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Msx1 controls inductive signaling in mammalian tooth morphogenesis

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SUMMARY

Members of the *Msx* homeobox family are thought to play important roles in inductive tissue interactions during vertebrate organogenesis, but their precise developmental function has been unclear. Mice deficient for *Msx1* exhibit defects in craniofacial development and a failure of tooth morphogenesis, with an arrest in molar tooth development at the E13.5 bud stage. Because of its potential for experimental manipulation, the murine molar tooth germ provides a powerful system for studying the role of *Msx* genes in inductive signaling during organogenesis. To further analyze the role of *Msx1* in regulating epithelial-mesenchymal interactions during tooth morphogenesis, we have examined the expression of several potential *Msx1* downstream genes in *Msx1* mutant tooth germs and we have performed functional experiments designed to order these genes into a pathway.

Our results show that expression of Bone Morphogenetic Protein 4 (BMP4), the HMG box gene *Lef1* and the heparan sulfate proteoglycan syndecan-1 is specifically reduced in *Msx1* mutant dental mesenchyme, while expression of the extracellular matrix protein tenascin is unaffected. BMP4 soaked beads can induce *Bmp4* and *Lef1* expression in explanted wild-type dental mesenchymes, but only *Lef1* expression in *Msx1* mutant dental mesenchyme. We thus conclude that epithelial BMP4 induces its own expression in dental mesenchyme in a manner that requires *Msx1*. In turn, we show that addition of BMP4 to *Msx1* deficient tooth germs bypasses the requirement for *Msx1* and rescues epithelial development from the bud stage to the E14.5 cap stage. Lastly, we show that FGFs induce syndecan-1 expression in dental mesenchyme in a manner that also requires *Msx-1*. These results integrate *Msx1* into a regulatory hierarchy in early tooth morphogenesis and demonstrate that *Msx1* is not only expressed in dental mesenchyme in response to epithelial signals, but also in turn regulates the reciprocal expression of inductive signals in the mesenchyme which then act back upon the dental epithelium. We propose that *Msx* genes function repetitively during vertebrate organogenesis to permit inductive signaling to occur back and forth between tissue layers.

Key words: BMP4, epithelial-mesenchymal interaction, homeobox gene, inductive signalling, knockout mice, *Msx1*, tooth development

INTRODUCTION

Inductive tissue interactions between apposed epithelial and mesenchymal tissue layers are used recurrently throughout vertebrate development to direct the formation of many organs (reviewed by Thesleff et al., 1995). An initial instructive signal, provided by one of the tissue layers, is followed by a reciprocal exchange of inductive signals, resulting in a stepwise determination of both tissue components. Classical developmental studies have shown that this process, termed secondary induction, applies to vertebrate organs as disparate as the kidney, mammary and salivary glands, tooth and hair follicle (Saxén, 1987; Grobstein, 1967; Kratochwil, 1969; Kollar, 1970; Hardy, 1992).

A paradigm for inductive interactions between tissue layers has been elucidated for mesodermal induction in *Xenopus*, where it has been shown that peptide growth factors of the TGF-β superfamily are critical signals in the specification of mesoderm by endoderm (reviewed by Kessler and Melton, 1994; Jessell and Melton, 1992). In the context of *Drosophila* organogenesis, expression of *decapentaplegic* (*dpp*), which encodes a member of the TGF-β superfamily, is directly regulated in visceral mesoderm by the Ultrabithorax (Ubx) homeoprotein, and Dpp is instrumental in signaling between mesoderm and endoderm in specifying midgut constriction (Capovilla et al., 1994; reviewed by Bienz, 1994). In a separate example in *Drosophila*, ectodermal Dpp regulates the mesodermal expression of *tinman* and the induction of visceral and cardiac mesoderm (Frasch, 1995). In vertebrate embryos, morphogenetic signaling pathways have been partly elucidated in limb bud (Riddle et al., 1993; Niswander et al., 1994; Laufer et al., 1994; Yang and Niswander, 1995), somite (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Munsterberg et al., 1995; Pourquié et al., 1996) and neural tube (Echelard et al., 1993; Roelink et al., 1995; Liem et al., 1995). In mammalian organogenesis, however, the regulatory pathways which 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 have begun to be defined (Vainio

