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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 and that of the Msx1 homeobox transcription factor in the dental mesenchyme. The requirement for *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; Vaahtokari 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 Vaahtokari, 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 $Msx1^{+/-} \times Msx1^{+/-}$ 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 used 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 *Cola1-(nls) 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 *Cola1* gene (coding for the α1 chain of collagen type I) 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

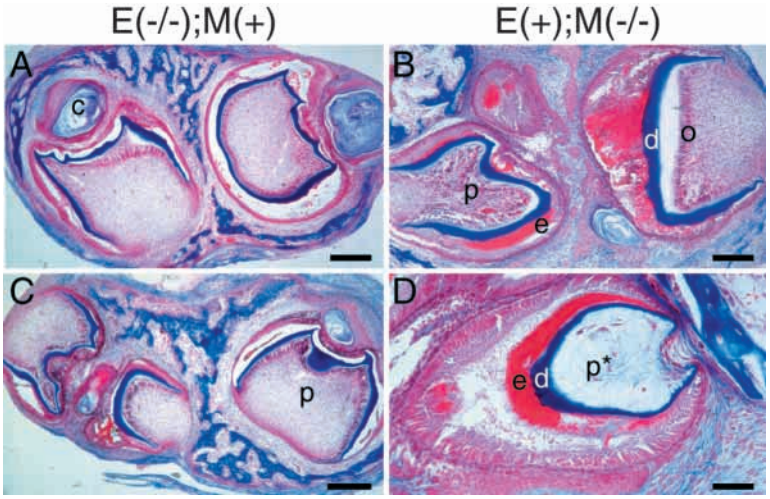

Fig. 1. Molar tooth formation becomes *Msx1* independent after the cap stage. Molar tooth development in *Msx1* mutant (–/–)/wild-type (+/+ or +/–, denoted +) combinations of dental epithelium (E) and mesenchyme (M). (A,C) Combinations of mutant epithelium with wild-type mesenchyme form teeth irrespective of the stage of donor embryos. (B,D) Combinations of wild-type epithelium and mutant mesenchyme develop teeth only when created on or after E15.5. Although smaller (note differences in scale), these teeth exhibit normal cytodifferentiation of dentin-secreting odontoblasts and enamel-secreting ameloblasts. (D) In some cases, degeneration of the odontoblast cell layer and the dental pulp was observed, even after dentin deposition. Sections were stained with Azan dichromic stain to differentiate between blue dentin and red enamel matrix. c, keratinized epithelial cyst; d, dentin; e, enamel; o, odontoblasts; p, dental pulp; p*, degenerating dental pulp. Scale bars, 200 μm in A,C; 100 μm in B; 65 μm in D.

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 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

Stage	Tissue source	Epi (–/–); Mes (+)	Epi (+); Mes (–/–)
E10.5	Entire mandible	6/11	0/11
E11.5	Entire mandible	4/5	0/4
E12.5	Entire mandible	5/17	0/17
E13.5	Mandibular molar	11/9*	2/10‡
E14.5	Mandibular molar	25/23*	1/14‡
E15.5	Mandibular molar	21/21	34/94
E16.5	Mandibular molar	15/18	2/19

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.

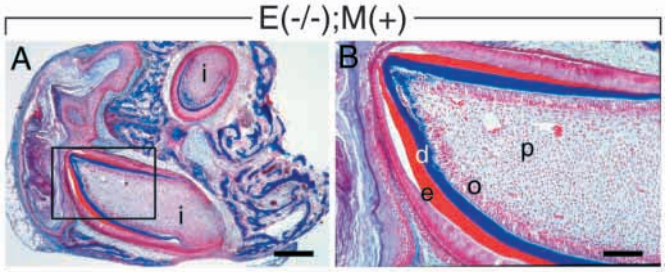

Fig. 2. Incisor tooth formation does not depend on early *Msx1* epithelial function. (A,B) Combinations of E14.5 mutant epithelium (E–/–) and wild-type mesenchyme (M+) form incisor teeth (i) that exhibit normal cytodifferentiation of dentin-secreting odontoblasts and enamel-secreting ameloblasts. Sections were stained with Azan dichromic stain to differentiate between blue dentin and red enamel matrix. The boxed region in A is shown at higher power in B. d, dentin; e, enamel; i, incisor tooth; o, odontoblasts; p, dental pulp. (C) Incisor teeth formed in experimental tissue recombinations between *Msx1* mutant dental epithelium and mesenchyme performed at different stages of development. Scale bars, 200 μm in A; 40 μm in B.

