

## FGFs and BMP4 induce both *Msx1*-independent and *Msx1*-dependent signaling pathways in early tooth development

Marianna Bei<sup>1,2</sup> and Richard Maas<sup>1,\*</sup>

<sup>1</sup>Genetics Division, Department of Medicine, Brigham & Women's Hospital and Harvard Medical School, 20 Shattuck Street, Boston, MA 02115, USA

<sup>2</sup>Harvard School of Dental Medicine, Department of Oral Biology, 188 Longwood Avenue, Boston, MA 02115, USA

\*Author for correspondence (e-mail: maas@rascal.med.harvard.edu)

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### SUMMARY

During early tooth development, multiple signaling molecules are expressed in the dental lamina epithelium and induce the dental mesenchyme. One signal, BMP4, has been shown to induce morphologic changes in dental mesenchyme and mesenchymal gene expression via *Msx1*, but BMP4 cannot substitute for all the inductive functions of the dental epithelium. To investigate the role of FGFs during early tooth development, we examined the expression of epithelial and mesenchymal *Fgfs* in wild-type and *Msx1* mutant tooth germs and tested the ability of FGFs to induce *Fgf3* and *Bmp4* expression in wild-type and *Msx1* mutant dental mesenchymal explants. *Fgf8* expression is preserved in *Msx1* mutant epithelium while that of *Fgf3* is not detected in *Msx1* mutant dental mesenchyme. Moreover, dental epithelium as well as beads soaked in FGF1, FGF2 or FGF8 induce *Fgf3* expression in dental mesenchyme in an *Msx1*-dependent manner. These results indicate that, like BMP4, FGF8 constitutes an epithelial inductive signal capable of inducing the expression of downstream signaling molecules in dental mesenchyme via *Msx1*. However, the BMP4 and FGF8 signaling pathways are distinct. BMP4 cannot induce *Fgf3* nor can FGFs induce *Bmp4* expression in dental mesenchyme, even though both signaling molecules can induce *Msx1* and *Msx1* is necessary for *Fgf3* and *Bmp4* expression in dental mesenchyme.

In addition, we have investigated the effects of FGFs and BMP4 on the *distal-less* homeobox genes *Dlx1* and *Dlx2* and we have clarified the relationship between *Msx* and *Dlx* gene function in the developing tooth. *Dlx1, Dlx2* double

mutants exhibit a lamina stage arrest in maxillary molar tooth development (Thomas B. L., Tucker A. S., Qiu M., Ferguson C. A., Hardcastle Z., Rubenstein J. L. R. and Sharpe P. T. (1997) *Development* 124, 4811-4818). Although the maintenance of molar mesenchymal *Dlx2* expression at the bud stage is *Msx1*-dependent, both the maintenance of *Dlx1* expression and the initial activation of mesenchymal *Dlx1* and *Dlx2* expression during the lamina stage are not. Moreover, in contrast to the tooth bud stage arrest observed in *Msx1* mutants, *Msx1, Msx2* double mutants exhibit an earlier phenotype closely resembling the lamina stage arrest observed in *Dlx1, Dlx2* double mutants. These results are consistent with functional redundancy between *Msx1* and *Msx2* in dental mesenchyme and support a model whereby *Msx* and *Dlx* genes function in parallel within the dental mesenchyme during tooth initiation. Indeed, as predicted by such a model, BMP4 and FGF8, epithelial signals that induce differential *Msx1* and *Msx2* expression in dental mesenchyme, also differentially induce *Dlx1* and *Dlx2* expression, and do so in an *Msx1*-independent manner. These results integrate *Dlx1*, *Dlx2* and *Fgf3* and *Fgf8* into the odontogenic regulatory hierarchy along with *Msx1*, *Msx2* and *Bmp4*, and provide a basis for interpreting tooth induction in terms of transcription factors which, individually, are necessary but not sufficient for the expression of downstream signals and therefore must act in specific combinations.

Key words: mouse, FGFs, tooth development, *Dlx*, *Msx*, inductive signaling

### INTRODUCTION

Organogenesis is a complex process that results from a series of instructive and permissive cell-cell inductive interactions (Saxén, 1977; Wessels, 1977). In chicken embryos, morphogenetic signaling pathways have been partly elucidated in the limb bud (reviewed in Johnson and Tabin, 1997), somite (reviewed in Tajbakhsh and Spörle, 1998) and neural tube

(reviewed in Jessell and Goodman, 1996). In mammalian organogenesis, however, the regulatory pathways that control inductive signaling are largely unknown.

Murine molar tooth development provides an example of an organ in which some of the early inductive interactions and molecular signaling events are beginning to be defined (Vainio et al., 1993; Chen et al., 1996; Kratochwil et al., 1996; Vaahtokari et al., 1996; Neubüser et al., 1997; Jernvall et al.,

1998; reviewed in Thesleff et al., 1995; Maas and Bei, 1997; Thesleff and Sharpe, 1997). In the mouse embryo, molar tooth development commences morphologically at mouse embryonic day 11.5 (E11.5), with a thickening of the dental epithelium to form the dental lamina. Before E12.5, the dental epithelium can elicit tooth formation when recombined with neural-crest-derived second branchial arch mesenchyme, while the reciprocal combination fails (Mina and Kollar, 1987; Lumsden, 1988). Hence, at this time, the tooth-forming inductive potential resides in the prospective dental epithelium. The epithelium invaginates to form a tooth bud in the underlying dental mesenchyme, which proliferates and condenses. Subsequent to E12.5, dental mesenchyme can induce tooth formation when recombined with second arch epithelium, while recombinants containing dental epithelium and second arch mesenchyme fail. Thus a shift in odontogenic potential from dental epithelium to dental mesenchyme occurs at E12.5.

Recent studies have suggested that the TGF- $\beta$  superfamily member Bone Morphogenetic Protein 4 (BMP4) constitutes one component of the inductive signal that transfers tooth inductive potential from dental epithelium to mesenchyme (Vainio et al., 1993). *Bmp4* expression is first observed in the molar tooth at E11.5 in the dental lamina epithelium but then shifts at E12.5 to the dental mesenchyme, coincident with the shift in tooth developmental potential between tissue layers and BMP4 can induce morphologic changes in dental mesenchyme (Vainio et al., 1993; Turecková et al., 1995). *Msx* genes are also implicated in the epithelial-mesenchymal interactions involved in tooth development. *Msx1* is strongly expressed in the dental mesenchyme throughout the lamina, bud, cap and bell stages of odontogenesis (MacKenzie et al., 1991a,b, 1992; Maas et al., 1996). *Msx2* expression is initially restricted to the mesenchyme directly beneath the prospective dental lamina, thereafter localizing to the dental papilla mesenchyme and the epithelial enamel knot (MacKenzie et al., 1992). The involvement of *Msx* gene function in tooth development is demonstrated by *Msx1* knockout mice, which exhibit a highly penetrant arrest at the bud stage of molar tooth development (Satokata and Maas, 1994).