et al., 1993; reviewed by Thesleff et al., 1995). The first observable morphogenetic event in molar tooth development occurs at E11.5, when the oral epithelium thickens in the presumptive tooth forming region to form the dental lamina. At E12.5, the lamina invaginates into the surrounding dental mesenchyme to form a tooth bud. The mesenchyme proliferates and condenses around the bud, where two cell adhesion molecules, the heparan sulfate proteoglycan syndecan-1 and the extracellular matrix protein tenascin, are expressed in the dental mesenchyme. Both molecules are believed to be involved in mesenchymal cell condensation (Thesleff et al., 1995). The tooth bud then progresses to the cap (E14) and bell (E16) stages. Postnatally, these epithelial-mesenchymal interactions culminate in the differentiation of the epithelium into enamel-secreting ameloblasts and of the mesenchyme into dentin-secreting odontoblasts, pulp and alveolar bone (Palmer and Lumsden, 1987).

Prior to E12.5, mandibular arch 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. After E12.5, this potential shifts to the dental mesenchyme, which can now induce tooth formation when recombined with second arch epithelium, while recombinants containing dental epithelium and second arch mesenchyme fail. Recent studies have suggested that the TGF-β superfamily member Bone Morphogenetic Protein 4 (BMP4) constitutes one component of the inductive signal that transfers tooth inductive potential from dental epithelium to mesenchyme. *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 (Vainio et al., 1993; Turecková et al., 1995). Moreover, BMP4 can function as an inductive signaling molecule in early tooth morphogenesis, able to cause morphologic changes and to induce its own expression in dental mesenchyme. BMP4 can also induce the expression of two members of the mammalian *Msx* homeobox gene family, *Msx1* and *Msx2*, in dental mesenchyme (Vainio et al., 1993).

Msx1 and *Msx2* are implicated in the epithelial-mesenchymal interactions involved in tooth development. *Msx1* is strongly expressed in the dental mesenchyme and excluded from the dental epithelium throughout the bud, cap and bell stages of odontogenesis (MacKenzie et al., 1991a,b; 1992). *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). Mice deficient for the HMG box gene *Lef1* exhibit a phenotypically similar bud stage arrest in molar tooth development (van Genderen et al., 1994), suggesting the possibility that *Msx1* and *Lef1* reside within the same genetic pathway.

Msx expression in various embryonic mesenchymes can be induced by epithelial contact or by inductive signaling molecules (Davidson et al., 1991; Robert et al., 1991; Takahashi et al., 1991; Pavlova et al., 1994; reviewed by Davidson, 1995). Dental mesenchymal expression of *Msx1* and *Msx2* can be induced by dental epithelium until the E16 bell and E13 bud stages respectively (Jowett et al., 1993). In addition, heterospecific grafts between chick and mouse limb and facial primordia have shown that *Msx1* expression is activated when facial mesenchyme is grafted into the limb bud, although the converse experiment fails (Brown et al., 1993). Lastly, *Msx1* and *Msx2* expression can be induced in dental mesenchyme by recombinant BMP4 (Vainio et al., 1993) and in limb bud mesenchyme by BMPs and FGFs (Vogel et al., 1995; Wang and Sassoon, 1995). Thus, mesenchymal *Msx* gene expression can be induced by signaling molecules known to be expressed by dental and other epithelia.

Despite information about the regulation of *Msx* gene expression, the downstream targets of *Msx* gene regulation in organogenesis remain unknown. Here we show that *Msx1* controls a regulatory hierarchy in the dental mesenchyme, and we order *Msx1*, *Bmp4*, *Lef1* and syndecan-1 in this hierarchy. In addition, we show that *Msx1* responds not only to signaling molecules from an inducing tissue, in this case the dental epithelium, but that in the induced dental mesenchyme *Msx1* regulates the expression of the signaling molecule BMP4, which may then act reciprocally upon the epithelium.