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.

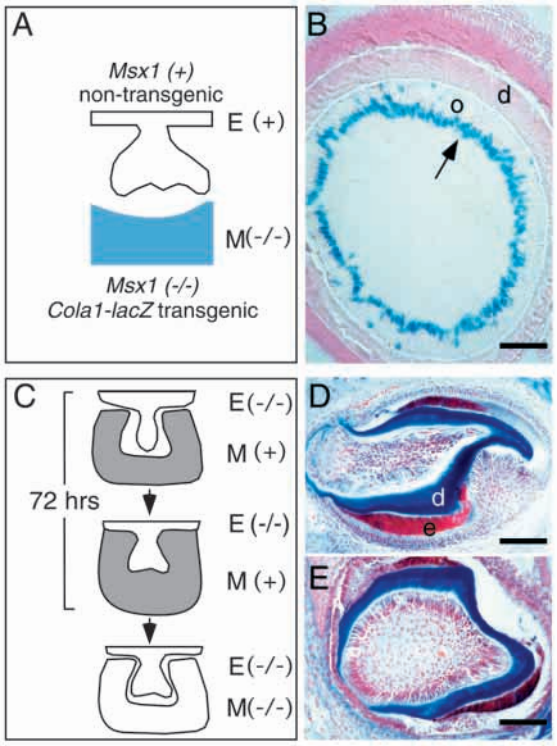

Fig. 3. Use of the *Cola1-(nls) lacZ* transgene identifies *Msx1* mutant odontoblasts by X-gal staining. (A,B) Combinations of E15.5 wild-type, nontransgenic dental epithelium with mutant mesenchyme carrying the *Cola1-(nls) lacZ* transgene result in teeth in which all nuclei of the odontoblast layer stain for *lacZ* activity (arrow, counterstained with Eosin). (C-E) Teeth can form from entirely *Msx1*-deficient tissues. (C) Scheme of double-recombination experiments. In the first recombination, mutant epithelium (white) is combined with wild-type mesenchyme (gray) and cultured for 72 hours. Next, induced mutant epithelium (white) is combined with mutant mesenchyme (white) which replaces the wild-type mesenchyme. (D,E) Double recombinations of mutant epithelium and mutant mesenchyme produce teeth with enamel (e) and dentin (d), although neither tissue contains a functional *Msx1* gene. Sections were stained with Azan dichromic stain to differentiate blue dentin and red enamel. E, epithelium; M, mesenchyme. Scale bars, 40 μm in B; 200 μm in D,E.

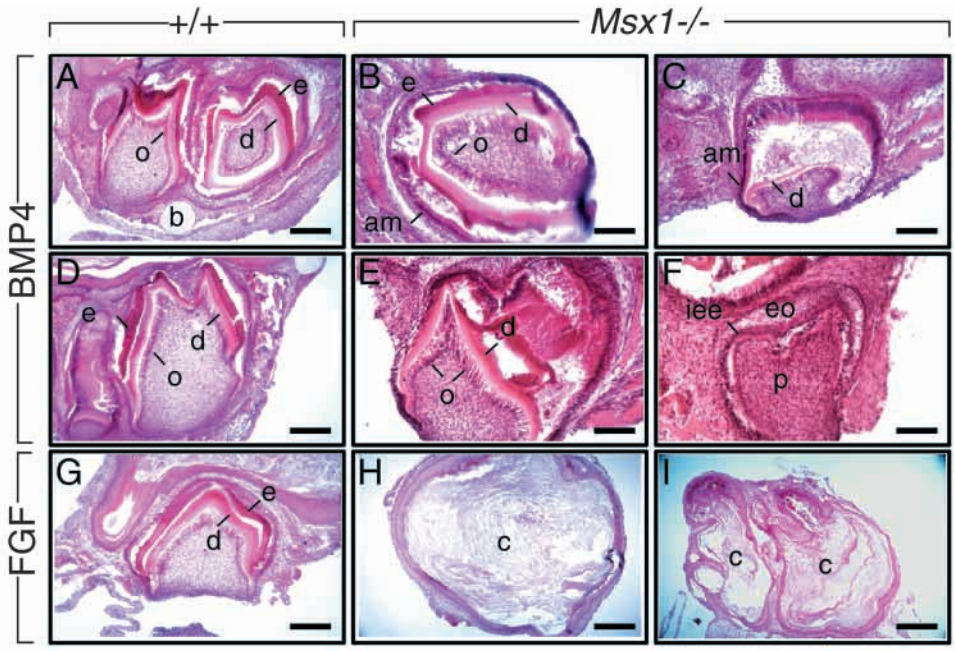

Fig. 4. BMP4 but not FGF rescues the *Msx1* mutant tooth phenotype. Following exposure to BMP4 or FGF-soaked beads, tooth germs were transferred under the kidney capsule of syngeneic mice for 9-12 days, then assessed by histology. (A,D,G) Wild-type tooth germs treated with BMP4, FGF or BSA (not shown) 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). (H,I) *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.

