth development in *Wnt1Cre;BmprIa*$^{F/-}$ mutants, we carried out BrdU labeling and TUNEL assays to examine cell proliferation rate and apoptosis. In the developing palatal shelves of mutants at E13.5, we found a significantly reduced level of cell proliferation in the mesenchyme of the anterior palate, as compared to that in the controls (Fig. 4E, F, I). However, cell proliferation rates in the palatal epithelium and the posterior palatal mesenchyme remained unchanged (data not shown). Similarly, in the developing molar of mutants at this stage, a dramatic reduction in cell proliferation rate was also found in the dental mesenchyme, but not in the dental epithelium (Fig. 4G, H, I; and data not shown). The reduction in cell proliferation rate correlates with a reduced level of pSmad1/5/8 in mutants, indicating a positive role for BMP/Smad signaling in the regulation of cell proliferation. On the other hand, TUNEL assays did not reveal enhanced/ectopic cell apoptosis in the tooth germs and palatal shelves of mutants at this stage (data not shown). Thus this reduced cell proliferation rate in the mesenchymal compartment represents one defective cellular mechanism contributing to a cleft palate formation and an arrest of tooth development in *Wnt1Cre;BmprIa*$^{F/-}$ mutants.

Altered gene expression in the developing palate and tooth of Wnt1Cre;BmprIa$^{F/-}$ mice

Mutations in several genes that are expressed in the mesenchymal compartment of developing tooth and palate, including *Msx1* and *Pax9*, cause cleft palate defect as well as arrested tooth development at the bud stage (Satokata and Maas, 1994; Peters et al., 1998). *Bmp4* is expressed and forms a positive regulatory loop with *Msx1* in the anterior palatal mesenchyme and the dental mesenchyme (Chen et al., 1996; Peters et al., 1998; Zhang et al., 2000; Zhang et al., 2002). We therefore set to examine the expression of these genes in the developing palate and tooth of *Wnt1Cre;BmprIa*$^{F/-}$ mice. Since *Shox2* expression pattern overlaps with that of *BmprIa* in the anterior palatal mesenchyme and inactivation of *Shox2* leads to the formation of anterior cleft of the secondary palate in mice (Yu et al., 2005; Gu et al., 2008), we also examined *Shox2* expression. Our results revealed a significant down-regulation of *Bmp4*, *Msx1*, and *Pax9* expression in the anterior palatal mesenchyme of E13.5 *Wnt1Cre;BmprIa*$^{F/-}$ mice (Fig. 5). *Shox2* expression was also reduced in mutants (Fig. 5). Similarly, in the developing tooth germ of mutants, the expression of *Bmp4*, *Msx1*, and *Pax9* was significantly down-regulated in the mesenchyme at the bud stage (Fig. 6). However, residual expression for all these genes was present in the mutant dental mesenchyme.

Interestingly, the upper molars in mutants appeared to retain a relatively higher level of *Msx1* expression in the mesenchyme as compared to the lower molars (Fig. 6B). This observation correlates with a stronger *BmprIb* expression in the upper molar mesenchyme (Fig. 1F) and an arrest of upper molar development at a relatively advanced stage (the early cap stage) in the majority of mutants, suggesting a partially functional redundancy between *BmprIa* and *BmprIb*. Nevertheless, our results indicate that BMPR-IA is a major mediator of BMP signaling in the regulation of *Msx1* and *Pax9* expression, which in turn is required for *Bmp4* expression in the palatal and dental mesenchyme (Chen et al., 1996; Peters et al., 1998; Zhang et al., 2000; 2002).

caBMPR-IB partially rescues tooth development but not cleft palate defect in Wnt1Cre;BmprIa$^{F/-}$mice

To determine if BMPR-IB-mediated BMP signaling can substitute for BMPR-IA-mediated signaling in regulating tooth and palate development, we created a conditional transgenic allele that expresses a constitutively active form of BMP receptor-IB (*caBmprIb*) upon crossing to a Cre mouse line. While activation of the *caBmprIb* allele in the epidermis by a *K14-Cre* transgenic allele causes severe ichthyosis skin disease (Yu et al., unpublished), expression of *caBmprIb* in the neural crest cells activated by *Wnt1-Cre* does not produce any visible phenotype (data not shown). *Wnt1Cre;caBmprIb* mice survived normally and were indistinguishable from their wild type littermates. In situ hybridization assay confirmed a wide spread expression of *BmprIb* in the cranial neural crest-derived mesenchyme, including the palate shelf and tooth germ at E13.5 (Supplemental Fig. 2). We compounded the *caBmprIb* transgenic allele onto the *Wnt1Cre;BmprIa*$^{F/-}$ background to generate mice lacking *BmprIa* but expressing *caBmprIb* in the neural crest cells (*Wnt1Cre;BmprIa*$^{F/-}$;*caIb*). Grossly, *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice exhibited similar craniofacial abnormalities indistinguishable from *Wnt1Cre;BmprIa*$^{F/-}$ mice, including hypoplastic mandible and anterior clefting of the secondary palate (data not shown). Most *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* embryos died at mid-gestation if the drinking water of dams did not contain isoproterenol and ascorbic acid, except one that was identified at the birth (data not shown). These results indicate that *caBmprIb* fails to substitute for the loss of *BmprIa* to regulate craniofacial and peripheral nervous system development.

Histological analyses confirmed the phenotype of anterior palate clefting in *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice (data not shown), consistent with failed restoration of *Bmp4*, *Msx1*, *Pax9* and *Shox2* expression in the anterior palatal mesenchyme (Fig. 5). In contrast, although we still could not find any residual structure of lower incisors, we observed tooth structures of upper incisors and both upper and lower molars in *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* newborn mice (Fig. 7A-D). While slight delay in development and smaller in size, the molars in *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice indeed formed and appeared comparable to the wild type controls in developmental stage and patterning (Fig. 7B). The rescued tooth development is consistent with an almost completely restored *Msx1* expression and partially rescued *Bmp4* and *Pax9* expression in molar mesenchyme at the bud stage (Fig. 6). However, while the upper incisors developed, they formed adjacently, appeared much smaller in size, and did not extend to the lateral sides of the nasal cavity (Fig. 7D). Closer examination of the upper incisors also revealed a mis-patterned incisor structure. In normal developing incisors, ameloblasts form only in the future labial side (Fig. 7C). However, in the upper incisors of *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice, ameloblasts were found around the dental pulp (Fig. 7D').

