Distinct functions for Bmp signaling in lip and palate fusion in mice

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Summary

Previous work suggested that cleft lip with or without cleft palate (CL/P) is genetically distinct from isolated cleft secondary palate (CP). Mutations in the Bmp target gene *Msx1* in families with both forms of orofacial clefting has implicated Bmp signaling in both pathways. To dissect the function of Bmp signaling in orofacial clefting, we conditionally inactivated the type 1 Bmp receptor *Bmpr1a* in the facial primordia, using the *Nestin cre* transgenic line. *Nestin cre*; *Bmpr1a* mutants had completely penetrant, bilateral CL/P with arrested tooth formation. The cleft secondary palate of *Nestin cre*; *Bmpr1a* mutant embryos was associated with diminished cell proliferation in maxillary process mesenchyme and defective anterior posterior patterning. By contrast, we observed elevated apoptosis in the fusing region of the *Nestin cre*; *Bmpr1a* mutant medial nasal process. Moreover, conditional inactivation of the *Bmp4* gene using the *Nestin cre* transgenic line resulted in isolated cleft lip. Our data uncover a *Bmp4-Bmpr1a* genetic pathway that functions in lip fusion, and reveal that Bmp signaling has distinct roles in lip and palate fusion.

Key words: Bmp, Palate, Morphogenesis

Introduction

Orofacial clefting, a common congenital malformation with a prevalence of from 1 in 500 to 1 in 2,500 live births, has a complex etiology that includes strong genetic and environmental components. Clinically, orofacial clefting has been categorized based on the presence or absence of cleft lip and whether or not it is part of a more extensive syndrome. Moreover, cleft lip with or without cleft palate (CL/P) and isolated cleft palate (CP) have been considered to be separate entities based on genetic and developmental grounds (Murray and Schutte, 2004; Schutte and Murray, 1999). Fusion of the lip occurs earlier in development, complete by 11.5 days post-coitum (dpc) in the mouse, and involves the fusion of the medial and lateral nasal processes with the maxillary process (MP) (Moore and Persaud, 1993). Secondary palate formation, completed in the mouse by 14.5 dpc, is a complex process that involves downward growth of the palatal shelves, derived from the MP, followed by elevation and midline fusion (Ferguson, 1988).

Recent findings have shown that in some families CL/P and CP may result from the same genetic lesion. For example, a nonsense mutation (ser104stop) in the *Msx1* homeobox gene, a transcriptional regulator that is a downstream component of bone morphogenetic protein (Bmp) signaling pathways, resulted in both cleft palate (CP) and cleft lip/palate (CL/P) in a Dutch family (van den Boogaard et al., 2000). Moreover, common mutations in *p63* have also been shown to cause CL/P and CP within a family (Barrow et al., 2002). Recent evidence suggests that *p63* is a direct downstream target of Bmp signaling in zebrafish (Bakkers et al., 2002). Taken together, these data suggest that Bmp-signaling has a role in two forms of orofacial clefting that were previously considered to be distinct.

Bmp ligands are expressed in the facial primordia (Ashique et al., 2002; Barlow and Francis-West, 1997; Francis-West et al., 1994), and are known to signal through broadly expressed type I and type II serine/threonine kinase receptors (von Bubnoff and Cho, 2001). Bead-implantation experiments in chick embryos led to the conclusion that both reduction and enhancement of Bmp signaling within facial primordia caused defective lip fusion (Ashique et al., 2002). Although underscoring the importance of tightly regulated Bmp signaling in lip and palate fusion, the transient nature of bead implantation experiments, as evidenced by the requirement for multiple rounds of bead insertion, may fail to uncover the complete requirements for Bmp function in orofacial development.

Direct investigation of the type 1A Bmp receptor gene *Bmpr1a* (also referred to as *Alk3*), and *Bmp4* in craniofacial development has been hampered by the early embryonic lethality of the germline null mutant mice (Mishina et al., 2002; Mishina et al., 1995; Winnier et al., 1995). To circumvent this

early lethality, we used conditional null alleles of *Bmpr1a* and *Bmp4* to directly investigate Bmp signaling in lip and palate fusion in mouse embryos. We report here that the *Bmpr1a*-dependent pathway is a major regulator of lip and palate fusion and tooth morphogenesis. Inactivation of *Bmpr1a* in the craniofacial primordia resulted in CL/P with tooth agenesis. However, we provide evidence that the mechanisms underlying lip clefting and cleft secondary palate are distinct. We also report that deficiency of *Bmp4* resulted in isolated cleft lip. Taken together, our results reveal that Bmp signaling has distinct functions in lip fusion versus secondary palate development.

Materials and methods

Generation of the conditional *Bmpr1a* and *Bmp4* alleles

The *Bmpr1a* and *Bmp4* conditional null alleles have been previously described (Liu et al., 2004; Mishina et al., 2002). Both alleles have been demonstrated to have wild-type activity prior to recombination and to revert to a null allele after recombination. The *Nestin cre* transgenic line, previously described, directs cre activity in the craniofacial region, as reported here, but is imprinted in somatic tissues when maternally inherited (Trumpp et al., 1999). For our crosses, the *Nestin cre* transgene was derived from the male.

Palate in vitro organ culture

Palatal shelves were harvested at 13.0 dpc and 14.0 dpc from wild-type and mutant embryos. The dissected palatal shelves were cultured on 6.5-mm transwell (Costar), keeping the paired shelves with their medial edge epithelia (MEE) in close apposition without apparent distortion of the tissue shape. Paired palatal shelves were cultured overnight using DMEM supplemented with 1% penicillin/streptomycin (Chai et al., 1998).

Separation of epithelium from mesenchyme

Branchial arches were dissected and placed in a Petri dish containing HBSS (Sigma) with 10% FCS. After dissection, HBSS was replaced with dispase II (0.8 U/ml from Roche). Explants were incubated for 1 hour at room temperature. Forceps were used to gently separate the epithelium from mesenchyme.

Histologic analysis

Mouse tissues were fixed in buffered formalin overnight, dehydrated through graded alcohols, and embedded in paraffin wax. Paraffin blocks were sectioned at 7-10 μm and stained with Hematoxylin and Eosin.

Skeletal preparations

After scalding, mice were eviscerated, fixed in 95% ethanol and stained with Alcian Blue (0.015% Alcian Blue dissolved in 20% acetic acid and 76% ethanol). Following two washes with 95% ethanol, the sample was cleared by 2% KOH at room temperature. Bone was stained with Alizarin Red (50 mg Alizarin Red in one liter of 2% KOH).

