BMP4 rescues a non-cell-autonomous function of Msx1 in tooth development

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SUMMARY

The development of many organs depends on sequential epithelial-mesenchymal interactions, and the developing tooth germ provides a powerful model for elucidating the nature of these inductive tissue interactions. In Msx1-deficient mice, tooth development arrests at the bud stage when Msx1 is required for the expression of Bmp4 and Fgf3 in the dental mesenchyme (Bei, M. and Maas, R. (1998) Development 125, 4325-4333). To define the tissue requirements for Msx1 function, we performed tissue recombinations between wild-type and Msx1 mutant dental epithelium and mesenchyme. We show that through the E14.5 cap stage of tooth development, Msx1 is required in the dental mesenchyme for tooth formation. After the cap stage, however, tooth development becomes Msx1 independent, although our experiments identify a further late function of Msx1 in odontoblast and dental pulp survival. These results suggest that prior to the cap stage, the dental epithelium receives an Msx1-dependent signal from the dental mesenchyme that is necessary for tooth formation.

To further test this hypothesis, Msx1 mutant tooth germs were first cultured with either BMP4 or with various FGFs for two days in vitro and then grown under the kidney capsule of syngeneic mice to permit completion of organogenesis and terminal differentiation. Previously, using an in vitro culture system, we showed that BMP4 stimulated the growth of Msx1 mutant dental epithelium (Chen, Y., Bei, M. Woo, I., Satokata, I. and Maas, R. (1996). Development 122, 3035-3044). Using the more powerful kidney capsule grafting procedure, we now show that when added to explanted Msx1-deficient tooth germs prior to grafting, BMP4 rescues Msx1 mutant tooth germs all the way to definitive stages of enamel and dentin formation. Collectively, these results establish a transient functional requirement for Msx1 in the dental mesenchyme that is almost fully supplied by BMP4 alone, and not by FGFs. In addition, they formally prove the postulated downstream relationship of BMP4 with respect to Msx1, establish the non-cell-autonomous nature of Msx1 during odontogenesis, and disclose an additional late survival function for Msx1 in odontoblasts and dental pulp.

Key words: Msx, BMP4, Organogenesis, Rescue, Tooth, Mice

INTRODUCTION

The development of many vertebrate organs depends on sequential and reciprocal inductive signaling between epithelium and mesenchyme (Grobstein, 1967; Wessells, 1977; Saxén, 1977; Gurdon, 1992). Experiments in which the epithelium and mesenchyme are systematically separated and recombined have provided insight into the nature of signaling interactions that operate between tissue layers.

In the developing mouse molar tooth germ, such experiments have shown that early (embryonic day (E) 9.5-E12.0) oral epithelium possesses odontogenic potential and can elicit tooth formation in non-dental mesenchyme. With the early bud stage, developmental dominance shifts to the mesenchyme, which can now induce non-oral epithelium to form an enamel organ (Kollar and Baird, 1969; Mina and Kollar, 1987; Lumsden, 1988). Expression and functional analysis have implicated the TGF-β superfamily member bone morphogenetic protein 4 (BMP4) as a possible inductive signal that transfers tooth inductive potential from dental epithelium to mesenchyme (Vainio et al., 1993). Bmp4 expression in the molar is first observed in the thickened E11.5 dental lamina epithelium, but then shifts to the dental mesenchyme by E12.5. Moreover, BMP4 induces its own expression, indicating that mesenchymal Msx1 function is demonstrated by Msx1 knockout mice, which exhibit an 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 Msx1-deficient dental mesenchyme but is preserved in Msx1 mutant epithelium (Bei et al., 1996; Chen et al., 1996). Moreover, in Msx1 mutant dental mesenchyme, BMP4 cannot induce its own expression, indicating that mesenchymal Bmp4 expression requires Msx1 function. In addition, recombinant BMP4 weakly stimulates dental epithelial growth in in vitro culture (Chen et al., 1996). These results indicate that Msx1 is
required for the expression of Bmp4 in the dental mesenchyme and suggest that mesenchymal BMP4 is likely to function downstream of Msx1. However, the absence of specific markers to identify the developmental stages attained in these experiments, the limitations of the in vitro culture system – which does not efficiently support tooth development beyond intermediate stages of odontogenesis – and the fact that a variety of growth factors promote the proliferation of embryonic epithelia somewhat nonspecifically, indicate that the question of the odontogenic function of Msx1 remains open.