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 or untreated *Msx1* mutant tooth germs showed evidence of further development beyond the original bud stage, instead forming in all cases keratinized cysts (n=11, data not shown). Strikingly, however, in 39% (7/18) of cases, *Msx1* mutant teeth were rescued with BMP4 to the stage of dentin formation (Fig. 4B,C,E), while others showed various degrees of developmental progression (Fig. 4F). Although the rescued teeth that formed were approx. 50% of wild type in size, in all cases a functional odontoblast layer had developed, as shown by the deposition of a dentin matrix (Fig. 4B,C,E). Moreover, a well-differentiated ameloblast layer was present in all teeth that formed, and in one case (Fig. 4B), enamel matrix was present. These results formally establish that BMP4 acts downstream of *Msx1* in the dental

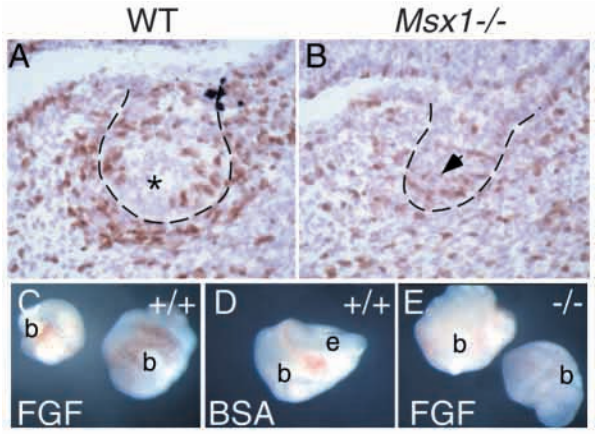

Fig. 5. Altered cell proliferation in *Msx1*-deficient tooth germs. (A,B) Compared with wild type, reduced numbers of BrdU-incorporated cells are observed in the *Msx1* mutant dental mesenchyme. In wild-type dental epithelium, reduced cell proliferation is observed in the prospective enamel knot region (*), whereas in *Msx1* mutant epithelium, proliferative cells are observed in this region (arrow). Broken lines indicate the epithelial-mesenchymal junction. (C-E) FGFs induce cell proliferation in dental mesenchyme by an *Msx1*-dependent mechanism. (C) FGF4- (right) or FGF7 (left)-soaked beads (b) each induce cell proliferation in wild-type dental mesenchyme. (D) BSA beads fail to induce proliferation in wild-type mesenchyme. (E) In contrast to their effect on wild-type mesenchyme, neither FGF4 (not shown) nor FGF7 induce proliferation in *Msx1* mutant dental mesenchyme. e, epithelium.

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 mensenchyme. 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 *Msx1* 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 Thesleff, 1998; Vainio et al., 1993; Kettunen et al., 1998). The reduced size of the teeth that formed in our tissue recombinations 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 epithelia, 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 (Jernvall et al., 1998). In contrast, in *Msx1*-deficient tooth germs, the prospective enamel knot either does not form or, if it does, its cells fail to cease 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 *Lef1* mutant teeth (van Genderen et al., 1994; Kratochwil, 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 (HSPGs) to induce dimerization of FGFRs and biological responses (Schlessinger et al., 1995; Plotnikov et al., 1999). Interestingly, the potential low-affinity receptor syndecan 1 is not expressed

in *Msx1*-deficient dental mesenchyme (Bei et al., 1996; Chen et al., 1996). Thus, the inability of FGFs to induce cell proliferation in *Msx1* mutant dental mesenchyme may relate to a requirement for Msx1 for expression of one or more components of the FGF signal transduction pathway. Alternatively, Msx1 may interact directly with components of this pathway via protein-protein interactions. In either case, the *Msx1* 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 dental epithelium, *Msx1* 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 *Msx1* in odontoblasts and dental pulp cells (http://bite-it.helsinki.fi/), and with the emerging view that *Msx* 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 *Msx1* 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 *Msx1*-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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