Analysis of BrdU incorporation

Pregnant mice were intraperitoneally injected with BrdU (100 mg/kg body weight) 1 hour prior to sacrifice. Embryos were fixed overnight in 10% formalin at 4°C, dehydrated through an ethanol series, cleared in xylene, embedded in paraffin wax and sectioned at 5 μm. BrdU was detected immunohistochemically with a cell proliferation kit (Invitrogen), according to the manufacturer's instructions.

Whole-mount β-gal staining

After dissection, the embryos were fixed in fresh fix buffer [(0.2 glutaraldehyde, 2% formalin, 5 mM EGTA, 2 mM $MgCl_2$, in 0.1 M phosphate buffer (pH 7.3)] for 30 minutes. Following three washes with rinse buffer [0.1% sodium dexycholate, 0.2% NP40, 2 mM $MgCl_2$, in 0.1 M phosphate buffer pH 7.3)], the samples were stained with staining buffer (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, in rinse buffer) overnight at room temperature.

Results

Inactivation of *Bmpr1a* in the craniofacial primordia

The $Bmpr1a^{flox}$ allele, a conditional null allele of *Bmpr1a*, has LoxP sites flanking exon 2, which is essential for *Bmpr1a* function (Mishina et al., 2002). We crossed $Bmpr1a^{flox}$ homozygous mice, which are normal and fertile, to *Nestin cre;*$Bmpr1a^{null}$ heterozygous mice (Trumpp et al., 1999), to generate embryos that were deficient for *Bmpr1a* in the craniofacial primordia, the *Nestin cre;*$Bmpr1a^{null/flox}$(*n/f*) mutant embryos. Crosses to *Rosa26 Reporter* (*R26R*) mice revealed that the *Nestin cre* transgene directed cre activity in the oral and dental epithelium, in epithelium of the medial and lateral nasal processes, and in the MP mesenchyme (Fig. 1A-D). At 13.5 dpc, *Nestin cre;R26R* compound heterozygous embryos had cre activity in the mesenchyme and epithelium of the palatal shelves (Fig. 1E,F). In situ experiments using the *Bmpr1a exon2* probe, which is deleted in the *Nestin cre;Bmpr1a n/f* mutant embryos, failed to provide reproducible data. Therefore, to understand the timing and completeness of the *Bmpr1a* recombination, we performed a microdissection experiment followed by PCR.

Microdissection of craniofacial tissues, followed by PCR, indicated that *Bmpr1a* had been deleted from the MP mesenchyme and epithelium in *Nestin cre;Bmpr1a n/f* mutant embryos by 10.5 dpc (Fig. 1G). The PCR data also consistently showed that recombination at the *Bmpr1a* locus was incomplete in the epithelium of the mandibular and lateral and medial nasal processes, as a low intensity PCR product was obtained from these tissues. Taken together, the PCR data and the *R26R* data reveal that the *Nestin cre;Bmpr1a n/f* embryos have complete removal of *Bmpr1a* from the epithelium and mesenchyme of the MP by 10.5 dpc, and mosaic deletion in the epithelium of the mandibular and nasal processes.

Signaling through *Bmpr1a* is required for lip and secondary palate fusion

At 18.5 dpc, we found that all *Nestin cre;Bmpr1a n/f* mutant embryos showed a strong phenotype with bilateral cleft lip and palate (Fig. 2A-D). Histological sections of the forming secondary palate at multiple time points revealed that at 13.5 dpc, both the wild-type and the *Nestin cre;Bmpr1a n/f* palatal shelves grew vertically down the side of the tongue, although the mutant shelves were slightly smaller than the control (Fig. 2E,F). Moreover, at 14.5 dpc, the palatal shelves elevated correctly in both wild-type and mutant embryos (Fig. 2G,H). However, after palatal shelf elevation, the mutant palatal shelves failed to grow together and fuse (Fig. 2I,J). These results suggest that the cleft secondary palate in *Nestin cre;Bmpr1a n/f* embryos was secondary to failure of palatal shelf growth.

To investigate this, we studied cell proliferation in the MP mesenchyme of wild-type and *Nestin cre;Bmpr1a n/f* mutant

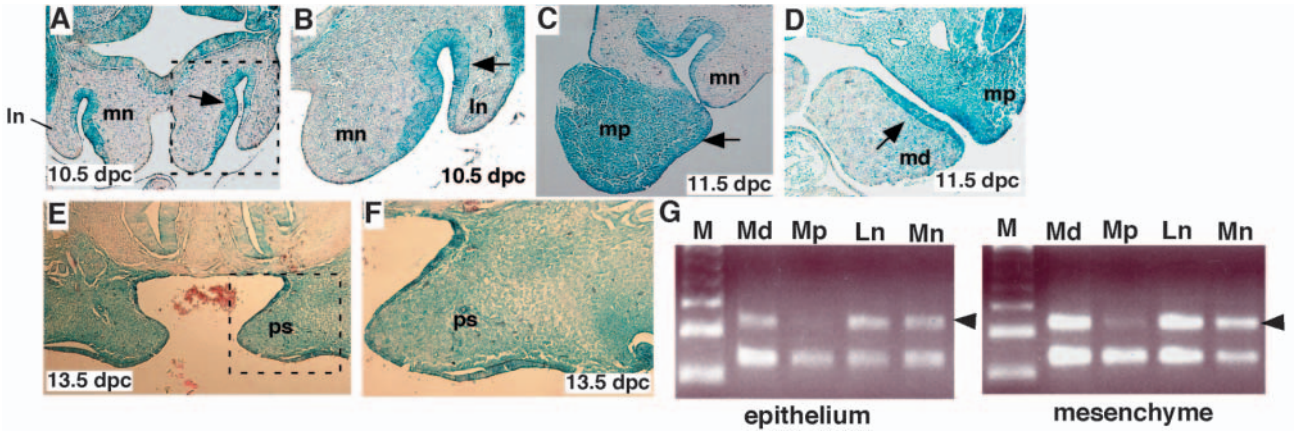

Fig. 1. Spatiotemporal analysis of cre activity directed by the Nestin cre transgene. (A-F) Coronal sections through whole-mount β-gal staining of E10.5 (A,B), 11.5 (C,D) and 13.5 (E,F) dpc *Nestin cre*$^{+/-}$*;R26R*$^{+/-}$ mice showing cre activity in *Nestin cre* transgenic mice. Cre activity is denoted by the blue color (arrows). Boxed areas in A and E are shown at higher magnification in B and F, respectively. In E and F, β-gal-positive cells are found throughout the palatal shelves. (G) PCR analysis of dissected tissues from *Nestin cre;Bmpr1a n/f* embryos at 10.5 dpc. Primers flanking the second LoxP site were used to determine the extent of recombination at the *Bmpr1a* locus. The upper band is the product of *Bmpr1a*^{flox} allele (arrowhead). The lower band is the product of *Bmpr1a*^{null} allele and serves as an internal control. M, DNA marker; Md, mandibular process; Mp, maxillary process; Ln, laternal nasal process; Mn, medial nasal process; ps. palatal shelf.