The genetic hierarchies that operate during early tooth development also involve other genes besides Bmp4 and Msx1 (Kratochwil et al., 1996; Vahtokari et al., 1996; Neubüser et al., 1997; Jernvall et al., 1998; Bei and Maas, 1998; reviewed in Thesleff et al., 1995; Maas and Bei, 1997; Thesleff and Sharpe, 1997; Peters and Balling 1999). Members of the fibroblast growth factor family (FGFs) are expressed in the early molar dental lamina (E11.5), and one of them, FGF8, appears to act antagonistically with BMP4 to specify sites of tooth initiation (Neubüser et al., 1997). Another member of the FGF family, Fgf3, is expressed in the dental mesenchyme, in this case from the bud stage (Thesleff and Vahtokari, 1992). Fgf8 expression is preserved in Msx1 mutant epithelium, while that of Fgf3 is not detected in Msx1 mutant dental mesenchyme (Bei and Maas, 1998). Moreover, dental epithelium as well as FGF-soaked beads induce Fgf3 expression in the dental mesenchyme in an Msx1-dependent manner. These results indicate that epithelial BMP4 and FGF8 act in an Msx1-dependent fashion to induce expression of members of their respective gene families in the dental mesenchyme (Bei and Maas, 1998). However, the precise role of Msx1 in tooth morphogenesis and mesenchymal signaling and the function of mesenchymally expressed BMP4 and FGF3 in tooth development is unclear. The present study was undertaken to address these issues.

MATERIALS AND METHODS

Embryos and genotyping
Embryos were collected from matings of Msx1+/− × Msx1+/− mice maintained in N7-N8 BALB/c (Boston, MA; for bead rescue experiments) and C57BL/6 (Boston, MA – for bead rescue experiments; and Salzburg, Austria – for tissue recombination) backgrounds. The day of plug discovery was designated as E0.5. Genotyping and fixation of Msx1 mutant embryos in the bead rescue and BrdU experiments were performed as previously described (Bei and Maas, 1998). For tissue recombinations, Msx1−/− embryos were unambiguously identifiable by E13.5 from their incisor phenotype, and from E14.75 also by their cleft palate. Msx1+/− and Msx1−/− embryos are phenotypically indistinguishable and were both used as donors of wild-type tissues.

Tissue dissection and recombination
At E10.5 to E12.5 the entire jaw (oral aspect) was used, from E13.5 onwards, mandibular and maxillary molars were dissected individually. Epithelium and mesenchyme were separated after 30-60 minutes incubation in 0.1% collagenase (type I, Sigma, St Louis, MO) in Dulbecco’s minimal essential medium (D-MEM) at 37°C, and subsequently recombined on Nuclepore filters (type 110405; with 0.1 μm pore size; Costar) in a manner faithful to their original orientation (Kratochwil et al., 1996). After 2-3 days’ incubation in vitro, in D-MEM supplemented with 10% FCS and 10% chick embryo extract, recombinants were transplanted under the kidney capsule of syngeneic male hosts where they were allowed to develop for 9-12 days. The stage of tooth development was assessed histologically (Kratochwil et al., 1996). Mutant (+/−) wild-type (+/+ or −/−) combinations were usually done with tissues taken from littermates, except for the experiments using a Colal1 (+/+) lacZ transgene as a marker. Transgenic mice carrying three to five copies of a reporter gene construct containing the promoter and 8.7 kb of 5’ upstream sequences of the mouse Colal1 gene (coding for the α1 chain of collagen type 1) fused to the lacZ reporter gene with the SV40 nuclear localization signal, were produced and kindly provided by N. Ghaffari-Tabrizi (Institute of Molecular Biology, Salzburg). This transgene is faithfully expressed in cells producing collagen I, and thus at particularly high levels in differentiated odontoblasts. These transgenic (tg) mice were crossed into the Msx1 colony to produce tg/tg; Msx1−/− males, whose offspring all carried the reporter transgene.