MATERIALS AND METHODS

Embryo isolation and genotyping

Embryos were collected from matings of *Msx1* (+/–) × *Msx1* (+/–) mice maintained in a N4-5 BALB/c background, taking the day of vaginal plug discovery as day 0.5. Genomic DNA was isolated from extra-embryonic membranes of E9.5-E14.5 embryos or from tails of 4-5-week-old mice. For *Msx1* genotyping, 250 ng to 500 ng of genomic DNA was analyzed by PCR using (in a single reaction) the two forward primers 5′-CCAGCATGCACCTACGCAA-3′ (wild-type *Msx1* sequence) and 5′-TCTGGACGAAGAGCATCAGG-3′ (*neo* sequence present in the mutant), and the reverse primer 5′-AGCAGGCGGCAACATGGGTT-3′ (wild-type *Msx1* sequence). The primers amplify a 270 bp fragment from the wild-type allele and a 490 bp fragment from the mutant allele. Samples were subjected to PCR using 35 cycles of 1 minute each at 94°C, 66°C and 72°C, followed by analysis on a 1.5% agarose gel.

Probes

A 285 bp fragment of murine *Bmp4* cDNA in pGEM3Z (Genetics Institute, Cambridge, MA) was digested with *Pst*I or *Eco*RI and transcribed with T7 or SP6 RNA polymerase for sense and antisense riboprobes, respectively. A 494 bp fragment of murine *Lef1* cDNA in pBluescript SK (gift of Hans Clevers, University Hospital, Utrecht), was digested with *Pvu*II and transcribed with T7 RNA polymerase for an antisense riboprobe. A 1,377 bp fragment of murine syndecan-1 cDNA in pGEM3Z (gift of Drs M. Hinkes and M. Bernfield, Childrens' Hospital, Boston, MA), was digested with *Hind*III or *Eco*RI and transcribed with T7 or SP6 RNA polymerase for sense and antisense riboprobes, respectively. A 295 bp fragment of murine *Egr1* cDNA (p3.6 T7; a gift from Dr Vikas Sukhatme, Beth Israel Hospital, Boston) was digested with *Xba*I or *Eco*RI and transcribed with T3 or T7 RNA polymerase for sense and antisense riboprobes, respectively.

Tissue section and whole-mount in situ hybridization

Embryos were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) for approximately 5 hours and dehydrated through increasing concentrations of ethanol before embedding in paraffin wax. Sections of 7 μm were cut from paraffin-embedded embryos and

dried at 37°C overnight. In situ hybridizations were performed as described (Sassoon and Rosenthal, 1993). After drying, slides were dipped in Kodak NTB2 radiographic emulsion diluted 1:1 with dH_2O. Following 10 days exposure at 4°C, slides were developed and counterstained with either hematoxylin or Hoechst dye. For whole-mount in situ hybridization, both sense and antisense digoxygenin-labeled RNA probes were generated by in vitro transcription and checked for size and yield by electrophoresis. Whole mount in situ hybridization was performed as previously described (Chen et al., 1996).

Bead implantation and tissue recombinations

The mandibular arch of individual E11.5 embryos was removed and the first molar tooth forming region, visible as a shallow depression, was excised in PBS. To separate the dental epithelium from the mesenchyme, the tissues were incubated for 2 minutes in 2.25% trypsin/0.75% pancreatin on ice and then transferred to PBS plus horse serum (1:1) on ice for 10 minutes. The tissues were then microsurgically separated. Bead implantation and tissue recombination were performed according to the procedures described previously (Vainio et al., 1993). 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 protein (Genetics Institute, Cambridge, MA) at 37°C for 30 minutes, or heparin acrylic beads (Sigma, St Louis, MO) were incubated with 710 ng/μl recombinant human FGF1, FGF2 (R&D Systems, Minneapolis, MN) or FGF4 (Genetics Institute, Cambridge, MA) 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. For tissue recombinations, separated epithelium from one embryo was recombined, either on top of or adjacent to dental mesenchyme from another embryo without prior knowledge of the genotype. 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, the explants were fixed and processed for whole-mount in situ hybridization or immunostaining.