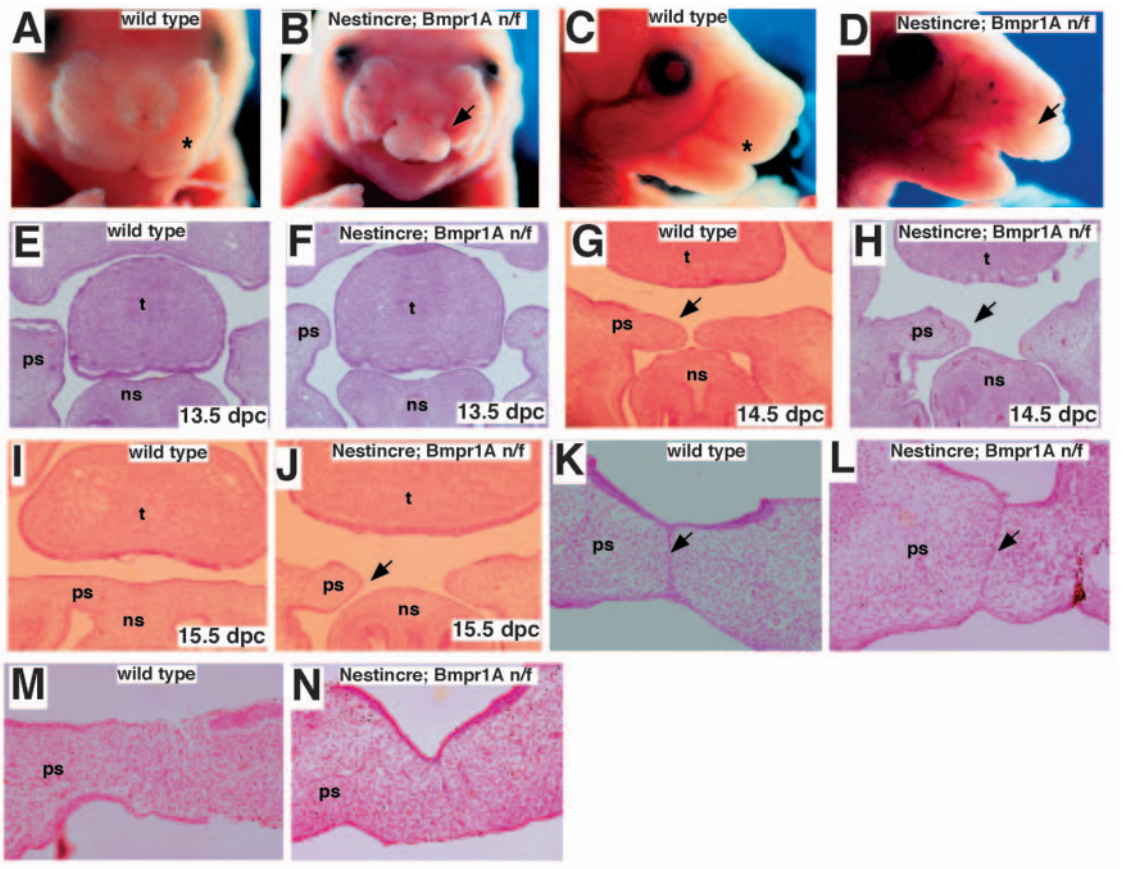

Fig. 2. Cleft lip and palate in *Nestin cre;Bmpr1a n/f* mutant embryos. (A-D) Frontal (A,B) and lateral (C,D) views of 16.5 dpc wild-type (A,C) and (B,D) *Nestin cre;Bmpr1a n/f* mutants showing the bilateral cleft lip in mutant embryos (arrow). Star in A and C indicates area of lip fusion in wild-type embryos. (E-J) Hematoxylin and Eosin staining of coronal sections through the palatal region of wild-type (E,G,I) and *Nestin cre;Bmpr1a n/f* mutant embryos (F,H,J) at the labeled developmental stages. Arrows denote unfused palatal shelves. (K-N) Coronal sections stained with Hematoxylin and Eosin of wild-type (K,M) and *Nestin cre;Bmpr1a n/f* mutant (L,N) secondary palate explants cultured overnight. Palatal shelves were harvested at 14.5 dpc (K,L) and E13.5 (M,N). Arrows denote the degenerating medial edge epithelium (MEE) in K and L. ns, nasal septum; ps, palatal shelf; t, tongue.

embryos using a BrdU-incorporation assay. At 10.5 dpc, prior to lip fusion, we found that the cell proliferation rates in the maxillary mesenchyme were reduced in *Nestin cre;Bmpr1a n/f* mutant embryos. In 11.5 dpc embryos, the proliferative index in MP mesenchyme was 43.1% in *Nestin cre;Bmpr1a n/f* mutants, whereas in wild-type embryos it was 49.3% ($P<0.05$, χ^2). In the epithelium and mesenchyme of the medial and lateral nasal processes, the proliferative index in the mutant was similar to that in the wild-type embryo (data not shown). From these data, we conclude that *Bmpr1a* function is required for normal cell proliferation in MP mesenchyme.