Biological rescue assays
For bead implantation, Affi-Gel blue agarose beads (100-200 mesh, 75-150 μm diameter, Bio-Rad) were incubated with 100 ng/μl recombinant human BMP4 (Genetics Institute, Cambridge, MA) at 37°C for 30 minutes. Because sufficient recombinant FGF3 was unavailable, owing to poor solubility when bacterially expressed, other FGFS were employed as surrogates. FGF7 and FGF10 share high homology in their sequences with FGF3, and also interact efficiently with the same FGFR2 receptor as FGF3 (Ornitz et al., 1996; Igarashi et al., 1998). Therefore, heparin acrylic beads (Sigma, St. Louis, MO) were incubated with recombinant human FGF7 (200 ng/μl) or human FGF10 (200 ng/μl), or with human FGF4 (250 ng/μl) or mouse FGF8 (250 ng/μl) (R&D Systems, Minneapolis, MN) at 37°C for 1 hour. Control beads were soaked with similar concentrations of BSA under the same conditions. Freshly isolated wild-type and Msx1−/− tooth rudiments were collected from E13.5 embryos and placed on Nuclepore filters with the dental epithelium facing down. 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 (D-MEM) supplemented with 10% FCS, 10% chick embryo extract at 37°C for 48 hours. After culture, explants, still implanted with beads, were transplanted under the kidney capsule of adult syngeneic mice for 9-12 days, to allow development of teeth, which were processed for histology.

BrdU labeling of paraffin sections and cultured explants
Female Msx1−/− E13.5 pregnant mice were injected i.p. with 50 μg/gm body weight of 5-bromo-2′-deoxyuridine (BrdU) (Sigma). After 4 hours, embryo heads were collected, fixed in 10% neutral buffered formalin overnight, processed for histology and sectioned at 10 μm. Sections were incubated with BrdU antibody (Boehringer Mannheim) overnight at 4°C. BrdU incorporation was detected using horseradish-peroxidase (HRP) conjugated mouse IgG with a diaminobenzidine color reaction. BrdU labeling of cultured explants as whole mounts was performed as described (Kettunen et al., 1998). For tissue sections, a series of 18 sections from four Msx1 mutant and 18 sections from four wild-type mice were stained with BrdU, photographed by digital camera, imported into Adobe Photoshop and the total number of labeled cells determined.

RESULTS

Msx1 is transiently required in dental mesenchyme and functions in a non-cell-autonomous manner in tooth development

To define the tissue requirements for Msx1 function, we
performed reciprocal tissue combinations between dental epithelium and mesenchyme from Msx1 mutant and wild-type (+/+ or +/−) embryos. Lower jaws (E10.5-E12.5) or molar rudiments (E13.5-E16.5) were dissected at different stages of development and the epithelium was enzymatically separated from the mesenchyme. Experimental tissue combinations were first cultured for two days in vitro and then grown under the kidney capsule of syngeneic mice to permit completion of organogenesis and terminal differentiation. Tooth development was assessed in histological sections.

At all stages tested, homochronic recombinations of mutant epithelium and wild-type mesenchyme produced fully differentiated teeth at high frequency (Table 1; Fig. 1A,C). In addition, a more limited set of tissue recombinations of Msx1 mutant prospective incisor epithelium and wild-type mesenchyme at E13.5 and E14.5 also resulted in efficient formation of incisor teeth (Fig. 2A-C). Transient Msx1 expression in presumptive incisor epithelium has been demonstrated at E10.5 by in situ hybridization (Tucker et al., 1998). However, our recombination experiments indicate that Msx1 function is not required in dental epithelium for tooth development.