RT-PCR

First molar tooth germs were isolated by careful excision from E14.5 embryos obtained from *Msx1* (+/–) × *Msx1* (+/–) crosses, and the dental epithelium and mesenchyme separated as described above. The dental mesenchyme was frozen in liquid nitrogen and stored at –80°C. After genotyping, mesenchymes having the same genotype were pooled and subjected to RNA extraction using RNAzolB (Cinna/Biotecx Laboratories, Houston, TX). RNA was also extracted from E11.5 mouse limbs with the same method. About 100 ng RNA was heated at 75°C for 3 minutes prior to reverse transcription (Superscript, Life Sciences, Gaithersburg, MD) at 42°C for 1 hour. PCR was performed at 94°C for 1 minute, 60°C for 2 minutes and 72°C for 1 minute in the presence of [α-^{32}P]dCTP. Samples were withdrawn at 25, 35 and 45 cycles, analyzed by 6% non-denaturing PAGE and subjected to autoradiography to determine the logarithmic range of PCR amplification. The following primers were used: *Bmp4* (gift of Dr Stephen Harris, University of Texas San Antonio), 5′-GAAGGCAAGAGCGCGAGG-3′, 5′-CCCGGTCTCAGGTATCA-3′; *Lef1* (Travis et al., 1991), 5′-CACCTAAGCGACGAGCACT-3′ (nt 1,457-1,476), 5′-CGTGTTGAGGCTTCACGTGC-3′ (nt 1,842-1,821); mouse β-actin (Hu et al., 1986) 5′-GCTGTGTTCCCATCCATCGTGG-3′ (nt 1,875-1,896), 5′-GACGCATGATGGCGGTGTGGCA-3′ (nt 2,561-2,540). The two different *Lef1* PCR products were confirmed by DNA sequencing.

Immunostaining

The lower jaws of E13.5 wild-type and *Msx1* mutant embryos were removed and embedded in OCT. Frozen sections were made at 15 μm and stored at –80°C until use. Sections were rinsed in PBS for 10 minutes and incubated with 10% normal rabbit serum for 1 hour at room temperature prior to incubation with a rat monoclonal antibody against chick tenascin (Sigma, St Louis, MO) or a rat monoclonal antibody against mouse syndecan (clone 281-2, from Pharmingen, San Diego, CA) at 4°C overnight. After a 20 minute wash with PBS, sections were incubated with goat anti-rat IgG antibody conjugated with FITC at room temperature for 45 minutes. Sections were mounted with Vectashield (Vector Labs, Burlingame Labs, CA) after PBS washing. For whole-mount immunostaining, cultured bead-implanted dental mesenchymes or isolated mandibles were fixed in methanol/DMSO (4:1) and then cleared with 10% H_2O_2. A mouse monoclonal antibody (h3b2/17.8.1) which detects both BMP2 and BMP4 was provided by Genetics Institute (Cambridge, MA) and used in a whole-mount immunostaining protocol. Immunostaining was performed according to the method of Dent et al. (1989). After staining, isolated mandibles were embedded, sectioned and counterstained with hematoxylin.

In vitro rescue cultures

Mandibles from E13.5 embryos obtained from *Msx1* (+/–) × *Msx1* (+/–) crosses were dissected out. The first molar tooth germs and immediately surrounding tissue were isolated from each mandible with a needle in PBS containing Ca^{2+} and Mg^{2+}. Prior to culture, tooth germs were stored on ice in a serum-free chemically defined medium as previously described (Yamada et al., 1980). All tooth germs were placed in organ culture within 1 hour after isolation. Tissues were placed on filters supported by a metal grid in the chemically defined medium consisting of Eagle's minimum essential medium, 2.05 mM L-glutamine, 0.66 mM L-glycine, 0.056 mM vitamin C, and 15 mM Hepes buffer, pH 7.4. For rescue experiments, defined medium was supplemented with recombinant BMP4 protein at a final concentration of 100 ng/ml. Tissues were cultured for 6 days with medium changes every two days. Tissues were fixed in Bouin's fixative, embedded in wax, sectioned and stained with hematoxylin and eosin.