Bmpr1a deficient palatal shelves fuse in vitro

If the cleft secondary palate of *Nestin cre;Bmpr1a n/f* mutants was caused by a proliferation defect in the maxillary mesenchyme, then mutant palatal shelves may still retain the ability to fuse in vitro. To investigate this notion, organ culture experiments were performed. Palatal shelves were harvested at 13.0 and 14.0 dpc and cultured overnight with the medial edge epithelium (MEE) in contact. The *Nestin cre;Bmpr1a n/f* palatal shelves harvested at both 13.0 and 14.0 dpc fused in organ culture (Fig. 2K-N). Moreover, in the palatal shelves harvested at 13.0 dpc, MEE degeneration was clearly visible (Fig. 2M,N). These data indicate that the *Nestin cre;Bmpr1a n/f* palatal shelves retain the ability to fuse and support the hypothesis that the mechanism underlying cleft secondary palate in the *Nestin cre;Bmpr1a n/f* embryos was a failure of palatal shelf outgrowth. However, further experiments are required to prove that the MP proliferation defect caused cleft secondary palate in *Nestin cre;Bmpr1a n/f* embryos.

Defective patterning of the secondary palate in *Nestin cre;Bmpr1a* deficient embryos

Recent work has suggested that Bmp signaling plays a role in anterior posterior (AP) patterning of the palatal shelves (Zhang et al., 2002). To investigate whether AP patterning was defective in the *Nestin cre;Bmpr1a* mutant palatal shelves, we examined the expression of *Msx1* in the wild-type and *Nestin cre;Bmpr1a* mutant secondary palates. *Msx1* continued to be expressed in the anterior secondary palate of *Nestin cre;Bmpr1a* mutant embryos (Fig. 3A-B). The expression of *Msx1* in the MP of mutant embryos suggests that Bmp signals are transduced to the MP mesenchyme in the absence of *Bmpr1a*. This is likely to reflect the contribution of other type I receptors, such as *Bmpr1b* (also known as *Alk6*), to Bmp signaling in the MP.

In contrast to *Msx1*, we found that expression of the *paired box* gene *Pax9*, required for normal palate development (Peters et al., 1998), was expanded in the *Nestin cre;Bmpr1a* mutant embryos (Fig. 3C,D). Similarly, *Barx1* expression was also expanded in the secondary palates of *Nestin cre;Bmpr1a* mutant embryos at 12.5 dpc (Fig. 3G,H). Other genes important for palate development, such as *Tbx22* and *Osr2* (Lan et al., 2004; Marcano et al., 2004), were expressed normally in *Nestin cre;Bmpr1a n/f* embryos (Fig. 3G-J). From these data, we conclude that signaling through *Bmpr1a* is necessary for correct spatiotemporal expression of crucial transcriptional regulators of palate development, such as *Barx1* and *Pax9*.

Elevated apoptosis in the fusing lip region of *Nestin cre* mutant embryos

To gain insight into the molecular mechanism underlying cleft lip in *Nestin cre;Bmpr1a* mutants, we examined markers of the edge epithelium of the MP and medial nasal process (MNP). *Fgf8* is expressed in the epithelium of the nasal processes and the MP in chick embryos (Helms et al., 1997). Moreover, *Fgf* signaling is known to closely interact with Bmp signaling pathways to regulate craniofacial and tooth development (Neubuser et al., 1997; Tucker et al., 1998). In addition to *Fgf8*, we examined expression of *p63* and *Pitx1*, which are expressed in the edge ectoderm of the fusing lip. *P63* has also been implicated in cleft lip and palate in human patients (Celli et al., 1999), and *Pitx1* is a known target gene for Fgf8 in mandibular development (St Amand et al., 2000).

To precisely define the onset of the lip fusion defect, we examined *Fgf8* expression in embryos that had been carefully staged by counting somite number (Fig. 4A-D). In 29-somite embryos (10.0 dpc), *Fgf8* expression was similar in the wild-type and *Nestin cre;Bmpr1a* mutant embryos (Fig. 4A,B). *Fgf8* was expressed in the proximal part of the nasal process ectoderm but was not yet expressed in the fusing region. By contrast, 31-somite embryos (10.25 dpc) expressed *Fgf8* in the ectoderm at the lip fusion point, whereas *Nestin cre;Bmpr1a* mutants failed to upregulate *Fgf8* in this region (Fig. 4C,D). This difference in *Fgf8* expression was more pronounced in 10.5 dpc (34-35 somites) embryos (Fig. 4E,F). In addition to diminished *Fgf8* expression, we found that *Nestin cre;Bmpr1a* mutants also had downregulated expression of both *p63* and *Pitx1* in the fusing ectoderm of the nasal processes in 10.5 dpc embryos (Fig. 4G-L).

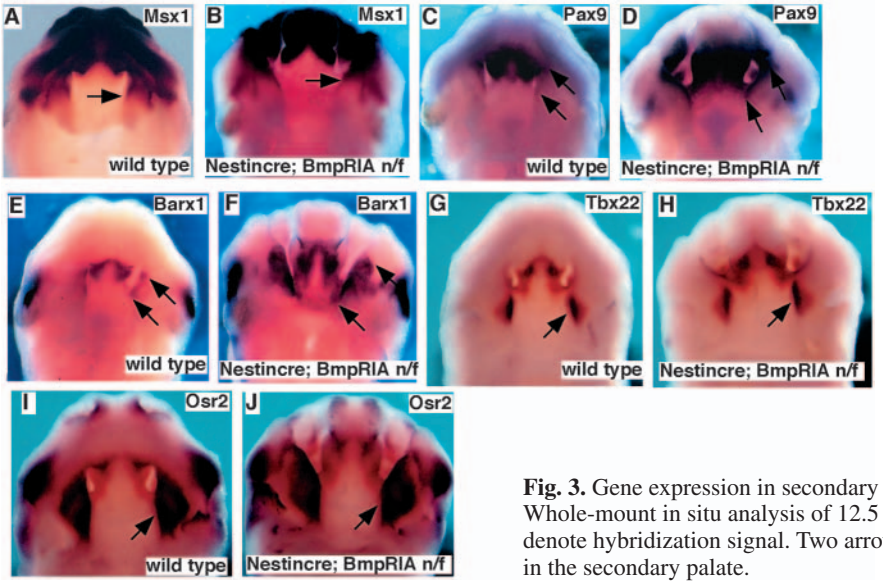

Fig. 3. Gene expression in secondary palates of *Nestin cre;Bmpr1an/f* mutant embryos. Whole-mount in situ analysis of 12.5 dpc embryos with the labeled probes. Arrows denote hybridization signal. Two arrows (C-F) mark the limits of the hybridization signal in the secondary palate.