In contrast, the developmental capacity of reciprocal recombinants of wild-type dental epithelium and Msx1 mutant mesenchyme depended on the stage at which the tissues were isolated: up to E14.5, such recombinations failed to develop teeth, the epithelium forming keratinized cysts instead (Table 1, data not shown). The same combinations done with E15.5 tissues, however, yielded teeth in 36% of cases. Although these teeth were significantly smaller than their reciprocal counterparts, they exhibited well-differentiated odontoblast and ameloblast layers that had deposited both a dentin and an enamel matrix (Fig. 1B,D). Remarkably, however, in several cases degeneration of the odontoblast layer and dental pulp was

Table 1. Recombination experiments performed between wild-type and Msx1 mutant tissues

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tissue source</th>
<th>Epi (+/−): Mes (+)</th>
<th>Epi (+): Mes (+/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10.5</td>
<td>Entire mandible</td>
<td>6/11</td>
<td>0/11</td>
</tr>
<tr>
<td>E11.5</td>
<td>Entire mandible</td>
<td>4/5</td>
<td>0/4</td>
</tr>
<tr>
<td>E12.5</td>
<td>Entire mandible</td>
<td>5/17</td>
<td>0/17</td>
</tr>
<tr>
<td>E13.5</td>
<td>Mandibular molar</td>
<td>11/9*</td>
<td>2/10</td>
</tr>
<tr>
<td>E14.5</td>
<td>Mandibular molar</td>
<td>25/25*</td>
<td>1/14*</td>
</tr>
<tr>
<td>E15.5</td>
<td>Mandibular molar</td>
<td>21/21</td>
<td>34/94</td>
</tr>
<tr>
<td>E16.5</td>
<td>Mandibular molar</td>
<td>15/18</td>
<td>2/19</td>
</tr>
</tbody>
</table>

Teeth formed in experimental tissue recombinations between wild-type (Msx1+/+ or +/−; designated as +) and mutant (Msx1−/−) dental epithelium and mesenchyme performed at different stages of development. From E10.5 to E12.5, entire mandibles were used for tissue recombinations; from E13.5 onwards only mandibular molars.

*The E13 and E14 molar rudiments give rise to both first and second molars; hence more teeth were obtained than combinations created.
†These sporadic teeth may be due to small amounts of contaminating wild-type mesenchymal cells.
observed, with marked hypocellularity (Fig. 1D). The fact that substantial amounts of dentin were present in these teeth suggests that the loss of odontoblasts and pulpal cells occurred relatively late during the in vivo culture period.

**A tissue-specific marker verifies that Msx1 mutant mesenchyme can form odontoblasts**

To exclude the possibility that the wild-type epithelium used in the previous experiments was contaminated with small amounts of wild-type mesenchyme, owing to incomplete enzymatic separation prior to recombination, we employed a transgene marker for the identification of Msx1 mutant odontoblasts. A transgenic line carrying a Cola1-(nls) lacZ reporter gene that is strongly expressed in odontoblasts was crossed into the Msx1 mutant strain for the production of transgenic Msx1/− embryos. Wild-type embryos used in these experiments were derived from a nontransgenic C57BL/6 colony.

As in the previous experiments, recombinations of E15.5 wild-type epithelium with Msx1 mutant mesenchyme carrying the Cola1-(nls) lacZ transgene yielded small but well differentiated teeth. In seven out of eight teeth examined, the entire odontoblast layer stained strongly with X-gal, providing evidence not only for its mutant origin but, owing to the expression of the lacZ reporter in odontoblasts, also for proper odontoblast differentiation (Fig. 3A,B).

**Teeth can form in double recombinants with both tissues being Msx1 mutant**

The fact that normal tooth development requires Msx1 function only in the mesenchyme is consistent with the restriction of Msx1 expression to dental mesenchyme between E11.5 and E17.5 (http://bite-it.helsinki.fi/). Surprisingly, however, the requirement for Msx1 is both transient and non-cell-autonomous, with Msx1 function becoming dispensable by E15.5. This suggests that prior to E15.5, an Msx1-dependent signal elaborated by dental mesenchyme acts on the epithelium in a manner that that is both necessary and sufficient for further tooth development. According to this hypothesis, recombinations of E15.5 wild-type dental epithelium and Msx1 mutant dental mesenchyme form teeth because the epithelium has already received this signal from its prior contact with wild-type mesenchyme in vivo, before its separation and recombination with mutant mesenchyme. Once this signal is received, tooth development can proceed in an Msx1-independent manner to stages of dentin and enamel synthesis.

