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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.

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 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 et al., 2006; 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

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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 *Bmprlb* null mice (Baur et al., 2000; Yi et al., 2000), tissue-specific inactivation of *Bmprla* 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 *Bmprla* in the epithelial component for tooth and palate development, the requirement of *Bmprla* in the mesenchymal component remains unknown. This is likely due to the unavailability of a dental and palatal mesenchyme-specific Cre deletor mouse line and the fact that mice bearing deletion of *Bmprla* 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 *Bmprla* 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*;*Bmprla*$^{F/-}$ embryos survive to term, making it possible to examine the role of *Bmprla* throughout development of neural crest-derived tissues. Taking this advantage, we investigated the role of *Bmprla* in the mesenchymal tissue and further tested if BMPR-IB-mediated signaling is able to substitute for the loss of *Bmprla* in tooth and palate development.

Materials and methods

Animals and embryo collection

The generation and genotyping of transgenic and gene-targeted animals, including *Wnt1-Cre*, *Bmprla*$^{+/-}$, *Bmprla*$^{F/F}$, have been described previously (Mishina et al., 1995; Danielian et al., 1998). The *pMes-caBmprlb* conditional transgenic line contains a constitutively active form (with Gln203 to Asp change) of BMPR-IB (named *caBmprlb*), 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 *Bmprla* in their neural crest cells (*Wnt1Cre*;*Bmprla*$^{F/-}$) were obtained by crossing *Wnt1Cre*;*Bmprla*$^{+/-}$ mice with *Bmprla*$^{F/F}$ line. To obtain embryos carrying *Wnt1Cre*;*Bmprla*$^{F/-}$ alleles and a *pMes-caBmprlb* transgenic allele, *Wnt1Cre*;*Bmprla*$^{+/-}$ mice were crossed with *Bmprla*$^{F/+}$;*pMes-caBmprlb* mice. Mice containing such compounded alleles are referred as *Wnt1Cre*;*Bmprla*$^{F/-}$;*caIb*.

Embryos with *Bmprla* deficiency in their neural crest cells (*Wnt1Cre*;*Bmprla*$^{F/-}$) 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*;*Bmprla*$^{F/-}$ embryos to survive to term (Morikawa et al., 2009). This was done by supplementing the drinking water of dams with 200 μg/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*;*Bmprla*$^{F/-}$, and *Wnt1Cre*;*Bmprla*$^{F/-}$;*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*;*Bmprla*$^{+/-}$ mice with *Bmprla*$^{F/+}$;*pMes-caBmprlb* mice, and placed in PBS on ice. Embryos carrying *Wnt1Cre*;*Bmprla*$^{F/-}$ or *Wnt1Cre*;*Bmprla*$^{F/-}$;*caIb* compounded alleles exhibited craniofacial abnormalities and could be easily distinguished from embryos with other genotypes. *Wnt1Cre*;*Bmprla*$^{F/-}$;*caIb* embryo could be further distinguished from *Wnt1Cre*;*Bmprla*$^{F/-}$ 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*;*Bmprla*$^{F/-}$;*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.