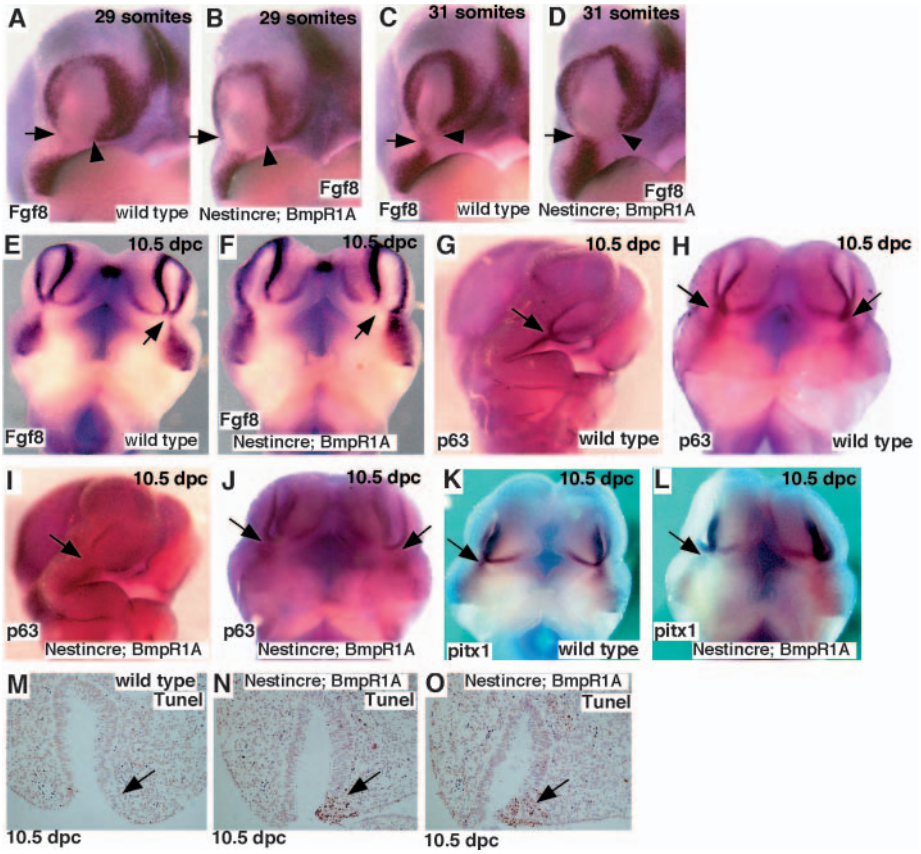

Fig. 4. Analysis of gene expression in the fusing lip of *Nestin cre;Bmpr1a n/f* mutant embryos. (A-D) *Fgf8* expression in the fusing lip region of wild-type and *Nestin cre;Bmpr1a n/f* at the labeled stage. (E-L) *Fgf8* (E,F), *p63* (G-J) and *pitx1* (K,L) expression in the edge epithelium of the MNP and MP of wild-type and *Nestin cre;Bmpr1a n/f* mutant 10.5 dpc embryos. Arrows denote areas of hybridization that are absent in the mutant embryos. (E,F,H,J-L) Ventral views; (G,I) lateral oblique views. (M-O) Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) studies on sections from wild-type and *Nestin cre;Bmpr1a n/f* mutant embryos. Arrows denote the area of elevated apoptosis in the mutant embryo. Two examples of mutant embryos are shown (N,O).

We next studied apoptosis in the fusing lip region of wild-type and *Bmpr1a* mutant embryos at 10.5 dpc (34-35 somites). In wild-type embryos, little apoptosis was detected in fusing ectoderm or underlying mesenchyme, but in mutant embryos upregulation of apoptosis was detected in the ectoderm and mesenchyme of the MNP (Fig. 4M-O). Taken together, these data indicate that reduced Bmp signaling results in elevated apoptosis in the ectoderm and mesenchyme of the MNP. Moreover, Bmp signaling in the edge epithelium of the frontonasal process and the MP is required for the expression of markers of the edge epithelium of the MP and MNP, such as *Fgf8* and *p63*.

Bmpr1a deficiency in oral ectoderm results in defective tooth morphogenesis

Consistent with the role of *Msx1* in tooth agenesis in human patients, we detected tooth defects in *Nestin cre;Bmpr1a* mutants. At 16.5 dpc, mandibular and maxillary first and second molars are normally in the late and early cap stage of development, respectively (Fig. 5A,B). The inner and outer enamel epithelium in the developing molars are separated by the stellate reticulum. The maxillary molars in *Nestin cre;Bmpr1a n/f* 16.5 dpc embryos were arrested at the bud stage, with an invaginated dental lamina encircled by condensed mesenchyme (Fig. 5A,C). In contrast to the defective maxillary molars, *Nestin cre;Bmpr1a n/f* 16.5 dpc embryos had well-developed cap-staged mandibular molars (Fig. 5B,D). In 16.5 dpc wild-type embryos, there are two well-developed incisor tooth germs in each arch (Fig. 5E,F). In the *Nestin cre;Bmpr1a* mutant embryos, development of the mandibular incisors was normal but mutant embryos lacked maxillary incisor teeth (Fig. 5G,H).

At 18.5 dpc, the wild-type mandibular and maxillary molars have progressed to the bell stage and contain newly differentiated odontoblasts that secrete pre-dentine and contain polarized, presecretory ameloblasts (Fig. 5I,J). The bud-staged mutant maxillary molars had failed to progress at 18.5 dpc (compare Fig. 5C with 5K). The epithelial invagination lacked the typical cap morphology and was surrounded by dental papilla-like mesenchyme (Fig. 5K). Mandibular molars in 18.5 dpc mutant embryos were arrested at the cap stage of development. First and second mandibular molars were present but were in close proximity to each other, giving the appearance of fused tooth buds (Fig. 5J,L). Taken together, these data reveal that signaling through *Bmpr1a* is required for maxillary tooth development to progress beyond the bud stage.

Bmp4 deficiency resulted in isolated cleft lip

In order to gain insight into the ligands that would be involved in cleft lip and palate, we examined expression of *Bmp4* and *Bmp2*. At 10.5 dpc, we found that *Bmp2* and *Bmp4* are co-expressed in the edge epithelium at the point of fusion between the MNP and the MP (Fig. 6A,B). We next used a conditional null allele of *Bmp4*, the $Bmp4^{floxneo}$ allele, to address the functional role of Bmp4 in CL/P. The $Bmp4^{floxneo}$ allele has LoxP sites flanking exon 4 that encodes the mature Bmp4 ligand. Removal of the LoxP flanked region has been shown to result in a null allele (Liu et al., 2004).