Insight into the genetic relationship between *Msx1* and *Bmp4* comes from experiments showing that *Bmp4* expression is reduced in the *Msx1* mutant tooth mesenchyme but is preserved in *Msx1* mutant epithelium (Bei et al., 1996; Chen et al., 1996). These results indicate that *Msx1* is required for the expression of *Bmp4* in the dental mesenchyme and that *Bmp4* therefore functions downstream of *Msx1* in the dental mesenchyme. On the contrary, epithelial *Bmp4* expression does not require *Msx1* for its expression and therefore acts upstream of *Msx1*. Experiments have shown that BMP4 can induce the expression in the dental mesenchyme of *Msx1* and its own expression (Vainio et al., 1993). However, in *Msx1* mutant dental mesenchyme, BMP4 cannot induce its own expression indicating that mesenchymal *Bmp4* expression requires *Msx1* function. Furthermore, addition of recombinant BMP4 to chemically defined media partly rescues the *Msx1* mutant tooth bud phenotype to the cap stage of odontogenesis (Chen et al., 1996), further substantiating the view that mesenchymal *Bmp4* functions downstream of *Msx1* and suggesting that mesenchymal BMP4 acts back upon the dental epithelium to mediate the reciprocal epithelial-mesenchymal interactions that occur during tooth morphogenesis.

Nonetheless, despite the fact that epithelial BMP4 is able to induce its own expression and that of *Msx1* in the dental

mesenchyme, BMP4 cannot substitute for all the inductive functions of the dental epithelium. For example, dental epithelium, but not recombinant BMP4, induces the expression of the heparan sulfate proteoglycan *syndecan-1* and cell proliferation of dental mesenchyme (Jernvall et al., 1994). This suggests that other epithelial factors besides BMP4 are responsible for the induction of cell proliferation and of *syndecan-1* gene expression. Plausible candidates for this role are members of the fibroblast growth factor family (FGFs). For example, FGF4 can stimulate cell proliferation of dental mesenchyme (Jernvall et al., 1994) and FGF4 and other FGFs can induce *syndecan-1* in the dental mesenchyme (Chen et al., 1996). However, *Fgf4* is expressed in the dental epithelium at E14.5 in the enamel knot, too late to be the natural inducer of dental mesenchyme at the lamina stage. In contrast, *Fgf1*, *Fgf2*, *Fgf8* and *Fgf9* are expressed in the early molar dental lamina, and FGF8 can induce a translucent zone in dental mesenchyme and has been proposed to act antagonistically with BMP4 to specify the sites of tooth initiation (Cam et al., 1992; Heikinheimo et al., 1994; Neubüser et al., 1997; Kettunen and Thesleff, 1998). In the dental mesenchyme, another member of the FGF family, *Fgf3*, has also been found to be expressed as early as E12.5 (Thesleff and Vaahtokari, 1992). However, the relationship of epithelial FGF signaling and mesenchymal *Fgf3* expression to *Msx* function during early tooth development remains unknown.

Targeted mutations in either of two members of the *distal-less* homeobox gene family, *Dlx1* or *Dlx2*, affect development of skeletal elements derived from the proximal ends of first and second branchial arches (Qiu et al., 1995, 1997) but have no effect on tooth development. However, mice compounded for mutations in both *Dlx1* and *Dlx2* exhibit a selective absence of upper molars and an arrest at the lamina stage (Thomas et al., 1997). This has been explained on the basis of functional redundancy with other *Dlx* genes expressed in mandibular but not maxillary mesenchyme. Although mesenchymal *Bmp4* and *Msx1* expression are preserved in the arrested maxillary molar tooth germs in *Dlx1*, *Dlx2* knockout mice (Thomas et al., 1997), less is known about how *Dlx* genes might fit into the genetic pathway that includes *Msx1* and *Bmp4*.

Here we show that FGFs that are expressed in the dental epithelium can induce *Fgf3* expression in the dental mesenchyme via a *Msx1*-dependent mechanism distinct from that by which epithelial BMP4 induces its own gene expression in dental mesenchyme. The data indicate that *Msx1* is necessary but not sufficient for the expression of inductive signaling molecules in the dental mesenchyme. In addition, the activation of *Dlx1* and *Dlx2* expression is preserved in *Msx1* mutant tooth germs, and *Msx1*, *Msx2* double mutants exhibit a tooth phenotype that resembles that in *Dlx1*, *Dlx2* double mutants in terms of stage of developmental arrest. FGF8 and BMP4 differentially induce *Dlx1* and *Dlx2* expression in the dental mesenchyme in a *Msx1*-independent fashion, further supporting the conclusion that *Dlx* and *Msx* genes act in parallel during early induction of the dental mesenchyme in response to epithelial signals.

## MATERIALS AND METHODS

### Postimplantation embryos and genotyping

Embryos were collected from matings of *Msx1*<sup>+/-</sup> × *Msx1*<sup>+/-</sup> mice or *Msx1*, *Msx2* double heterozygous mice maintained in a N5-6 BALB/c background, and fixed in 4% paraformaldehyde/phosphate-buffered

saline (PBS) for approximately 5 hours, dehydrated through increasing concentrations of ethanol and embedded in paraffin wax. The preparation, genotyping and detailed phenotype analysis of the *Msx2* mutant mice and *Msx* compound mutant embryos will be described in detail elsewhere (R. Maas, unpublished data). Genotyping of *Msx1* mutant mice was performed as previously described (Chen et al., 1996).