To test this hypothesis further, we performed double recombinations in which, eventually, both tissues were Msx1 mutant. In the first step, E14.5 Msx1 mutant epithelium was associated with wild-type mesenchyme. These explants were cultured for 3 days in vitro, a time period chosen to permit the mutant epithelium to be induced by the hypothetical Msx1-dependent factor(s). Then the tissues were separated again, and the mutant epithelium was associated with fresh mutant mesenchyme (Fig. 3C). Thus, neither tissue in the final recombination possessed a functional Msx1 gene, but the epithelium had been transiently exposed to wild-type mesenchyme. These double recombinations yielded teeth at low frequency (7/65) and of small size, but with fully developed ameloblast and odontoblast layers and a clearly identifiable dentin matrix (Fig. 3D,E). We conclude that transient exposure of dental epithelium to Msx1-dependent mesenchymal factor(s) is sufficient to promote further tooth development in the absence of functional Msx1. However, the mesenchymal degeneration noted in some cases (even after odontoblast differentiation, as evidenced by the deposition of dentin), suggests an additional, late requirement for Msx1 function in odontoblast differentiation and/or survival. Similarly, when Cola1-(nls) lacZ transgenic mutant epithelial buds containing an adherent layer of mutant mesenchyme were combined with wild-type non-transgenic mesenchyme, X-gal staining mutant odontoblasts were underrepresented or absent (data not shown). This result suggests that in addition to its non-cell-autonomous function, Msx1 executes a cell-autonomous function in tooth development. However, only the non-cell-autonomous function of Msx1 appears to be essential for tooth formation.

![Fig. 3. Use of the Cola1-(nls) lacZ transgene identifies Msx1 mutant odontoblasts by X-gal staining.](image-url)
BMP4, but not FGFs, rescues the Msx1 mutant tooth phenotype

Previous work indicates that at the bud stage of tooth development, the expression of both Bmp4 and Fgf3 in the dental mesenchyme is Msx1 dependent. To determine whether either BMP4 or FGF3 might constitute the Msx1-dependent signal inferred from our single and double tissue recombination experiments, we co-cultured wild-type and Msx1-deficient E13.5 tooth germs with beads soaked in various recombinant FGFs, in recombinant BMP4 or with both types of beads. Following organ culture for 48 hours in the presence of growth factor-containing beads, the rudiments were transferred under the kidney capsule of syngeneic mice for 9-12 days after which the stage of tooth development was assessed by histology.

As expected, wild-type tooth germs, cultured with or without BMP4, or with BSA, yielded teeth that differentiated to advanced stages of enamel and dentin matrix deposition (Fig. 4A,D). In these cases, exposure of wild-type tooth germs to exogenous BMP4 had no apparent effect on their subsequent development. In addition, neither BSA-treated nor untreated Msx1 mutant tooth germs showed no development, and keratinized cysts formed instead (not shown). In 39% of cases, addition of BMP4 to Msx1 mutant tooth germs resulted in rescue to stages of dentin and enamel secretion, although the rescued teeth are smaller than controls (note differences in scale bar, below). (HL) Msx1 mutant teeth cannot be rescued with FGF4, 7, 8 or 10 and cysts (c) form instead. Sections were stained with H&E and are representative of multiple independent experiments. am, ameloblasts; b, bead; c, keratinized cyst; d, dentin; e, enamel; eo, enamel organ; iee, inner enamel epithelium; o, odontoblasts; p, dental pulp. Scale bars, 200 μm in A,D,G; 100 μm in B,C,E,F; 400 μm in H,I.

Fig. 4. BMP4 but not FGF rescues the Msx1 mutant tooth phenotype. Following exposure to BMP4 or FGF-soaked beads, tooth germs were transferred under the kidney capsule of syngeneic mice for 9-12 days, then assessed by histology. (A,D,G) Wild-type tooth germs treated with BMP4, FGF or BSA (not shown) produced teeth exhibiting normal cytodifferentiation and matrix deposition. In contrast, BSA-treated and untreated Msx1 mutant tooth germs showed no development, and keratinized cysts formed instead (not shown). (B,C,E,F) In 39% of cases, addition of BMP4 to Msx1 mutant tooth germs resulted in rescue to stages of dentin and enamel secretion, although the rescued teeth are smaller than controls (note differences in scale bar, below). (HL) Msx1 mutant teeth cannot be rescued with FGF4, 7, 8 or 10 and cysts (c) form instead. Sections were stained with H&E and are representative of multiple independent experiments. am, ameloblasts; b, bead; c, keratinized cyst; d, dentin; e, enamel; eo, enamel organ; iee, inner enamel epithelium; o, odontoblasts; p, dental pulp. Scale bars, 200 μm in A,D,G; 100 μm in B,C,E,F; 400 μm in H,I.
mesenchyme and is capable of bypassing the functional requirement for Msx1 in tooth formation. The results also support the conclusion that BMP4 constitutes the Msx1-dependent mesenchymal signal inferred from the recombination experiments.