Wild type molars at birth (P0) have formed differentiated ameloblasts and odontoblasts, as evidenced by their polarized (elongated) cell structure, expression of *Amelogenin* and *Dspp*, and formation of dentin (Fig. 7E, G, I). However, while elongated amebloblasts were observed in the molars of *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice at the same age, neither elongated

odontoblasts nor dentin were found (Fig. 7F). This was also true for the upper incisors in *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice (Fig. 7C, D). The lack of odontoblast differentiation was consistent with the absent expression of the odontoblast differentiation marker *Dspp* (Fig. 7J). Interestingly, despite their elongated morphology, ameloblasts in the rescued molars and upper incisors of *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice expressed an extremely low level, if there is any, of the ameloblast differentiation marker *Amelogenin* (Fig. 7H, and data not shown).

To determine if the absent *Dspp* and *Amelogenin* expression in the rescued teeth of *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice at birth represents a delay in tooth development and differentiation, we grafted mandibular molars from E13.5 *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* embryos and wild type controls underneath mouse kidney capsule. After 2 weeks in subrenal culture, the wild type grafts produced well-organized enamel and dentin (Fig. 7K). In *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* grafts, we observed elongated odontoblasts and ameloblasts, and dentin deposit (Fig. 7L). In situ hybridization analyses showed *Amelogenin* expression in the ameloblasts (Fig. 7L'), and *Dspp* expression in the odontoblasts (Fig. 7L"). These results confirm a delayed differentiation of odontoblasts and ameloblasts in *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice. Thus *caBmprIb* can substitute for the loss of *BmprIa* in the cranial neural crest cells to regulate tooth development, but it is unable to fully replace *BmprIa* function as evidenced by delayed odontoblast and ameloblast differentiation.

Discussion

In this study, we show detailed expression patterns of *BmprIa* and *BmprIb* in the developing tooth and secondary palate. While previous studies have uncovered a crucial role for *BmprIa* in the epithelial component in tooth and secondary palate development (Andl et al., 2004; Liu et al., 2005), our results demonstrate an absolute requirement of *BmprIa* in the mesenchymal compartment for the development of tooth and secondary palate as well. Cremediated loss of mesenchymal *BmprIa* leads to an anterior clefting of the secondary palate and an arrest of tooth development at the bud/early cap stage, which is accompanied by reduced expression of BMP signaling downstream genes and defective cell proliferation. Our rescue studies further show a limited functional redundancy between *BmprIa* and *BmprIb* in a tissue specific manner in the development of craniofacial organs.

Mesenchymal BmprIa is required for anterior palate development

Increasing evidence has demonstrated heterogeneity, at both the cellular and molecular levels, in the developing secondary palatal shelf along the A-P axis (reviewed in Hilliard et al., 2005; Okana et al., 2006; Gritli-Linde, 2007). At the molecular level, a number of genes exhibit differential expression in the developing palatal shelf along the A-P axis (Zhang et al., 2002; Alappat et al., 2005; Yu et al., 2005; He et al., 2008; Liu et al., 2008; Xiong et al., 2009). At the cellular level, cells from the anterior palate and posterior palate respond differentially to the induction of growth factors in terms of cell proliferation and gene expression (Hilliard et al., 2005). For example, exogenously applied BMP proteins induce cell proliferation and *Msx1* expression in the anterior palatal mesenchyme but not in the posterior palatal mesenchyme (Zhang et al., 2002; Hilliard et al., 2005). This could be explained by the restricted expression of both *BmprIa* and *BmprIb* in the anterior palatal mesenchyme (Fig. 1). Consistently, tissue-specific inactivation of *BmprIa* in the palatal mesenchyme results in defective cell proliferation only in the anterior palatal mesenchyme, and consequently leads to an anterior clefting of the secondary palate. These results indicate that *BmprIa* is a major player in mediating BMP signaling in the regulation of cell proliferation in the palatal mesenchyme. While *BmprIb* exhibits an expression pattern overlapping with *BmprIa* in the anterior palate, it apparently cannot compensate for the loss of *BmprIa*. Therefore, BMPR-IA and BMPR-IB appear to mediate different downstream signaling pathways during palatogenesis. In the anterior portion of palatal shelf, *Msx1* and

Bmp4 function in an autoregulatory loop to control a genetic cascade to regulate cell proliferation (Zhang et al., 2002). In the absence of *BmprIa*, expression of *Msx1* and *Bmp4*, in addition to *Pax9* which is a key regulatory gene in palate development, is dramatically down-regulated in the palatal mesenchyme. These results indicate an essential role for BMPR-IA mediated BMP4 signaling in the regulation of *Msx1* and *Pax9* expression. BMPR-IA is also required in the anterior palatal mesenchyme for normal expression of *Shox2* whose null mutation leads to the formation of anterior cleft of the secondary palate as well (Yu et al., 2005; Gu et al., 2008). This result is consistent with our previous report that BMP signaling is required for *Shox2* expression in the anterior palatal mesenchyme. However, based on the facts that BMP signaling is not sufficient for induction of Shox2 expression and a bioinformatic search fails to identify a potential Smad binding site in the 10-kb upstream region of the mouse *Shox2* gene (unpublished data), *Shox2* is unlikely a direct target of BMP signaling. Since loss of either *Msx1* or *Shox2* causes reduced cell proliferation in the anterior palatal mesenchyme (Zhang et al., 2002; Yu et al., 2005), the defective cell proliferation in the anterior palatal mesenchyme of *Wnt1Cre;BmprIa* mice could be attributed to a compounded effect of reduced expression of *Msx1* and *Shox2*.

It was reported previously that deletion of *BmprIa* in the palatal epithelium causes a complete clefting of the secondary palate (Andl et al., 2004; Liu et al., 2005). In this study, inactivation of *BmprIa* in the palatal mesenchyme also results in a cleft palate formation, indicating the requirement of BMPR-IA mediated signaling in both epithelium and mesenchyme for normal palatogenesis. Anterior clefting of the secondary palate is a rare type of cleft palate in humans, and was thought to be caused by a non-genetic mechanism known as post-fusion rupture (Fara, 1971; Mitts et al., 1981; Schupbach, 1983). Together with our previous report that *Shox2*-deficient mice exhibit anterior clefting of the secondary palate (Yu et al., 2005), our results provide direct evidence for a genetic involvement in the formation of such type of palate clefting.