### Probes

Murine *Fgf3* and *Fgf8* probes (gifts from Gail Martin, University of California at San Francisco, California), and *Dlx1* and *Dlx2* probes (gift from John L. R. Rubenstein, University of California, San Francisco, CA) were used. A 1300 bp fragment of murine *Fgf3* cDNA subcloned into pBluescript was digested with *Bss*HII and transcribed with T7 RNA polymerase for an antisense probe. An 800 bp fragment of murine cDNA for *Fgf8* subcloned into pBluescript SK+ was digested with *Pst*I and transcribed with T7 RNA polymerase for an antisense probe. A 240 bp fragment of murine cDNA for *Dlx1* subcloned into pBS KDD was digested with *Bam*HI or *Eco*RV and transcribed with T7 or T3 RNA polymerase for antisense or sense probes, respectively. A 560 bp fragment of murine cDNA for *Dlx2* subcloned into a variant of pBS SK- (E61) plasmid was digested *Eco*RI and *Not*I and transcribed with T3 and T7 RNA polymerase for antisense and sense probes, respectively. The RNA probes were radiolabeled with [ $\alpha$ - $^{35}$ S]UTP or digoxigenin using T3 or T7 RNA polymerase. The probes were reduced to an average size of 100-150 bp by hydrolysis.

### Bead implantation experiments

Bead implantation and tissue recombination experiments were performed according to previously described procedures (Vainio et al., 1993; Chen et al., 1996). For bead implantation, Affi-Gel blue agarose beads (100-200 mesh, 75-150  $\mu$ m diameter, Bio-Rad) were incubated with 70-100 ng/ $\mu$ l recombinant human BMP4 protein (Genetics Institute, Cambridge, MA) at 37°C for 30 minutes, or heparin acrylic beads (Sigma, St Louis, MO) were incubated recombinant human FGF1 (700 ng/ $\mu$ l), human FGF2 (100 ng/ $\mu$ l) or mouse FGF8 (250 ng/ $\mu$ l) (R&D Systems, Minneapolis, MN) proteins at 37°C for 1 hour. Control beads were soaked with similar concentrations of BSA under the same conditions. Protein-soaked beads were stored at 4°C and used within 1 week. Freshly isolated dental mesenchymes were placed on Nuclepore filters (pore size, 0.1 mm), and protein-soaked beads were washed in PBS and placed on the top of the mesenchyme. All explants were cultured on the filters, supported by metal grids in Dulbecco's minimal essential medium with 10% FCS at 37°C for 24 hours. After culture, explants were fixed and processed for whole-mount in situ hybridization.

### In situ hybridization

Whole-mount in situ hybridization experiments were performed as previously described (Xu et al., 1997). For tissue section in situ hybridizations with  $\alpha$ - $^{35}$ S-labeled probes, 7  $\mu$ m sections were cut from wax-embedded embryos, dewaxed through xylene, rehydrated and refixed in 4% paraformaldehyde/phosphate-buffered saline.

Further section preparation and hybridization were then performed as described (Sassoon and Rosenthal, 1993). Slides were dipped in Kodak NTB2 radiographic emulsion diluted 1:1 with distilled H<sub>2</sub>O. Following 6-10 days of exposure at 4°C the slides were developed using Kodak D19 developer and fixed using Kodak fixer and counterstained in Hoësch fluorescent dye. Photographs were taken using Kodak EPY 64T film on Zeiss Axiophot microscope using dark-ground illumination.

### Histology

Histology was performed by fixation in 10% formalin or 4% paraformaldehyde followed by dehydration and embedding in paraffin. Sections were cut by microtome at 7  $\mu$ m and stained with hematoxylin and eosin.

## RESULTS

### *Msx1* is required for mesenchymal *Fgf3* but not for epithelial *Fgf8* expression in early tooth development

To investigate the relationship between *Msx1* function and *Fgf* expression in early tooth development, *Fgf8* and *Fgf3* expression in wild-type and *Msx1*-deficient molar tooth germs was examined by in situ hybridization (Fig. 1). *Fgf8* expression is maintained in E11.5 dental epithelium in *Msx1* mutant embryos, indicating that epithelial expression of *Fgf8* does not require *Msx1* (Fig. 1A,B). In contrast, at E13.5, corresponding to the stage at which tooth development arrests in the *Msx1* mutants, *Fgf3* expression was undetectable in the *Msx1*-deficient molar tooth mesenchyme (Fig. 1C,D). Some *Msx1* mutant tooth buds arrest earlier than E13.5; however, in situ hybridization experiments performed at E12.5 confirmed the above result (data not shown). Thus, while *Fgf8* expression in the dental epithelium does not require *Msx1*, *Fgf3* expression in the dental mesenchyme is *Msx1* dependent.

### FGFs require *Msx1* to induce *Fgf3* expression in the dental mesenchyme

To determine if FGFs can induce their own expression in dental mesenchyme and, if so, whether this induction requires *Msx1*, microdissected wild-type and *Msx1* mutant E11.5 molar mesenchymes were implanted with beads soaked in recombinant FGF1, FGF2 or FGF8. Following organ culture for 24 hours, the specimens were analyzed for *Fgf3* expression by whole-mount in situ hybridization (Fig. 2; Table 1). Experiments employing concentrations between 100 and 700 ng/ $\mu$ l for all three FGF-soaked beads gave similar results. FGF1, FGF2 and FGF8 all induce *Fgf3* expression in wild-type

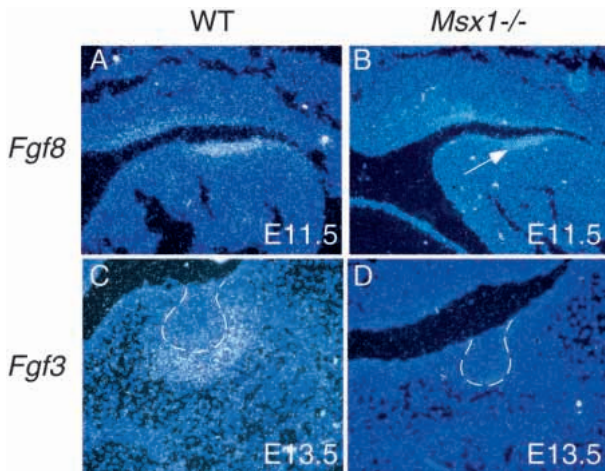
**Table 1. Induction of *Fgf3*, *Dlx1* and *Dlx2* expression in wild type and *Msx1***

Probe	Inducer								
	BMP4		aFGF		bFGF		FGF8		BSA
	+/+	-/-	+/+	-/-	+/+	-/-	+/+	-/-	
<i>Fgf3</i>	0/11	nd	4/6*	0/4	8/9	1/3*	9/15	0/3	0/5
<i>Dlx2</i>	18/18	2/3	nd	nd	5/6	1/1	20/23	8/9	0/7
<i>Dlx1</i>	0/3	nd	nd	nd	nd	nd	21/24	nd	0/2

nd, not done.