The fact that the BMP4 rescued teeth were smaller and formed less enamel than wild-type explants could suggest that additional factors also participate in the epithelial-mesenchymal interactions that result in tooth formation. Msx1 is required for the expression of both Bmp4 and Fgf3 in the dental mesenchyme. Owing to its poor solubility, recombinant human or mouse FGF3 is not available. However, FGF7 and FGF10 are mesenchymally expressed FGFs that use the same cognate epithelial FGF receptor isoform, FGFR2b, as FGF3. Therefore, we carried out a series of rescue experiments analogous to those for BMP4 with these FGFs, as well as with FGF4, which is normally expressed in the dental epithelium. However, when FGF4, 7 and 10 were employed, Msx1 mutant teeth were not rescued, there was no developmental progression, and cyst formation was again noted (Fig. 4H,I). These results indicate that FGFs alone are not sufficient to bypass the mesenchymal requirement for Msx1 in tooth development and therefore do not function analogously to BMP4. In addition, when FGF was added together with BMP4, the results were indistinguishable from those obtained with BMP4 alone (data not shown).

**FGFs require Msx7 to stimulate mesenchymal cell proliferation**

We next sought to determine the role of mesenchymal FGFs in tooth morphogenesis. FGFs can act as mitogens in the developing tooth (Jernvall et al., 1994; Kettunen and Theisleff, 1998; Vainio et al., 1993; Kettunen et al., 1998). The reduced size of the teeth that formed in our tissue recombination and in the BMP4 rescue experiments suggested the possibility of a cell proliferation defect that was not rescued by either of these experimental manipulations. To test this hypothesis, we analyzed BrdU incorporation in wild-type and Msx1 mutant tooth germs at E13.5. Cell proliferation was significantly reduced in Msx1-deficient dental mesenchyme with an average of 72±35 (mean±s.d.) BrdU-labeled cells per section, compared with 215±13 in wild-type embryos (P<0.001) (Fig. 5A,B). Interestingly, in the dental epithelium, the number of proliferating cells in Msx1 mutants was comparable with wild type, but their distribution differed. In wild-type dental epithelium, a zone of reduced cell proliferation corresponding to the prospective enamel knot was observed at the distal tip of the tooth bud (Fig. 5A). This cell population was not seen in the Msx1 mutant dental epithelium and BrdU-positive cells were evenly distributed (Fig. 5B).

To determine whether FGFs could rescue the cell proliferation defect in Msx1 deficient dental mesenchyme, beads soaked in recombinant FGFs were implanted in wild-type (Fig. 5C,D) and Msx1-deficient dental mesenchymes (Fig. 5E). Explants were cultured for 24-32 hours and assayed for BrdU incorporation. Neither epithelially (FGF4, 8) nor mesenchymally (FGF7) expressed FGFs were able to induce cell proliferation in Msx1 mutant dental mesenchyme (Fig. 5E). This result indicates that the mitogenic effect of FGFs on the dental mesenchyme is Msx1-dependent.

**DISCUSSION**

Our single and double tissue recombination results indicate that Msx1 is involved in a key signaling event between the dental mesenchyme and epithelium. The BMP4-rescued teeth provide compelling proof that BMP4 is a key Msx1-dependent mesenchymal signal required at the transition from the bud to the cap stage. The localization of the Type I BMP4 receptor Alk6 to bud-stage dental epithelium supports the view that BMP4 acts directly on this tissue (ten Dijke et al., 1994; Dewulf et al., 1995). Although the Msx1 mutant tooth phenotype is fully penetrant and Msx1 and Msx2 are not normally co-expressed in the dental mesenchyme, it is theoretically possible that compensatory upregulation of Msx2 could account for the rescue in our experiments. However, we have not found any evidence for compensatory Msx2 upregulation in Msx1 mutant tissues (Rauchman, M. and Maas, R., unpublished). Thus, the data are entirely consistent with the conclusion that BMP4 is a major effector of Msx1 mesenchymal function in odontogenesis.