Mesenchymal BmprIa is required for self-maintenance of Bmp4 expression in early tooth development

Previous studies have established a fundamental role for BMP signaling in many steps of tooth development. Our studies further reveal an essential role for BMPR-IA-mediated BMP signaling in the dental mesenchyme during tooth development. Tissue specific deletion of *BmprIa* in the cranial neural crest derived dental mesenchyme results in an arrest of molar development at the bud/early cap stage, which is associated with significant reduction in BMP activity and defective cell proliferation. Mesenchymally expressed BMP4 is required for progression of molar development from the bud stage to the cap stage (Chen et al., 1996; Jernvall et al., 1998; Zhang et al., 2000; Zhao et al., 2000). *Msx1* and *Pax9* act synergistically in dental mesenchyme to regulate *Bmp4* expression which in turn maintains *Msx1* expression (Chen et al., 1996; Peters et al., 1998; Ogawa et al., 2006; Nakatomi et al., 2010). Our gene expression analyses show dramatically down-regulated expression of *Bmp4*, *Msx1* and *Pax9* in *Wnt1Cre;BmprIa*$^{F/-}$ molar mesenchyme, indicating that BMPR-IA also functions as a major player in the positive regulatory loop involving *Bmp4*, *Msx1* and *Pax9*. Given the fact that *BmprIb* expression overlaps with that of *BmprIa* in molar germs before the cap stage, the residual expression of *Bmp4*, *Msx1* and *Pax9* in the *Wnt1Cre;BmprIa*$^{F/-}$ dental mesenchyme at the bud stage is likely attributed to *BmprIb* expression. Obviously, the residual *Bmp4* expression in the mutant dental mesenchyme is below the threshold that is required for progression of molar development from the bud stage to the cap stage. Interestingly, epithelial deletion of *BmprIa* also causes an arrest of tooth development at the bud stage (Andl et al., 2004; Liu et al., 2005), suggesting that BMPR-IA most likely also mediates mesenchymally derived BMP4 signaling in the dental epithelium for tooth progression to the cap stage.

Deletion of *BmprIa* in the mesenchymal compartment leads to different defects in incisors. The mandibular incisor buds never formed in *Wnt1Cre;BmprIa*$^{F/-}$ embryos. This phenotype is very likely due to the formation of a hypoplastic mandible which had severely defective distal region (Fig. 2B). However, the maxillary incisor buds fuse at the midline to form a single incisor bud which is also arrested at the early bud stage. This phenotype is considered a little bit severer than that observed in molars in the mutants. This could be explained by the complete lack of *BmprIb* expression in the upper incisor mesenchyme at the bud stage (Fig. 1B), or could be attributed to different requirement for levels of BMP signaling for development of different types of tooth.

BmprIb has limited redundant function with BmprIa in the regulation of craniofacial development

Bmp2 and *Bmp4* are expressed in developing craniofacial organs including palate and tooth, and play important roles in the development of these two organs (Nie et al., 2006). Both BMPR-IA and BMPR-IB show high affinity binding to BMP2 and BMP4 (Sieber et al., 2009), and exhibit partially overlapping but distinct expression patterns in the developing tooth and palatal shelf (this study). Despite primary structural differences in their kinase domains, BMPR-IA and BMPR-IB were shown previously to transduce similar intracellular signals in cell cultures (Wozney et al., 1988; ten Dijke et al., 1994; Hoodless et al., 1996; Kretzschmar et al., 1997). Several in vivo studies using gain-of or loss-of-function approaches seemed to be in favor of a functional similarity between BMPR-IA and BMPR-IB in craniofacial development. For examples, mice lacking *BmprIb* form normal craniofacial structures, suggesting that *BmprIa* may take over *BmprIb*'s function during craniofacial development (Baur et al., 2000; Yi et al., 2000). Expression of constitutively active forms of BMPR-IA or BMPR-IB in chicken craniofacial region gave rise to similar phenotypes, indicating that these two receptors may play similar role in regulating bone and cartilage formation during craniofacial development (Ashique et al., 2002). On the other hand, many lines of evidence argue for distinct roles of these two BMP type I receptors in embryogenesis. *BmprIa* deficient mice die prior to gastrulation, revealing a fundamental role for *BmprIa* in early embryonic development (Mishina et al., 1995). Expression of mutated forms of BMPR-IA and BMPR-IB in the chicken limb buds produces distinct phenotypes, suggesting that different BMP type I receptors are dedicated to specific functions during organogenesis (Kawakami et al., 1996; Yokouchi et al., 1996; Zou et al., 1997) In this study, we show that, despite their overlapped expression patterns, *BmprIb* does not share redundant function with *BmprIa* in the anterior palatal mesenchyme to regulate palate development. BMPR-IA-mediated signaling is irreplaceable for normal palatogenesis, as evidenced by the rescue experiment in which *caBmprIb* fails to substitute for the loss of *BmprIa*. In contrast, *BmprIb* appears to have limited functional redundancy with *BmprIa* in dental mesenchyme for molar development. This assumption is based on the facts: 1) *BmprIb* shows a much stronger expression level in the upper molar mesenchyme at the E13.5 bud stage as compared to the lower molar (Fig. 1F); at the cap stage, *BmprIb* expression is restricted to the dental epithelium of both upper and lower molars (Fig. 1I, J); 2) in *Wnt1Cre;BmprIa*$^{F/-}$ mice, all lower molars were arrested at the bud stage, but the majority of upper molars developed to the early cap stage (Fig. 3L); 3) *Msx1* retains a relatively higher level of expression in the upper molar mesenchyme as compared to its expression in the lower molar mesenchyme in *Wnt1Cre;BmprIa*$^{F/-}$ embryo at the bud stage (Fig. 6B); 4) *caBmprIb* was able to partially rescue tooth development (including molars and upper incisor) in *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice (Fig. 7). The differential rescue of palate and tooth phenotypes in *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice suggests that these two type I receptors mediate different signaling pathways in a tissue specific manner. Taken together, our results reveal an absolute requirement of *BmprIa* in the mesenchymal component for normal

palatogenesis and odontogenesis. *BmprIb* shares limited redundant function with *BmprIa* in a tissue specific manner during craniofacial development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr Yuji Mishina for providing *BmprIa* mutant mice, and Dr. Yang Chai for sharing in situ probes. This work was supported by NIH grant R01DE14044 to Y.P.C.