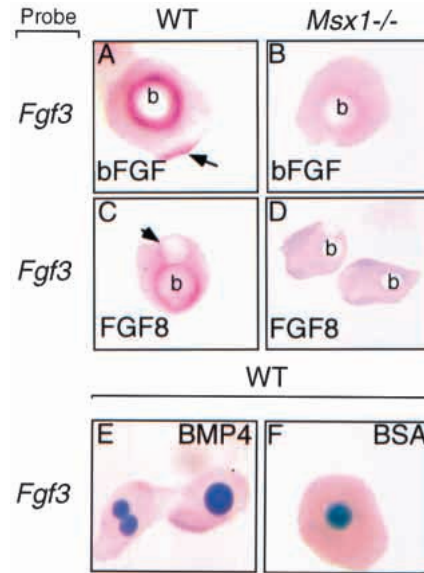
\*weak induction. The weak induction of *Fgf3* expression by aFGF is notable in that these experiments were performed employing a higher aFGF concentrations than used for other FGFs.





**Fig. 1.** *Msx1* is required for mesenchymal *Fgf3* but not for epithelial *Fgf8* expression in early tooth development. In situ hybridization analyses of *Fgf8* (A,B) and *Fgf3* (C,D) transcripts in wild-type (A,C) and *Msx1*-deficient (B,D) E11.5 (A,B) and E13.5 (C,D) first lower molar tooth germs at the dental lamina and bud stages of tooth development, respectively. The results demonstrate comparable expression of *Fgf8* in the mutant epithelium (B) relative to wild type (A). In contrast, reduced expression of *Fgf3* in the *Msx1* mutant mesenchyme (D) relative to wild type (C) is observed. The sections shown are representative of multiple independent experiments for each probe. The positions of the basal laminae in C and D, confirmed by hematoxylin staining, are indicated by dashed white lines.

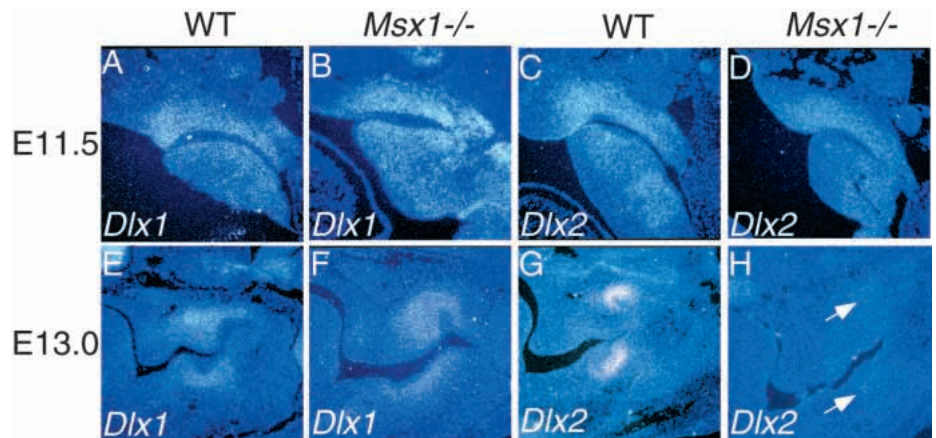
dental mesenchyme (Fig. 2A,C; Table 1). Experiments combining wild-type dental epithelium with wild-type dental mesenchyme also showed induction of *Fgf3* expression in the dental mesenchyme (Fig. 2A, arrow). However, while FGF1, FGF2 and FGF8 and dental epithelium all induced *Fgf3* expression in wild-type dental mesenchyme, none of these FGFs induced *Fgf3* expression above background in *Msx1* mutant dental mesenchyme (Fig. 2B,D; Table 1). Beads soaked in recombinant FGF8 were also tested, as a positive control, for their ability to induce *Msx1* expression in wild-type dental mesenchyme. As described (Kettunen and Thesleff, 1998),



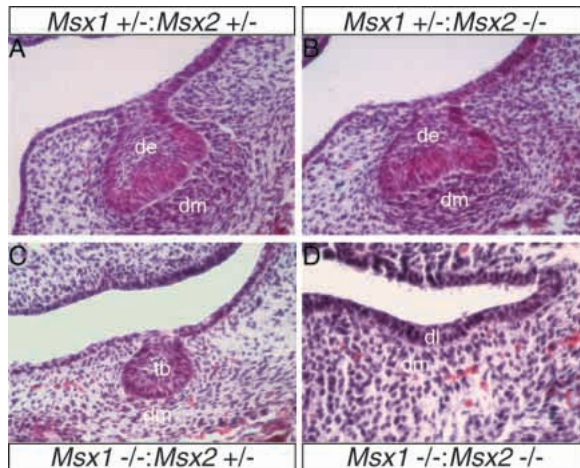
**Fig. 2.** FGFs but not BMP4 induce *Fgf3* expression in dental mesenchyme and induction by FGFs requires *Msx1*. FGF bead implantation experiments in wild-type (A,C,E,F) and *Msx1*-deficient (B,D) molar mesenchymes. bFGF (A,B) and FGF8 (C,D) agarose beads (soaked at 100 ng/μl and 250ng/μl, respectively) were implanted into microdissected E11.5 first lower molar tooth mesenchymes. After culture for 24 hours, the explants were analyzed by whole-mount in situ hybridization using a digoxigenin-labeled riboprobe for *Fgf3*. The white is the bead, the purple staining is the positive signal. A small piece of dental epithelium has been recombined with the mesenchyme at the edge of the rudiment (arrow in A). bFGF or FGF8 beads and epithelium induce *Fgf3* transcripts in the wild-type mesenchymes (A,C). However, neither FGF2 or 8 induces *Fgf3* expression in the *Msx1* mutant mesenchymes (B,D). BSA-soaked beads in wild-type mesenchyme (arrowhead in C) yield no staining for *Fgf3* expression. BMP4 beads (soaked at 70 ng/μl) were implanted in mesenchymal explants and analyzed by whole-mount in situ for *Fgf3*. Neither BMP4 nor BSA beads induce *Fgf3* expression in wild-type dental mesenchymes (E,F).