RESULTS

Msx1 is required for *Bmp4* and *Lef1* expression in dental mesenchyme

To begin a molecular epistasis analysis of *Msx* gene function, we examined *Bmp4* expression in both wild-type and *Msx1* deficient molar tooth germs by in situ hybridization (Fig. 1). These experiments show a significant reduction in *Bmp4* expression in the *Msx1* deficient molar mesenchyme at E13.5, the stage at which tooth bud development arrests in the *Msx1* and *Lef1* mutants. Some *Msx1* mutant buds arrest earlier than E13.5, at the early bud stage; however, results similar to those at E13.5 were also obtained at E12.5. At E14.5, the difference in expression became even more marked. In contrast, *Bmp4* expression was maintained in E11.5 dental epithelium in *Msx1* mutant embryos, indicating that dental epithelial *Bmp4* expression does not require *Msx1* (insets in Fig. 1B,E). In addition, in other tissues where *Msx1* is not normally expressed, such as gut mesenchyme and lung bud epithelium from E12.5 to E14.5, *Bmp4* expression was unaffected (data not shown). Immunostaining for BMP4 was performed using isolated wild-type and *Msx1* mutant mandibular rudiments at E13.5, using a monoclonal antibody that recognizes both BMP2 and BMP4. In the *Msx1* mutant tooth bud, immunoreactivity in the dental mesenchyme was reduced compared to wild-type mesenchyme (data not shown), consistent with the results obtained by in situ hybridization.

To test the hypothesis that *Msx1* and *Lef1* reside within the same genetic pathway, in situ hybridization experiments were performed with a murine *Lef1* riboprobe (Fig. 1). Although low levels of *Lef1* expression in the dental mesenchyme were noted in some experiments, *Lef1* expression was reduced in the *Msx1* mutant dental mesenchyme at E13.5 and E14.5 (Fig. 1). *Msx1* is therefore required for the normal level of expression of both *Bmp4* and *Lef1* in dental mesenchyme. Consistent with this, *Msx1* expression is preserved in the dental mesenchyme of *Lef1* deficient mice at E13.5 (Kratochwil et al., 1996).

Because of the qualitative nature of these results, we sought to confirm the reductions in *Bmp4* and *Lef1* expression in *Msx1* deficient dental mesenchyme by semi-quantitative RT-PCR. After enzymatically dissociating the dental epithelium, first molar tooth mesenchymes were isolated from lower mandibles of E14.5 embryos obtained from *Msx1* heterozygous crosses, and RNAs from wild-type and *Msx1* mutant mesenchymes were then analyzed for *Bmp4*, *Lef1* and β-actin transcripts (Fig. 2). After quantitation by phosphorimager, these experiments revealed an approximate 10-fold reduction in *Bmp4* and *Lef1* transcripts in *Msx1* mutant dental mesenchyme. The use of E14.5 mesenchymes in these experiments was mandated by the small amounts of mRNA isolable at E13.5. This may exaggerate the *Bmp4* and *Lef1* expression differences between wild-type and mutant compared to results which would be obtained at E13.5. Nonetheless, these experiments indicate that *Msx1* is required for *Bmp4* and *Lef1* expression in molar tooth mesenchyme.

Msx1 is required for syndecan-1 but not tenascin expression in dental mesenchyme

To test whether the reduction of *Bmp4* and *Lef1* expression in the *Msx1* deficient dental mesenchyme results from a general deficiency of neural crest derived dental mesenchyme, we analyzed other markers of early mesenchymal induction in the developing tooth. Expression of the mouse heparan sulfate proteoglycan syndecan-1 was analyzed by immunohistochemistry and by in situ hybridization. The immunochemical experiments consistently revealed a marked decrease in mesenchymal syndecan-1 immunoreactivity in the *Msx1* mutant at the E13.5 bud stage (Fig. 3A,B), although in some cases a weak residual signal was observed. A similar marked reduction in syndecan-1 gene expression in *Msx1* mutants was also observed by in situ hybridization in both molar and incisor tooth mesenchyme at E13.5 (data not shown).

In contrast, immunostaining for another marker of dental mesenchyme, tenascin, was indistinguishable in wild-type and *Msx1* mutant molar mesenchymes (Fig. 3C,D). The finding that tenascin is present at wild-type levels and distribution in the *Msx1* mutant tooth bud mesenchyme indicates that the reductions in *Bmp4*, *Lef1* and syndecan-1 expression are not due to a general deficiency in the amount of dental mesenchyme.