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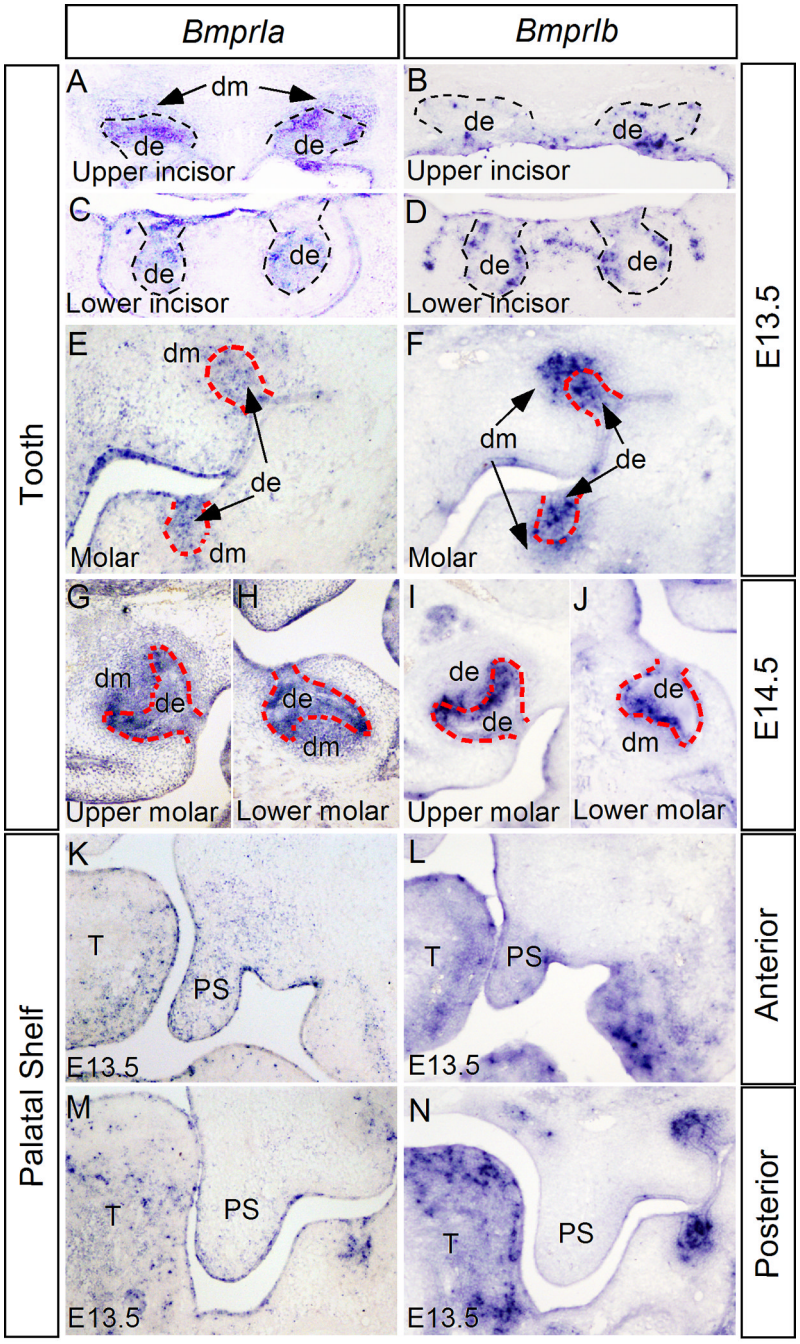

Figure 1.

Expression of *BmprIa* and *BmprIb* in developing tooth and palate. (A, E) At E13.5, *BmprIa* expression is detected in the epithelium and mesenchyme of upper incisor (A), but is restricted in the epithelium in lower incisor (E). (B, D) *BmprIb* is expressed in the epithelium of both upper (B) and lower (D) incisor at the same stage. (E, F) In the developing molars at E13.5, *BmprIa* (E) and *BmprIb* (F) is expressed in the epithelium and mesenchyme. Note that *BmprIb* exhibits a higher level of expression in the upper molar mesenchyme as compared to its expression in the lower molar (F). (G-J) At E14.5, *BmprIa* is continuously expressed in both the epithelial and mesenchymal compartments of upper (G) and lower (H) molars, but *BmprIb* expression is restricted to molar epithelium,

particularly in the inner enamel epithelium (I, J). (K-N) Expression of *BmprIa* (K, M) and *BmprIb* (L, N) in E13.5 palatal shelf. In the anterior palate, both *BmprIa* (K) and *BmprIb* (L) are expressed in the palatal epithelium and mesenchyme. In the posterior palate, *BmprIa* expression is observed in the epithelium and mesenchyme at relatively lower levels (M); however, no *BmprIb* expression is detected (N). Dashed lines demarcate dental epithelium. T, tongue; de, dental epithelium; dm, dental mesenchyme; PS, palatal shelf.