To determine when *Bmp4* had been removed from the

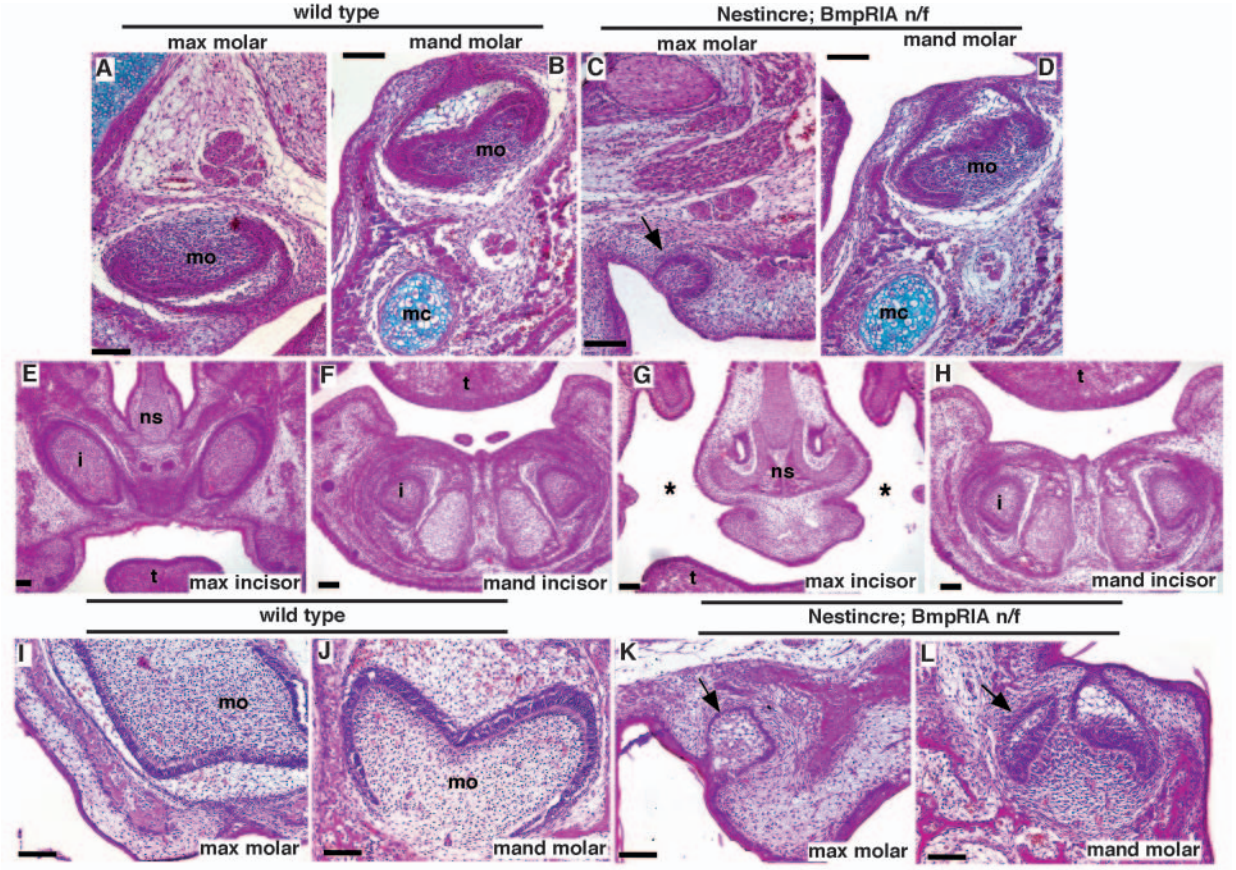

Fig. 5. Tooth abnormalities in *Nestin cre;Bmpr1a n/f* mutant embryos compared with stage-matched wild-type embryos. (A-D) Coronal sections of 16.5 dpc maxillary and mandibular molars. Genotypes and tooth types are labeled. In wild-type embryos (A,B), the maxillary and mandibular molars are in the cap stage of development, whereas in *Bmpr1a* mutants, the maxillary molar is at the bud stage of tooth development (arrow in C). The *Bmpr1a* mutant mandibular molars (D) are at the same stage of development as in the wild-type (B). Scale bar: 100 μm. (E-H) Coronal sections of maxillary and mandibular incisors at E16.5. Genotypes and tooth type are labeled. The developing maxillary and mandibular incisors are seen in the wild-type embryos but in the mutant the maxillary incisors are absent. In addition, there is a cleft of the primary palate (asterisk, G). Scale bar: 100 μm. (I,J) At E18.5 in the wild-type embryos, the maxillary and mandibular molars have progressed to the more advanced (bell) stage of development. (K) At 18.5 dpc, in the *Bmpr1a* embryos, the maxillary molars are still in the bud stage of tooth development (arrow). (L) The mandibular molars in 18.5 dpc mutants are at the cap stage of tooth development (arrow). I, incisor; mo, molars; mc, Meckel's cartilage; ns, nasal septum; t, tongue. Scale bars in I-L: 100 μm.

branchial arch ectoderm in *Nestin cre;Bmp4*$^{null/flox}$*(n/f)* embryos, we performed in situ analysis with the *Bmp4 exon 4* probe that hybridizes to the deleted region of the *Bmp4*^{floxneo} allele. At 10.5 dpc, we found that the *Bmp4 exon 4* probe hybridized to the edge ectoderm of the fusing lip in wild-type embryos, but, in the *Nestin cre;Bmp4 n/f* mutant embryos, no hybridization signal was detected (Fig. 6C,D). We conclude that in *Nestin cre;Bmp4 n/f* embryos, *Bmp4* has been deleted by 10.5 dpc in the edge epithelium of the MNP and MP.

We examined *Nestin cre;Bmp4 n/f* mutant embryos at two developmental timepoints. At 12.0 dpc, we found that all *Nestin cre;Bmp4 n/f* mutant embryos had a bilateral delay in fusion of the MNP and MP (Fig. 6E,F). At 14.5 dpc, two out of nine *Nestin cre;Bmp4 n/f* mutants had unilateral isolated cleft lip (Fig. 6G,H). Taken together, these data reveal that Bmp4 functions in the ectoderm of the nasal processes to regulate lip fusion. Moreover, these findings suggest that most *Nestin cre;Bmp4 n/f* mutants spontaneously repair the cleft lip that we observed at 12.0 dpc.