FGF8 induces *Msx1* expression in wild-type dental mesenchyme (data not shown). Conversely, control experiments

**Fig. 3.** *Msx1* is dispensable for initial *Dlx1* and *Dlx2* expression in dental mesenchyme, but is required for maintenance of *Dlx2* expression. In situ hybridization analyses of *Dlx1* transcripts in E11.5 and E13.0 wild-type (A,E) and *Msx1*-deficient (B,F) first upper and lower molar tooth germs. The results demonstrate that *Dlx1* expression is preserved in both upper and lower *Msx1* mutant dental mesenchyme at both dental lamina and tooth bud stages. In situ hybridization analyses of *Dlx2* transcripts in E11.5 and E13.0 wild-type (C,G) and *Msx1*-deficient (D,H) first upper and lower tooth germs. The results demonstrate that *Dlx2* expression is preserved in both upper and lower *Msx1* mutant dental mesenchyme at the dental lamina stage (D). Additional analyses through *Msx1* mutant lower tooth germs also show preserved *Dlx2* expression (data not shown). In contrast, reduced expression of *Dlx2* in upper and lower molar *Msx1* mutant mesenchyme relative to wild type is observed at the bud stage of tooth development (H). The sections shown are representative of multiple independent experiments for each probe.



Additional analyses through *Msx1* mutant lower tooth germs also show preserved *Dlx2* expression (data not shown). In contrast, reduced expression of *Dlx2* in upper and lower molar *Msx1* mutant mesenchyme relative to wild type is observed at the bud stage of tooth development (H). The sections shown are representative of multiple independent experiments for each probe.



**Fig. 4.** The *Msx* double mutant molar tooth arrests at the lamina stage of development. Arrest of molar tooth development at the dental lamina stage is observed in the *Msx* double mutant embryos. Histological sections (hematoxylin and eosin) of E14.5 *Msx1* and *Msx2* genetic compound mutant lower molar teeth show absence of a tooth phenotype in *Msx1*<sup>+/-</sup>, *Msx2*<sup>+/-</sup> and *Msx1*<sup>+/-</sup>, *Msx2*<sup>-/-</sup> embryos (A,B). In the *Msx1*<sup>-/-</sup>, *Msx2*<sup>+/-</sup> embryos an arrest of tooth development is observed at the bud stage (C), similar to *Msx1* mutants, while in the *Msx1*<sup>-/-</sup>, *Msx2*<sup>-/-</sup> embryos tooth development arrests at the dental lamina stage (D), or at an early bud stage (data not shown). Abbreviations: de, dental epithelium; dm, dental mesenchyme; dl, dental lamina; tb, tooth bud.

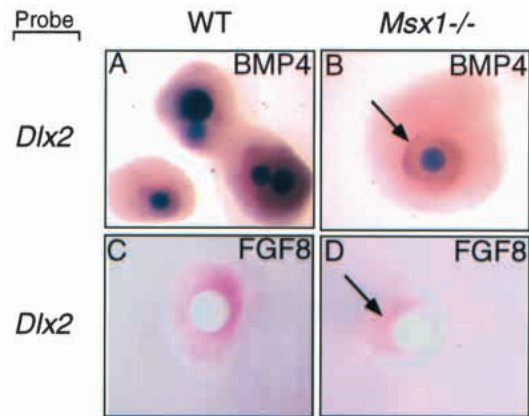
using beads containing BSA or sense riboprobes gave minimal if any signal (arrowhead in Fig. 2C and data not shown, respectively). These results indicate that *Msx1* is required for FGFs to induce *Fgf3* expression in dental mesenchyme.

#### BMP4 and FGFs act by independent pathways in inducing dental mesenchyme

Beads soaked in recombinant FGFs are not able to induce *Bmp4* expression in wild-type dental mesenchyme (data not shown, and Chen et al., 1996). To test whether BMP4 can induce *Fgf3* expression in dental mesenchyme, BMP4 beads were implanted in wild-type dental mesenchyme and the explants assayed by whole-mount in situ for *Fgf3* expression. Similar to control experiments using BSA beads, BMP4 beads cannot induce *Fgf3* expression in wild-type dental



**Fig. 5.** FGF8 and dental epithelium induce *Dlx1* expression in the wild-type dental mesenchyme. FGF8 beads (soaked at 50-70 ng/ $\mu$ l) were implanted into microdissected E11.5 first lower molar tooth mesenchymes. After culture for 24 hours, the explants were analyzed by whole-mount in situ hybridization using digoxigenin-labeled riboprobes for *Dlx1*. FGF8 beads and epithelium induce *Dlx1* transcripts in the wild type (A). Controls show that use of BSA-soaked beads (B) or sense riboprobes (data not shown) in wild-type mesenchyme yield no staining for *Dlx1*.



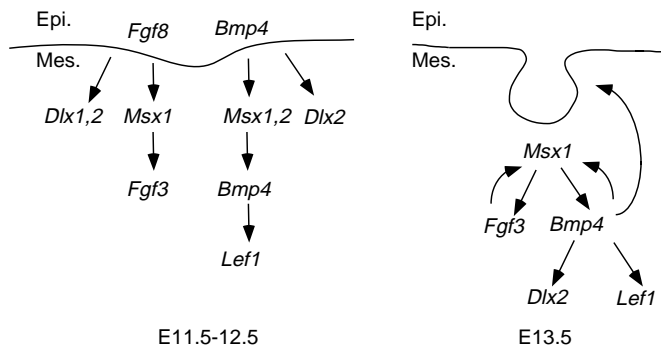
**Fig. 6.** BMP4 and FGF8 induce *Dlx2* expression in dental mesenchyme independent of *Msx1*. BMP4 and FGF8 bead implantation experiments in wild-type (A,C) and *Msx1*-deficient (B,D) molar mesenchymes. BMP4 and FGF8 beads (each soaked at 70 ng/ $\mu$ l) were implanted into microdissected E11.5 first lower molar tooth mesenchymes. After 24 hours, the explants were analyzed by whole-mount in situ hybridization using digoxigenin-labeled riboprobes for *Dlx2*. The blue or white is the bead, the purple staining is the positive signal. BMP4 and FGF8 beads and epithelium induce *Dlx2* transcripts in the wild-type and *Msx1* mutant dental mesenchymes (A-D). Controls show that use of BSA-soaked beads or *Dlx2* sense riboprobes yield no staining in wild-type mesenchyme (data not shown).

mesenchyme (Fig. 2E,F; Table 1). Thus, the BMP4- and FGF-signaling pathways are independent with respect to the induction of *Bmp4* and *Fgf3* expression in the dental mesenchyme. Since BMP4 and FGF8 can each induce *Msx1* expression and *Msx1* is necessary for *Fgf3* and *Bmp4* expression in dental mesenchyme, the failure of BMP4 and FGFs to respectively induce *Fgf3* and *Bmp4* expression indicates that *Msx1* is necessary but not sufficient for expression of these downstream genes in dental mesenchyme. Therefore, other factors may interact with the *Msx1* gene product, directly or indirectly, to provide sufficiency for downstream gene expression.