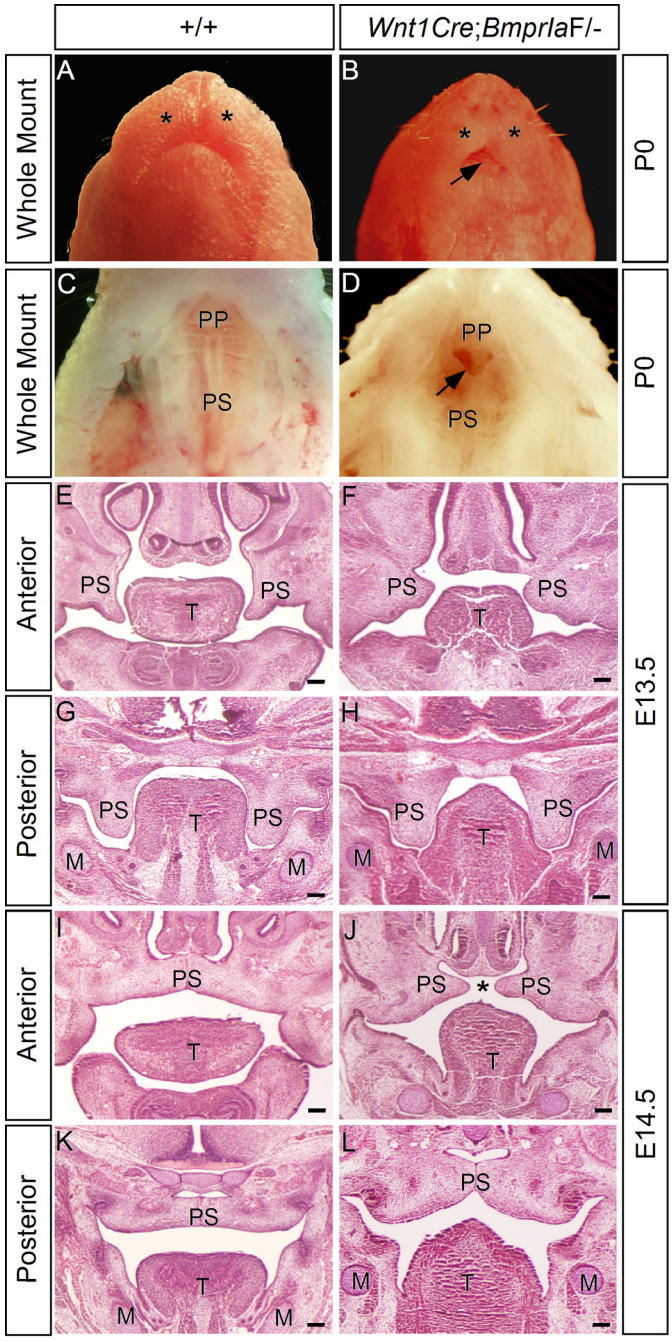

Figure 2.

Wnt1Cre;BmprIa$^{F/-}$ mice exhibit a unique anterior clefting of the secondary palate. (A-D) Gross examination of wild type (A, C) and *Wnt1Cre;BmprIa*$^{F/-}$ (B, D) mice at postnatal day 0 (P0) reveals craniofacial defects, including extremely shortened mandible and hypoplastic maxillary prominence (asterisks) (B), and an unusual type of anterior clefting of the secondary palate (arrow) (D). (E-H) coronal sections of E13.5 wild type control (E, G) and *Wnt1Cre;BmprIa*$^{F/-}$ (F, H) embryos reveal shortened and horizontally positioned palatal shelves in the anterior region of mutant (F). The posterior palatal shelves appear morphologically comparable in wild type control and mutant (G, H). (I-L) At E14.5 when the palatal shelves meet and fuse at the midline at the anterior (I) and posterior (K) domains

in wild type control, the mutant palatal shelves appear too short to meet in the anterior region (J), but do meet and fuse in the posterior region (L). M, Meckel's cartilage; T, tongue; PP, primary palate; PS, palatal shelf. Asterisks in (A) and (B) mark maxillary prominence. Asterisk in (J) marks palate clefting. Arrow in (B) points to exposed tongue, and in (D) points to the anterior clefting of the secondary palate. Scale bars represent 100 μm.

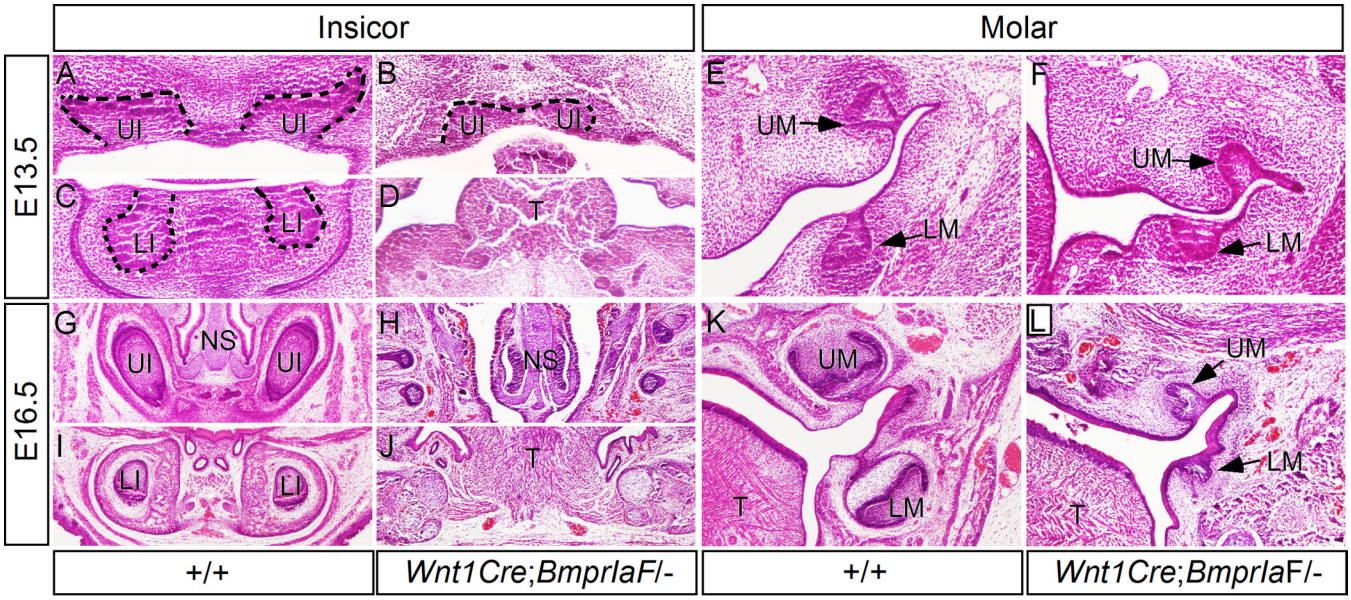

Figure 3.