Discussion

Our data uncover a crucial role for Bmp signaling in lip and secondary palate development. In the *Nestin cre;Bmpr1a n/f* embryos, defective proliferation was the likely cause for secondary palate clefting. In the lip, we provide evidence that premature apoptosis in the MNP edge epithelium resulted in clefting. Moreover, the phenotype of the *Nestin cre;Bmp4 n/f* embryos suggest that a Bmp4-BmpR1A planar signaling pathway functions in lip fusion. These findings provide insight into the mechanisms underlying the mixed clefting phenotypes observed in human patients.

Bmp signaling in secondary palate growth and AP patterning

Development of the secondary palate is a complex process that requires outgrowth, elevation and fusion of the palatal shelves (Ferguson, 1988). Our analysis revealed that *Bmpr1a* is required for proliferation of MP mesenchyme. The in vitro explants showed that the *Bmpr1a* mutant palatal shelves are

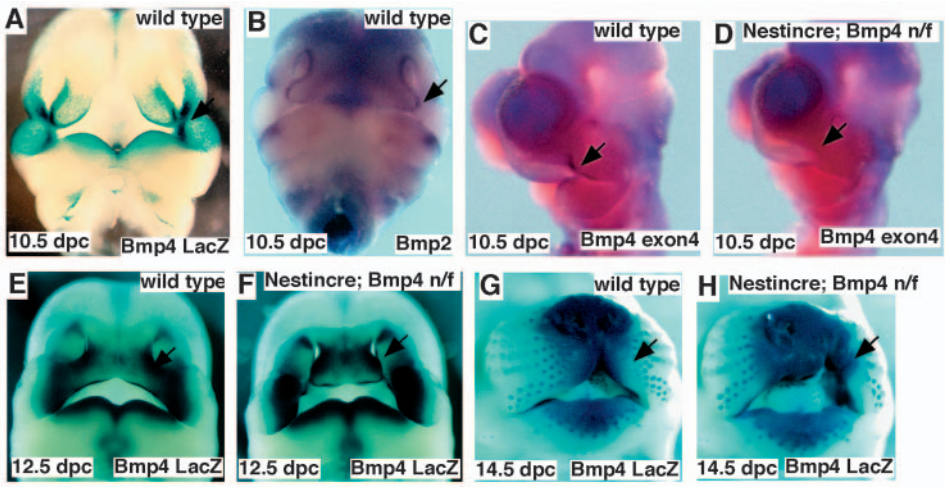

Fig. 6. Bmp4 function in lip fusion. (A,B) Whole-mount β-gal staining (A) and whole-mount in situ hybridization with *Bmp2* probe (B) in $Bmp4^{lacZ}$+/– and wild-type embryos, showing expression of *Bmp4* and *Bmp2* (arrow) in the fusing region of the lip. (C,D) Whole-mount in situ hybridization with a *Bmp4 exon 4* probe showing the absence of a hybridization signal in the *Nestin cre;Bmp4 n/f* mutant embryo (arrow). (E-H) β-Gal stained embryos showing cleft lip in the *Nestin cre;Bmp4 n/f* embryos (arrows). In this experiment the $Bmp4^{lacZ}$ allele was used instead of the $Bmp4^{null}$ allele. Stages and genotypes are labeled.

competent to fuse when placed together in culture. Previous work in secondary palate development uncovered a genetic pathway in which Msx1 and Bmp4 function in an autoregulatory loop to regulate proliferation in anterior palate mesenchyme (Zhang et al., 2002). Our data reveal that Bmp signaling is also required for cell proliferation at earlier stages, in the MP mesenchyme.

Two previous studies looking at Bmp signaling in chick embryos reached conflicting conclusions regarding the regulation of cell proliferation in the facial prominences. One study concluded that implantation of Bmp-soaked beads in MP mesenchyme resulted in elevated proliferation (Barlow and Francis-West, 1997). The second study, while concluding that *Noggin* reduced cell proliferation, found that implanted *Bmp2* beads failed to influence cell proliferation (Ashique et al., 2002). Our data support the notion that Bmp signaling promotes cell proliferation of MP mesenchyme.

We found that Bmp signaling is required to restrict expression of *Barx1* and *Pax9* in the forming secondary palate. It is interesting to note that in the developing mandible, Bmp signaling has been shown to repress expression of *Pax9* and *Barx1*, suggesting a conservation of pathways in the palate and mandible (Neubuser et al., 1997; Tucker et al., 1998; Zhang et al., 2002). Moreover, the changes in gene expression in the *Nestin cre;Bmpr1a* mutant secondary palates support the idea that cleft secondary palate is not a consequence of failed lip fusion.

We have found evidence for genetic redundancy among Bmp receptors in the MP. The *Nestin cre;Bmpr1a* mutant embryos still express *Msx1* in the palatal mesenchyme, suggesting that Bmp signals are transduced in the absence of *Bmpr1a*. Neural crest specific ablation of another type 1 Bmp receptor (*Alk2*) also resulted in cleft palate, although this may be secondary to mandibular defects (Dudas et al., 2004). Another possible candidate for redundancy is *Bmpr1b*, which is expressed in craniofacial processes (W.L. and J.F.M., unpublished). It will be important to explore the question of genetic redundancy between the type 1 Bmp receptors in future experiments.

Bmp signaling in lip fusion

Our data suggest that a *Bmp4-Bmpr1a* autoregulatory loop in the edge epithelium is necessary for lip fusion. These findings implicate the Bmp4-BmpR1A pathway in human clefting syndromes, and are consistent with previous observations showing the association between the Bmp-target *Msx1* and CL/P (van den Boogaard et al., 2000). It was previously shown that an Arg239Pro *Msx1* mutation resulted in tooth agenesis but no clefting in human patients (Vastardis et al., 1996). The second family with a Ser104stop mutation in *Msx1* had both CL/P and selective tooth agenesis (van den Boogaard et al., 2000). The defects in both families are likely to result from haploinsufficiency and suggest that the Arg239Pro *Msx1* mutation retained more residual function than the Ser104 stop mutation (Hu et al., 1998).