What is the specific function of BMP4 in tooth development? During tooth formation, Bmp4 expression in dental mesenchyme at the bud and cap stages coincides with formation of the enamel knot, a small population of cells at the tip of the epithelial bud that constitutes an important signaling center in tooth development (Vaahtokari et al., 1996). These cells are characterized by expression of the cyclin-dependent kinase inhibitor p21 (Cdkn1a – Mouse Genome Informatics) and by a cessation of cell proliferation. Considering that BMP4 induces p21 expression in isolated dental epithelia (Jernvall et al., 1998; Vaahtokari et al., 1996), it is attractive to suggest that BMP4 is transiently required to induce cell-cycle arrest in the localized area of the dental epithelium that will form the enamel knot. FGF signaling, however, does not rescue the Msx1 mutant tooth germs, which is of particular significance since FGFs but not BMP4 are able to rescue Left1 mutant teeth (van Genderen et al., 1994; Kratchowil, K. et al., unpublished). This specificity of action is consistent with the prevailing view that signaling molecules and transcription factors act during organogenesis in distinct molecular combinations. Here, we show that mesenchymal cell proliferation is Msx1-dependent and that mesenchymal FGFs, although downstream of Msx1 in terms of their expression, are unable to abrogate this proliferation defect. Thus, FGF signaling controls cell proliferation in an Msx1-dependent manner.

One potential explanation for this result would be if Msx1 function was required for appropriate expression of FGF receptors in the developing tooth. For example, recent data demonstrates that Fgfr2IIIb-deficient mice exhibit craniofacial phenotypes such as cleft palate and anodontia similar to those found in Msx1-deficient mice (De Moerlooze et al., 2000). This phenotypic similarity raises the possibility that both genes act in the same developmental pathway. A second explanation relates to the fact that FGFs act in coordination with FGF low-affinity heparin sulfate proteoglycan receptors (HSRGs) to induce dimerization of FGFs and biological responses (Schlessinger et al., 1995; Plotnikov et al., 1999). Interestingly, the potential low-affinity receptor syndecan I is not expressed
in \(Mx\)l-deficient dental mesenchyme (Bei et al., 1996; Chen et al., 1996). Thus, the inability of FGFs to induce cell proliferation in \(Mx\)l mutant dental mesenchyme may relate to a requirement for \(Mx\)l for expression of one or more components of the FGF signal transduction pathway. Alternatively, \(Mx\)l may interact directly with components of this pathway via protein-protein interactions. In either case, the \(Mx\)l dependence of FGF signaling in the dental mesenchyme provides a plausible explanation for the failure of FGF to improve upon the developmental rescue accomplished by BMP4.

An additional interesting finding in these experiments is that, in addition to its major role as an inductive signal acting on the epithelium, \(Mx\)l apparently executes a second function in the maintenance of odontoblast and dental pulp survival. Dentin matrix was typically present in recombinant teeth that formed from mutant dental mesenchyme, indicating that functional odontoblasts had initially formed. However, when analyzed, a significant proportion of these dentin-containing teeth lacked odontoblasts and dental pulp cells. This result is consistent with the expression of \(Mx\)l in odontoblasts and dental pulp cells (http://bite-it.helsinki.fi/), and with the emerging view that \(Mx\)l genes execute multiple, temporally distinct functions during the formation of individual organs (Satokata et al., 2000).

In summary, we show that BMP4 is a major effector of \(Mx\)l function during tooth development, responsible for the control of tooth morphogenesis, and that while FGFs may control mesenchymal cell proliferation, they do so in an \(Mx\)l-dependent manner. It becomes evident that these signaling factors evoke, individually or in combination, different biological functions controlling cell differentiation and proliferation in a time and tissue specific manner.

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