Wnt1Cre;BmprIa$^{F/-}$ mice show defective tooth development. (A, C) coronal sections of an E13.5 wild type embryo show upper incisors (A) and lower incisors (C) at the bud stage. (B, D) Coronal sections of an E13.5 *Wnt1Cre;BmprIa*$^{F/-}$ embryo show a fused upper incisor at the early bud stage (B) and lack of lower incisors (D). (E, F) Coronal sections of an E13.5 wild type embryo (E) and an E13.5 mutant show molar teeth at the bud stage. Note that the mutant molars appear slightly delay in development and have less condensed dental mesenchyme (F). (G, I) Sections show upper incisors (G) and lower incisors (I) in an E16.5 wild type embryo. (H, J) Sections from an E16.5 mutant embryo show complete lack of upper incisor (H) and lower incisor (J). (K) Coronal section from an E16.5 wild type embryo shows molar teeth at the bell stage. (L) Coronal section from an E16.5 mutant shows residual molar tooth structure. Note that the upper molar is arrested at the early cap stage, while the lower molar is arrested at the bud stage. Dashed lines demarcate dental epithelium. T, tongue; LI, lower incisor; LM, lower molar; NS, nasal septum; UI, upper incisor; UM, upper molar.

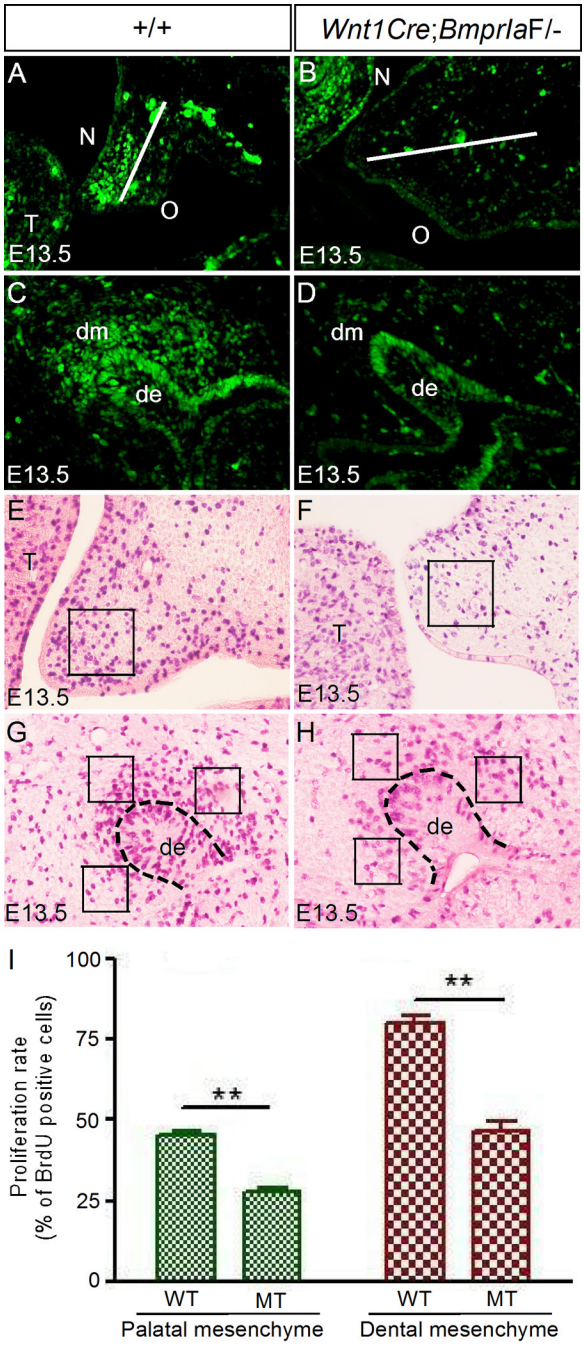

Figure 4.

Reduced levels of BMP/Smad signaling activity and cell proliferation in the palatal mesenchyme and dental mesenchyme of *Wnt1Cre;BmprIa*$^{F/-}$ embryo. (A, C) Immunostaining shows pSmad1/5/8 signals in the palatal shelf (A) and an upper molar (C) of an E13.5 wild type embryo. The white line in (A) bisects the palatal shelf into nasal and oral halves. Note that pSmad1/5/8 signals are mainly detected in the nasal half of the palatal shelf. (B, D) Immunostaining shows significantly reduced pSmad1/5/8 signals in the palatal mesenchyme (B) and dental mesenchyme (D) of an E13.5 mutant. (E-H) BrdU labeling shows reduced cell proliferation rates in the palatal mesenchyme (F) of anterior palate and molar mesenchyme (H) of E13.5 mutant embryos as compared to their wild type

counterparts (E, G). Squares indicate the areas where cells were counted. Dashed lines in (G, H) demarcate dental epithelium. (I) Comparison of BrdU-labeled cells in designated areas of palate and molar in controls and mutants. Standard deviation values were shown as error bars, and ** indicate $P < 0.001$. N designates nasal side of the palatal shelf, and O designates oral side of the palatal shelf. de, dental epithelium; dm, dental mesenchyme; MT, mutant; WT, wild-type.