#### *Msx1* is dispensable for initial *Dlx1* and *Dlx2* expression in dental mesenchyme, but is required for maintenance of *Dlx2* expression

Members of the *distal-less* gene family could act coordinately with *Msx* in tooth induction, since *Msx* and *Dlx* homeoproteins interact in vitro (Zhang et al., 1997) and *Dlx1*, *Dlx2* double mutants exhibit a lamina stage arrest in maxillary molar tooth development which, although earlier and more restricted than the bud stage arrest in *Msx1* mutants, could nonetheless be developmentally related (Thomas et al., 1997). To test whether *Msx1* is required for *Dlx1* and *Dlx2* expression in the dental mesenchyme, in situ hybridization was performed in wild-type and *Msx1* mutant upper and lower molar mesenchymes (Fig. 3). At E11.5 *Dlx1* expression was present in *Msx1* mutant dental mesenchyme, indicating that early mesenchymal *Dlx1* expression does not require *Msx1* (Fig. 3A,B). At E11.0, *Dlx2* expression in both dental epithelium and mesenchyme was observed in *Msx1* mutant embryos (data not shown) and, at E11.5, *Dlx2* expression continued to be present in *Msx1* mutant





**Fig. 7.** A genetic pathway for early tooth morphogenesis. At E11.5 epithelial BMP4 and FGF8 require *Msx1* to induce members of their own gene families in dental mesenchyme. While both BMP4 and FGF8 can induce *Msx1*, only BMP4 can induce *Msx2* expression (Vainio et al., 1993; Kettunen and Thesleff, 1998). In addition, while BMP4 and FGF8 both induce *Dlx2* expression in the dental mesenchyme, only FGF8 can induce *Dlx1* expression. Moreover, BMP4 cannot induce *Fgfs*, and FGFs cannot induce *Bmp4* expression, suggesting that BMP4 and FGF8 act through separate *Msx1*-dependent pathways to induce expression of these downstream genes. At the bud stage of development, *Fgf3* is placed downstream of *Msx1* since its expression is reduced in the *Msx1* mutant dental mesenchyme. *Dlx2* is placed downstream of mesenchymal *Bmp4* because BMP4 induces *Dlx2* expression even in the absence of *Msx1*, and *Dlx2* expression is reduced in the *Msx1* mutant dental mesenchyme. See text for details.

dental mesenchyme at wild-type levels (Fig. 3C,D). At the bud stage of tooth development, E12.5-13.5, *Dlx1* expression was maintained at control levels in the *Msx1* mutant dental mesenchyme (Fig. 3E,F, shown for E13.0). In contrast, in both *Msx1* mutant upper and lower molar tooth germs, *Dlx2* expression was not detected (Fig. 3G,H; arrows). Thus, *Msx1* is dispensable for the initial expression of *Dlx1* or *Dlx2* in dental mesenchyme at the lamina stage of tooth development while, at the subsequent bud stage, *Dlx2* mesenchymal expression but not that of *Dlx1* requires *Msx1*.

#### ***Msx1*, *Msx2* double homozygotes arrest at the lamina stage of tooth development, similar to *Dlx1*, *Dlx2* double homozygotes**

To further test whether *Msx* and *Dlx* genes might act in parallel and whether functional redundancy between *Msx1* and *Msx2* might mask an earlier requirement for *Msx* function during tooth initiation, tooth development in *Msx1*, *Msx2* compound mutant embryos was analyzed histologically (Fig. 4). At E14.5, tooth development in *Msx1*<sup>+/-</sup>-*Msx2*<sup>-/-</sup> embryos exhibits no phenotype and resembles that in *Msx1*<sup>+/-</sup>-*Msx2*<sup>+/-</sup> and wild-type control embryos (Fig. 4A,B). Thus, prior to E14.5, *Msx2* appears to play a non-essential role in tooth development. In *Msx1*<sup>-/-</sup>-*Msx2*<sup>+/-</sup> embryos, tooth development arrests at the bud stage, similar to the arrest observed in *Msx1* single mutants (Fig. 4C) (Satokata and Maas, 1994). In contrast, molar tooth development in some *Msx1*<sup>-/-</sup>-*Msx2*<sup>-/-</sup> double mutant embryos arrests at the dental lamina stage, with only a rudimentary thickening of the oral ectoderm (Fig. 4D). In other cases, immature dysplastic tooth buds were observed (data not shown). *Bmp4* and *Fgf8* expression is preserved in the *Msx1*<sup>-/-</sup>-*Msx2*<sup>-/-</sup> double mutant dental lamina (data not

shown). These results indicate that *Msx1* and *Msx2* function redundantly during tooth initiation in dental mesenchyme, where *Msx2* expression is superimposed transiently upon that of *Msx1* (MacKenzie et al., 1992). Moreover, the initial requirement for *Msx* gene function during tooth development coincides with the time when *Dlx1* or *Dlx2* gene function is required in prospective maxillary molar tooth development.

#### **FGF8 and dental epithelium but not BMP4 induce *Dlx1* expression in dental mesenchyme**

For *Msx* and *Dlx* to act in parallel in early tooth morphogenesis predicts that their mesenchymal expression should be coordinately regulated by dental epithelial factors. To determine whether FGF8 or BMP4 can induce *Dlx1* expression in the dental mesenchyme, beads containing FGF8 or BMP4 were implanted in wild-type E11.5 dental mesenchymes. Beads soaked in BMP4 produced minimal or no induction of *Dlx1* expression in the dental mesenchyme (data not shown, Table 1), while similarly treated beads strongly induced *Msx1* and *Dlx2* (see below). In contrast, FGF8 strongly induced *Dlx1* expression in wild-type dental mesenchyme at E11.5 (Fig. 5A, Table 1). Control experiments employing beads soaked in BSA or using a *Dlx1* sense riboprobe gave no signal (Fig. 5B, Table 1). Experiments combining wild-type dental epithelium with wild-type dental mesenchyme also showed induction of *Dlx1* expression in the dental mesenchyme (arrow in Fig. 5A).