Our observation that most *Nestin cre;Bmp4 n/f* mutant embryos repair their cleft lip also implicates *Bmp4* in clinical cases of microform cleft lip that have been observed in human patients. It is notable that congenital heart disease has been associated with microform clefting (Castilla and Martinez-Frias, 1995; Grech et al., 2000). We, and others, have recently reported that *Bmp4* has a direct role in cardiac morphogenesis, suggesting an important role for *Bmp4* in human congenital heart disease (Jiao et al., 2003; Liu et al., 2004).

Our findings reveal that *Bmpr1a* is required for survival of the edge epithelium and mesenchyme of the MNP. Work in chick embryos has shown that during lip fusion, the outermost periderm epithelium of the frontonasal mass and MP undergoes apoptosis. This exposes a fusion-competent basal epithelium, allowing basal cells to fuse through a process that may involve desmosomes. Cells within the fusing region, or the seam, undergo an epithelial to mesenchymal transition during remodeling of the lip (Sun et al., 2000). Our data suggest that, in *Bmpr1a* mutants, premature apoptosis in the edge epithelium of the MNP is responsible for clefting of the lip.

The role of programmed cell death in facial process fusion is controversial. The epithelial-mesenchymal transformation of the MEE to palate mesenchyme has been considered to be the critical determinant for secondary palate fusion (Fitchett and Hay, 1989). However, recent studies have challenged this model by suggesting that apoptosis is required for normal fusion to occur (Cuervo and Covarrubias, 2004; Cuervo et al., 2002). With regard to the data presented here, it is known that premature induction of apoptosis in palatal shelves inhibited fusion, perhaps as a result of reduced cell adhesion (Cuervo et al., 2002). Further experiments will be required to determine whether failed fusion of the edge epithelium in *Nestin*

cre;Bmpr1a n/f mutants is secondary to reduced cellular adhesion.

It is also notable that, in addition to elevated apoptosis in *Nestin cre;Bmpr1a n/f* mutant nasal ectoderm, there was elevated apoptosis in the underlying mesenchyme (Fig. 4M-O). This suggests that the defect in lip fusion may result from loss of the MNP mesenchyme in addition to the mechanisms discussed above. Further experiments will be required to investigate this.

The in situ analysis presented here revealed defects in the expression of ectodermal markers of the fusing lip, *Fgf8*, *Pitx1* and *p63*, in *Nestin cre;Bmpr1a n/f* mutants. *Fgf8* expression in the fusing lip region of the *Nestin cre;Bmpr1a n/f* mutants revealed a failure to upregulate *Fgf8* in 31-somite embryos prior to induction of apoptosis. In the mandibular process, *Fgf8* has an important role in promoting the survival of underlying mesenchyme (Trumpp et al., 1999). Therefore, in *Nestin cre;Bmpr1a n/f* mutants, the failure of *Fgf8* upregulation may directly result in elevated apoptosis and lip clefting. This notion is also supported by recent observations in chick embryos treated with retinoic acid inhibitors. In these retinoid deficient embryos, defective closure of the nasal pit was associated with elevated apoptosis and downregulation of *Fgf8* in the lateral nasal process (Song et al., 2004). However, further experiments will be required to definitively show that apoptosis is the cause of lip clefting in the *Nestin cre;Bmpr1a n/f* mutants.

An alternative hypothesis is suggested by the observation that *p63* has previously been shown to play an important role in differentiation of embryonic epidermis (Mills et al., 1999; Yang et al., 1999). Furthermore, recent work uncovered multiple, functional *Smad* regulatory elements in the zebrafish *p63* gene (Bakkers et al., 2002). Taken together with our observation that *p63* is reduced in the edge epithelium of the MNP and MP of *Nestin cre;Bmpr1a n/f* mutants, these data suggest the existence of a linear genetic pathway important for maturation and subsequent fusion of the edge epithelium. The connection of *p63* to clefting in humans makes this idea worthy of further investigation (Celli et al., 1999).

Bead implantation experiments performed in chick embryos concluded that, in the maxillary epithelium, Bmp signaling promoted cell death and loss of epithelium. Beads soaked in Noggin resulted in reduced cell death in the epithelium of the frontonasal mass, and thus epithelial integrity was maintained in the pre-fusion phases. Moreover, *Fgf8* expression in the frontonasal process was increased (Ashique et al., 2002). By contrast, our data suggest that Bmp signaling in the epithelium is required for cell survival and upregulation of *Fgf8* expression. The different results may stem from the large doses of Noggin that were necessary to obtain phenotypes in the chick experiments. It is also notable that we inactivated Bmp signaling in the nasal process epithelium, whereas Noggin was placed in the mesenchyme and so may have indirectly disrupted an unidentified signal from mesenchyme to epithelium.

Bmp signaling in tooth morphogenesis

It has been proposed that Bmp signaling has multiple roles in tooth development, including tooth type morphogenesis, tooth organ placement, and signaling within and from the enamel knot (Jernvall et al., 1998; Neubuser et al., 1997; Tucker et al., 1998). The *Nestin cre;Bmpr1a n/f* mutants display different tooth phenotypes in the mandible and maxilla. In the maxilla, molars arrest at the bud stage, while incisors arrest at an earlier stage. In the mandible, molars arrest at the cap stage, while incisor development is normal. Recently published observations showing that inactivation of *Bmpr1a* in dental epithelium resulted in arrest of tooth development at the bud stage (Andl et al., 2004) suggest that the distinct tooth phenotypes we observed are likely to result from variable cre activity. Nonetheless, our data also raise the possibility that different teeth may have different requirements for levels of Bmp signaling to complete organogenesis.

Our RT-PCR analysis showed that the *Nestin cre;Bmpr1a n/f* mutants mandibular ectoderm had mosaic cre activity, whereas maxilla had a more complete deletion of *Bmpr1a* at 11.5 dpc. The cap stage arrest is consistent with previous data showing that Bmp4 signaling to the forming enamel knot is crucial for the progression of tooth development (Bei et al., 2000; Chen et al., 1996; Jernvall et al., 1998).

Bmp4 in mesenchyme also has been proposed to induce expression of *p21*, and cell cycle arrest in cells of the prospective enamel knot. Once induced in enamel knot, Bmp4 functions to regulate tooth organ shape by controlling cell cycle progression and apoptosis. It is plausible that mosaic disruption of *Bmpr1a* in the dental epithelium would allow tooth germ development to progress beyond the bud stage and arrest at stages when enamel knot function would become important. Future experiments will be needed to investigate these ideas in more detail.

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