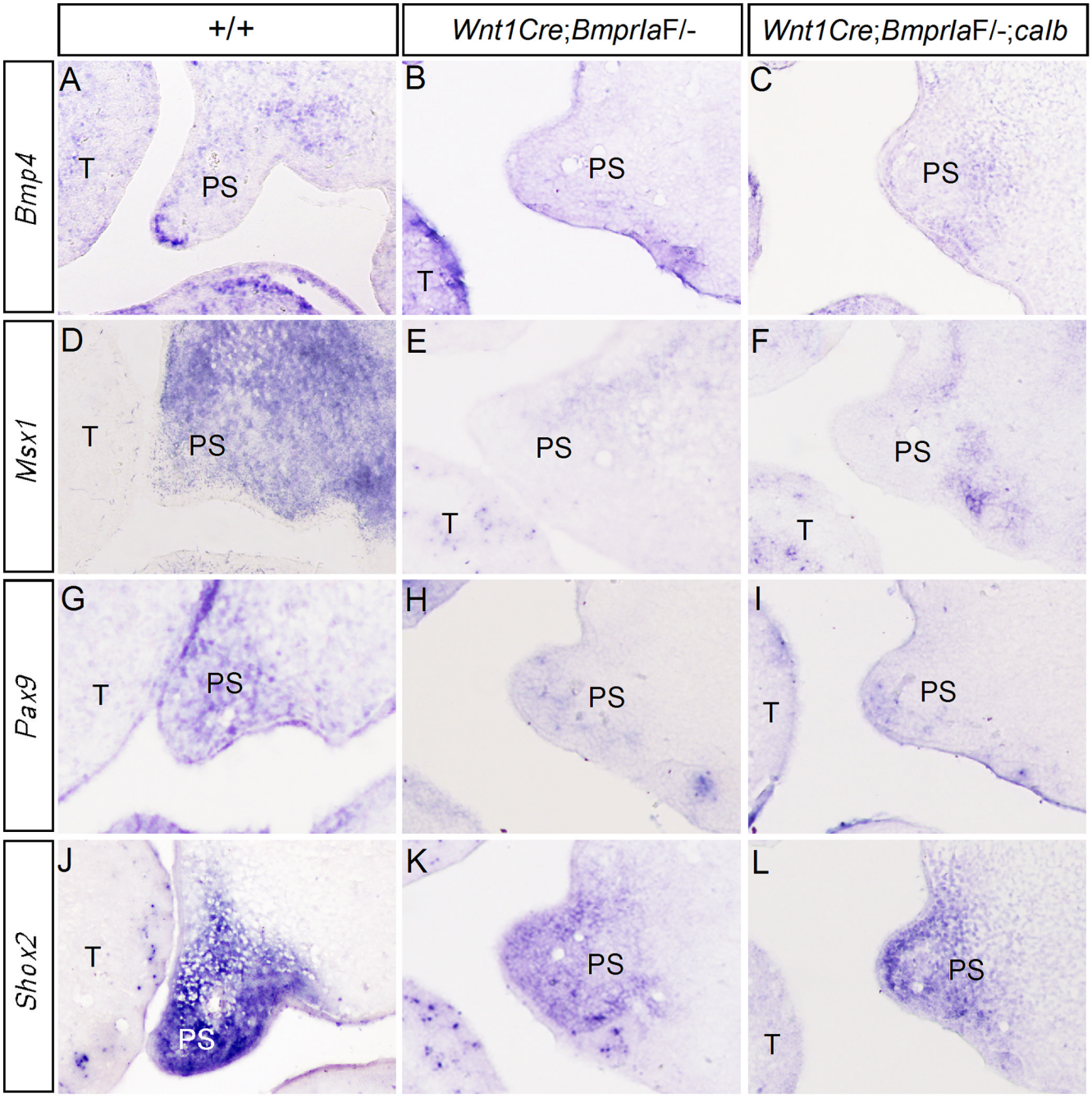

Figure 5.

Gene expression in the developing palatal shelves. (A, D, G, J) in situ hybridization shows expression of *Bmp4* (A), *Msx1* (D), *Pax9* (G) and *Shox2* (J) in the anterior palatal shelf of E13.5 wild type embryo. (B, E, H, K) Expression of *Bmp4* (B), *Msx1* (E), *Pax9* (H), and *Shox2* (K) is significantly reduced in the anterior palatal shelf of E13.5 *Wnt1Cre;BmprIa*$^{F/-}$ embryo. (C, F, I, L) Expression of *Bmp4* (C), *Msx1* (F), *Pax9* (I), and *Shox2* (L) remains down-regulated in the anterior palatal shelf of E13.5 *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* embryo. T, tongue; PS, palatal shelf.

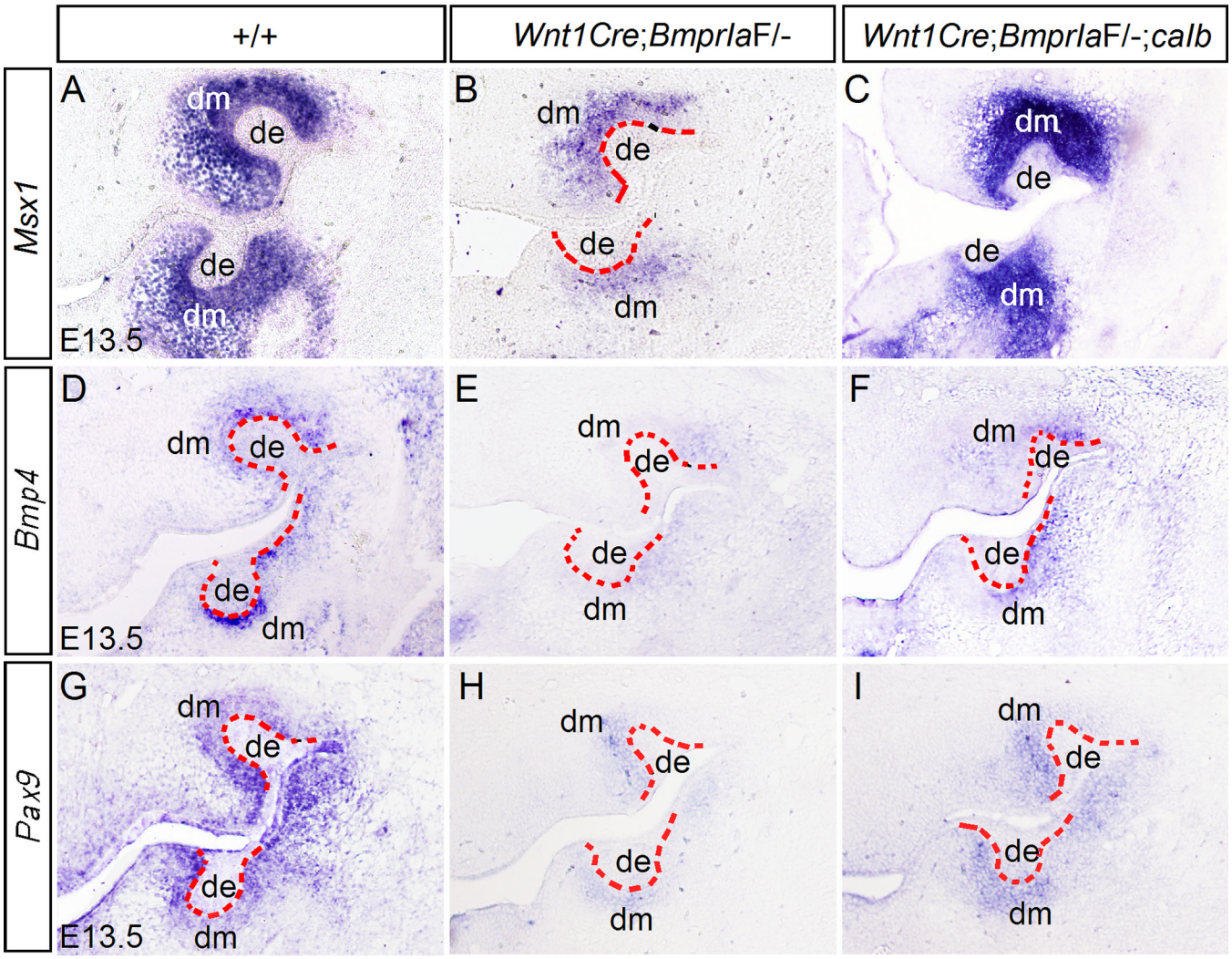

Figure 6.