#### **BMP4 and FGF8 induce *Dlx2* expression in dental mesenchyme independent of *Msx1***

To determine whether BMP4 or FGFs induce *Dlx2* expression in dental mesenchyme and whether this induction requires *Msx1*, beads containing BMP4, bFGF or FGF8 were implanted in isolated wild-type and *Msx1* mutant E11.5 dental mesenchymes. BMP4 induced *Dlx2* expression strongly and equally well in wild-type and *Msx1* mutant molar mesenchymes (Fig. 6A,B, Table 1). Control experiments employing beads soaked in BSA or using a *Dlx2* sense riboprobe gave no signal (Table 1). Induction of *Dlx2* expression in the dental mesenchyme was also observed when wild-type dental epithelium was recombined with wild-type dental mesenchyme (data not shown). FGF8 also induced *Dlx2* expression in wild-type dental mesenchyme and in *Msx1* mutant molar mesenchyme (Fig. 6C,D). Control experiments employing beads soaked in BSA gave no signal (data not shown and Table 1). Thus, *Msx1* is not required for *Dlx2* induction in the dental mesenchyme by either BMP4 or FGF8.

## **DISCUSSION**

#### **FGFs require *Msx1* to regulate epithelial-mesenchymal signaling during tooth development**

Based on the above results, a genetic model for early tooth development is presented (Fig. 7). At the dental lamina-early bud stage (E11.5-12.5), the model places epithelial *Fgf8* upstream of mesenchymal *Msx1* for the following reasons. *Fgf8* is known to be expressed as early as E10 in the oral ectoderm (Neubüser et al., 1997, and refs. cited therein), FGF8 is able to induce *Msx1* expression in the dental mesenchyme, mimicking a function of the oral ectoderm (Kettunen and Thesleff, 1998) and epithelial *Fgf8* expression is preserved in

both *Msx1* and *Msx1,Msx2*-deficient dental epithelia. Beginning at E12.5, *Fgf3* is expressed in dental mesenchyme (Thesleff and Vaahtokari, 1992), and *Msx1* is placed upstream of mesenchymal *Fgf3* because *Fgf3* expression is not detected in *Msx1* mutant dental mesenchyme. This model is further supported by bead implantation experiments demonstrating that FGF8 and other FGFs can induce *Fgf3* expression in the dental mesenchyme in a manner that requires *Msx1*. In addition, BMP4 is not able to induce *Fgf3* expression in wild-type dental mesenchyme and FGFs are not able to induce *Bmp4* expression (Chen et al., 1996). These results indicate that epithelial BMP4 and FGF8 act by different *Msx1*-dependent pathways to induce expression of members of their respective gene families in dental mesenchyme. Thus, the initiation step of tooth development may be considered as consisting of at least two separate *Msx1*-dependent pathways, corresponding to *Msx1* induction by either FGF8 or BMP4 (Fig. 7).

FGF8 is known to act as an inductive signal in different developmental systems (Vogel et al., 1995; Lee et al., 1997; Crossley et al., 1996; Richman et al., 1997). Our data suggest that an FGF-dependent signaling pathway participates in the regulation of tooth morphogenesis. Previously, BMP4 has been proposed to act as a signaling molecule mediating early epithelial-mesenchymal interactions during tooth morphogenesis (Vainio et al., 1993). However, since FGF8 is able to induce *Fgf3* expression in the dental mesenchyme at the lamina-bud transition stage when odontogenic potential shifts from the epithelium to the mesenchyme, FGFs may constitute an additional component of the signaling cascade mediating odontogenic epithelial-mesenchymal interactions.

For the moment, the role of mesenchymal FGFs such as FGF3 in odontogenesis remains unclear. *Fgf3* knockout mice do not exhibit an overt tooth phenotype (Mansour et al., 1993) and *FGF3* has been excluded as a gene defect in human hypodontia (Nieminen et al., 1996). However, this could be explained by functional redundancy between FGF3 and mesenchymally expressed FGF7 (Finch et al., 1995). FGF receptors are expressed during tooth development in both dental mesenchyme and epithelium. The FGFR2b splice variant, encoding a potential receptor for FGF3, FGF7 and FGF10, is expressed in the cap stage enamel organ, while FGFR1, capable of binding several FGFs, is expressed in dental papilla mesenchyme (Orr-Urtreger et al., 1991, 1993; Peters et al., 1992; Ornitz et al., 1996; Igarashi et al., 1998). It is thus possible that, analogous to the function proposed for mesenchymal BMP4 (Chen et al., 1996), mesenchymal FGFs bind to epithelial receptors to promote further epithelial development. A similar model where an epithelially expressed FGF ligand binds to a receptor that is expressed in adjacent mesenchyme and vice versa has been proposed for the limb bud (Xu et al., 1998).

It is also possible that FGF3 has direct effects within the dental mesenchyme. Since the FGF bead implantation experiments are performed at E11.5 but culture continues for an additional 24 hours, the induction of *Msx1* expression by FGFs could mimic either the effects of epithelial FGF8 or the effects of endogenous mesenchymal FGF3. The model presented here is compatible with mesenchymal FGF3 helping to maintain *Msx1* expression by a positive feedback loop. In addition, *Fgf3* was originally isolated as the *int-2* proto-oncogene, and thus is implicated in the regulation of cellular

proliferation (Dickson and Peters, 1987). Other FGFs have been shown to induce cell proliferation in dental mesenchyme (Jernvall et al., 1994) and FGF3 could represent an endogenous FGF that performs this function. Further experiments are required to address whether mesenchymal FGF3 acts directly upon the dental epithelium or acts autonomously within the dental mesenchyme.

### ***Msx* and *Dlx* genes are coordinately regulated and function in parallel in dental mesenchyme during tooth initiation**