Gene expression in developing molars. (A, D, G) In situ hybridization shows expression of *Msx1* (A), *Bmp4* (D), and *Pax9* (G) in the molar mesenchyme of E13.5 wild type embryo. (B, E, H) In situ hybridization shows reduced expression of *Msx1* (B), *Bmp4* (E), and *Pax9* (H) in the molar mesenchyme of E13.5 *Wnt1Cre;BmprIa*$^{F/-}$ embryo. (C, F, I) In situ hybridization shows a wild type level expression of *Msx1* (C), and a partially rescued *Bmp4* (F) and *Pax9* (I) expression in the molar mesenchyme of E13.5 *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* embryo. Dashed lines demarcate dental epithelium. de, dental epithelium; dm, dental mesenchyme.

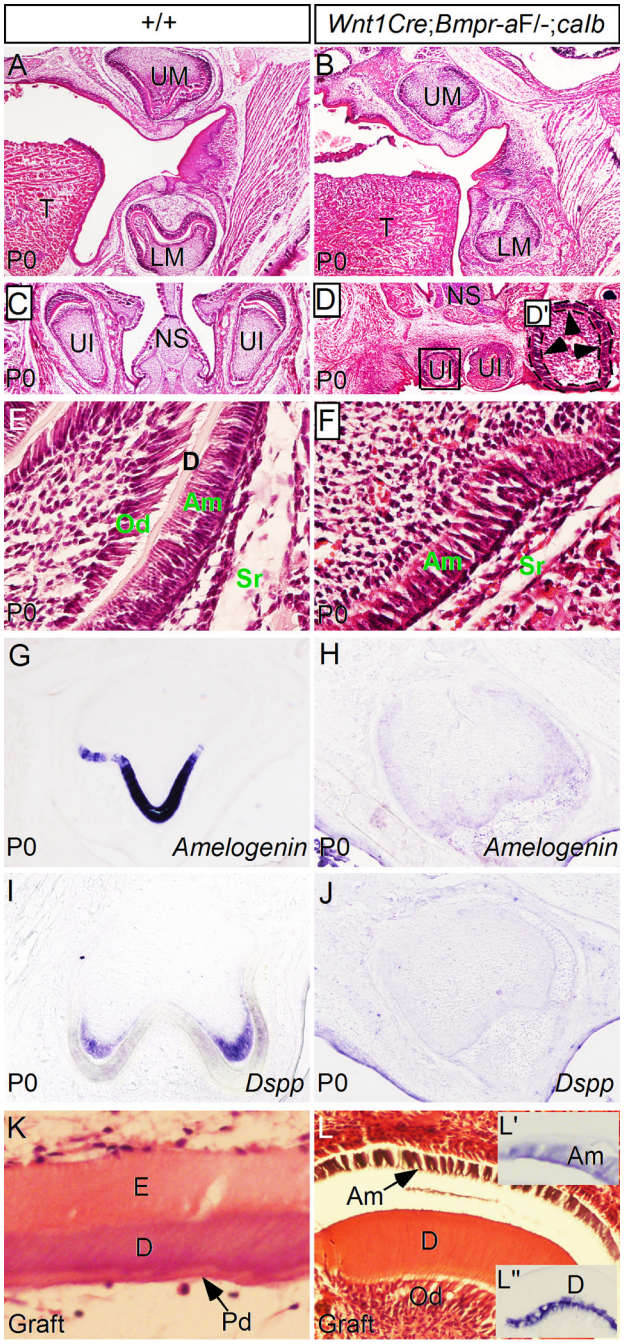

Figure 7.

Rescue of tooth development in *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mice. (A, C) Histological sections show morphology of molars (A) and upper incisors (C) in postnatal day 0 (P0) wild type mice. (B) A histological section from a P0 *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mouse shows molar structures that are comparable to wild type control in developmental stage and patterning. (D) A P0 *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* mouse shows upper incisor teeth. These two incisors form adjacently and underneath the nasal septum. (D') Higher magnification of an upper incisor from (D) shows mis-patterned incisor structure, with ameloblasts (arrowheads) forming all around the dental pulp. Black lines demarcate the ameloblast layer. (E) A higher magnification image shows formation of elongated odontoblasts and ameloblasts, and

deposit of dentin in a molar of a P0 wild type mouse. (F) A higher magnification image shows formation of elongated ameloblasts, but lack of odontoblasts and dentin deposit in a molar of *Wnt1Cre;BmprIa*$^{F/-}$;c*aIb* mouse at P0. (G, I) Expression of *Amelogenin* in ameloblasts (G) and *Dspp* in odontoblasts (I) is detected in P0 wild type molars. (H, J) Molars from P0 *Wnt1Cre;BmprIa*$^{F/-}$;c*aIb* mouse fail to express *Amelogenin* (H) and *Dspp* (J). (K) A molar germ graft from E13.5 wild type embryo shows formation of enamel and dentin after 2-week in subrenal culture. (L) A molar graft from E13.5 *Wnt1Cre;BmprIa*$^{F/-}$;c*aIb* embryo shows differentiation of ameloblasts and odontoblasts and dentin deposit. (L') In situ hybridization shows *Amelogenin* expression in ameloblasts of molar graft from *Wnt1Cre;BmprIa*$^{F/-}$;c*aIb* embryo. (L") *Dspp* expression is detected in odontoblasts of molar graft from *Wnt1Cre;BmprIa*$^{F/-}$;*caIb* embryo. D, dentin; E, enamel; Am, ameloblasts; LM, lower molar; NS, nasal septum; Od, odontoblasts; Pd, pre-dentin; Sr, stellate reticulum; UI, upper incisor; UM, upper molar.