Mice homozygous deficient for both *Dlx1* and *Dlx2* genes exhibit an arrest of upper molar tooth development at the dental lamina stage, and in situ hybridization experiments show that epithelial *Fgf8* and mesenchymal *Msx1* and *Bmp4* expression is preserved in the *Dlx* double mutant upper molar mesenchyme (Thomas et al., 1997). Conversely, in *Msx1* mutants, we find that at the initiation stage, epithelial *Fgf8* and mesenchymal *Dlx1* and *Dlx2* expression is preserved, while subsequent mesenchymal *Bmp4* expression is absent. Moreover, BMP4 and FGF8 are able to induce both *Msx1* and *Dlx2* expression in the dental mesenchyme while *Dlx1* expression is induced by FGF8. These results suggest that *Msx* and *Dlx* might act in parallel at the dental lamina stage (Fig. 7). Analysis of tooth development in *Msx1,Msx2* double mutants further supports this idea. While the *Msx1* mutant arrests at the bud stage and the *Msx2* mutant exhibits only later defects in tooth development (M. B., unpublished data), *Msx1,Msx2* double homozygotes exhibit an arrest at the dental lamina stage, which affects all molar teeth, but otherwise phenocopies the *Dlx1,Dlx2* double mutant phenotype in the maxillary molar dentition. This suggests that *Msx1* and *Msx2* function redundantly in early dental mesenchyme, and indicates that *Msx1* or *Msx2* function is required as early as tooth initiation, despite the later bud stage arrest in *Msx1* mutants. Furthermore, recombination experiments confirm that *Dlx1,Dlx2* and *Msx1* function is required solely in the dental mesenchyme for tooth morphogenesis (Thomas et al., 1997; K. Kratochwil, personal communication). Thus, at the initiation stage, we propose that *Dlx1,Dlx2* and *Msx1,Msx2* act in parallel in the dental mesenchyme, subject to differential induction by epithelial BMP4 and FGF8. Although these results do not show whether *Msx* or *Dlx* expression is directly activated by BMPs or FGFs in mouse tooth mesenchyme, in *Xenopus Msx1* expression is an immediate early response to BMP4 (Suzuki et al., 1997).

In contrast to the pathway presented for the initiation stage of tooth development, at the bud stage, *Dlx2* is placed downstream of mesenchymal *Bmp4* because *Dlx2* is reduced in *Msx1* mutant dental mesenchyme and bead implantation experiments show that BMP4 is able to induce *Dlx2* expression even in the absence of *Msx1*. These results suggest that, while *Dlx1* and *Dlx2* are likely to function in parallel with *Msx1* and *Msx2* at the lamina stage, *Dlx2* expression at the bud stage resides downstream of *Msx1*. This latter relationship suggests a requirement for mesenchymal *Fgf3* and *Bmp4* to maintain *Dlx2*, but not that of *Dlx1*, in the dental mesenchyme. However, the data do not exclude the possibility that, at the bud stage, *Dlx2* is a direct target for regulation by the *Msx1* gene product.

In *Dlx1,Dlx2* mutants, an abnormal area of *Barx-1*-negative mesenchymal cells underlying the maxillary molar tooth germ

coincides with an area of ectopic *Sox9* expression and with ectopic cartilage formation (Thomas et al., 1997). This result has been interpreted as a patterning defect resulting from mis-specification of migratory or premigratory odontogenic neural crest, distinct from mutants in epithelial-mesenchymal inductive tissue interactions such as *Msx1* and *Lef1* (Thomas et al., 1997). We suggest two provisos to this interpretation of the *Dlx1,Dlx2* mutant phenotype. First, the conclusion that the *Dlx1,Dlx2* mutant is unique as a patterning mutant must acknowledge that the most likely basis for the selective loss of maxillary molars is redundant function with *Dlx3*, *Dlx5* and *Dlx6* which are expressed in mandibular but not maxillary mesenchyme (Thomas et al., 1997; Qiu et al., 1997). Thus, there is no particular reason why the *Dlx* and *Msx* mutants need to be mechanistically distinct. Second, it is by no means clear that similar findings might not also pertain to tooth development in *Msx1,Msx2* double mutant embryos. For example, in the absence of proper induction of the dental mesenchyme, odontogenic mesenchyme might well be expected to acquire an altered fate such as cartilage or bone. In fact, newborn *Msx1* mutants exhibit ectopic bone formation in the region normally fated to form the molar tooth (see Fig. 3f, Satokata and Maas, 1994). Thus, we favor the interpretation that similar to the *Msx1,Msx2* mutant, the *Dlx1,Dlx2* mutant represents an early inductive failure within the dental mesenchyme.

### ***Msx1* must act with other transcription factors to regulate gene expression in dental mesenchyme**

Factors such as BMP4 are able to induce *Msx1* expression in dental mesenchyme, but they are not able to induce the expression of genes such as *Fgf3* for which *Msx1* function is required (Fig. 7). This indicates that *Msx1* is necessary but not sufficient for the induction of these signaling molecules in dental mesenchyme, and suggests that additional, coordinately induced factors are needed for *Msx1* to regulate gene expression. Thus, it is attractive to think that additional factors might interact directly with *Msx1* or, alternatively, might act separately from but simultaneously with *Msx1* on target gene promoters to regulate transcription. Recent in vitro data show that *Msx1* and *Dlx2* can form homodimeric and heterodimeric complexes via dimerization through their homeodomains (Zhang et al., 1997). Although it is unclear whether these homeoproteins interact or regulate transcription in vivo, a model has been proposed whereby *Msx* and *Dlx* gene products inhibit each others transcriptional properties (Zhang et al., 1997).

Other potential candidates for this role are members of the Lim-homeodomain family, *Lhx6* and *Lhx7*, which are specifically expressed in the oral aspect of the maxillary and mandibular mesenchyme in response to FGF8 but not BMP4, and are subsequently expressed in tooth mesenchyme (Grigoriou et al., 1998). In addition, *Pax9*, a member of the *Pax* family, is strongly expressed in the dental mesenchyme from E12-14, and *Pax9* knockout mice exhibit an arrest of tooth development that phenocopies the *Msx1* mutant (Peters et al., 1998).

Interestingly, *Pax9*, like *Msx*, *Dlx* and *Lhx* is differentially regulated by BMP4 and FGFs (Neubüser et al., 1997). It has been shown that *Bmp4* and *Fgf8* exhibit wide but overlapping expression domains in the mandibular arch and that BMP2 and

BMP4 antagonize the induction of mesenchymal *Pax9* by FGF8, thus helping to establish the position of prospective tooth mesenchyme. Although our bead implantation experiments were not analyzed for such antagonistic effects, it is clear that FGF and BMP epithelial signals provide different molecular functions in induction of the dental mesenchyme. In contrast to its repressive effects on *Pax9*, BMP4 activates the mesenchymal expression of *Msx1*, *Msx2* and *Dlx2*. Thus, the specific combination of factors that are induced in dental mesenchyme will differ depending upon whether the overlying ectoderm is expressing FGF8, BMP4 or both. The existence of spatially and molecularly distinct signaling pathways could provide a mechanism for the conversion of distinct ectodermal expression domains into unique combinations of transcription factors that are expressed in spatially distinct domains in the underlying mesenchyme. This would serve to preserve – as also occurs in the developing limb – the information that will provide polarity and pattern during subsequent organ development.

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