Msx1 is required for BMP4 to induce its own expression in dental mesenchyme

To determine if BMP4 induces its own expression in dental mesenchyme through the action of *Msx1*, microdissected wild-type and *Msx1* mutant E11.5 molar mesenchymes were implanted with beads containing recombinant BMP4. Following organ culture for 24 hours, the specimens were analyzed for *Bmp4* expression by whole-mount in situ hybridization (Fig. 4A,B). These experiments confirm that BMP4 is able to induce its own expression in wild-type dental mesenchyme, as previously shown by Thesleff and colleagues (Vainio et al., 1993). Control experiments employing beads containing BSA or using sense riboprobes gave no signal. Induction of *Bmp4* expression was also observed when small pieces of wild-type dental epithelium were recombined with wild-type dental mesenchyme (arrows, Fig. 4). While BMP4 induced its own expression in wild-type mesenchyme, neither BMP4 nor wild-type dental epithelium induced *Bmp4*

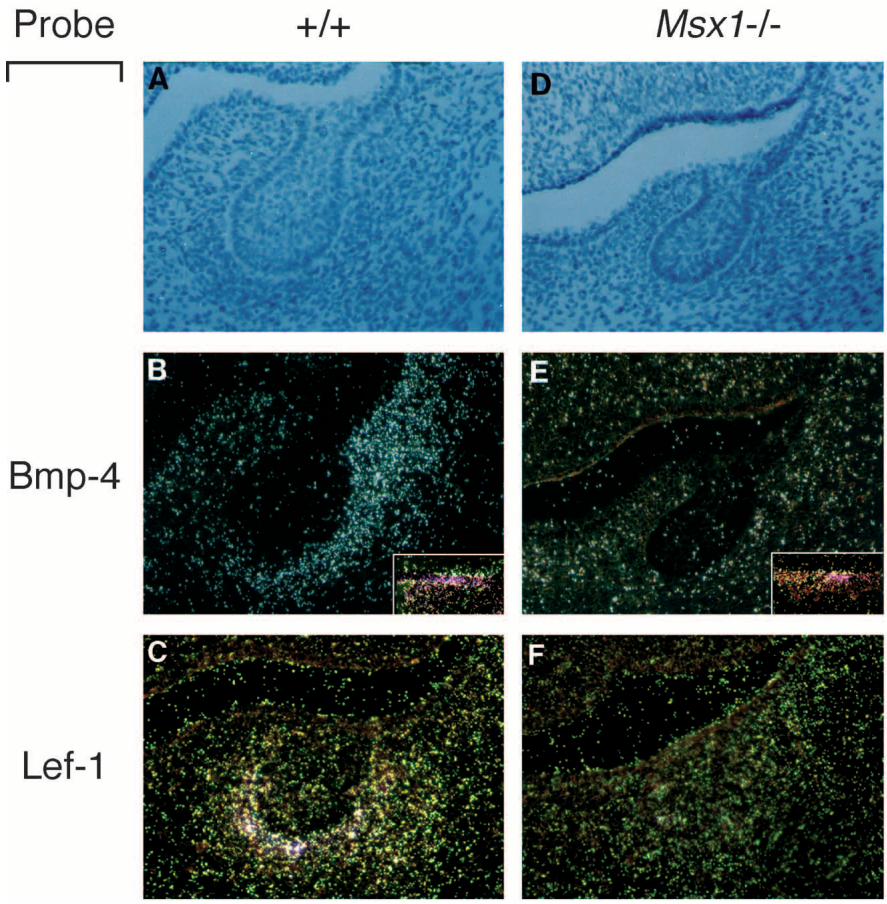

Fig. 1. *Bmp4* and *Lef1* expression in dental mesenchyme requires *Msx1*. Brightfield histology and in situ hybridization analyses of *Bmp4* and *Lef1* transcripts in wild-type (A-C) and *Msx1* deficient (D-F) E13.5 first lower molar tooth germs at the bud stage. The results demonstrate reduced expression of *Bmp4* (E) and *Lef1* (F) in the mutant mesenchyme relative to wild type (B,C). The insets shown in B and E show comparable expression of *Bmp4* in wild-type (B) and *Msx1* deficient (E) dental lamina at E11.5. The sections shown are representative of 5 independent experiments for each probe.

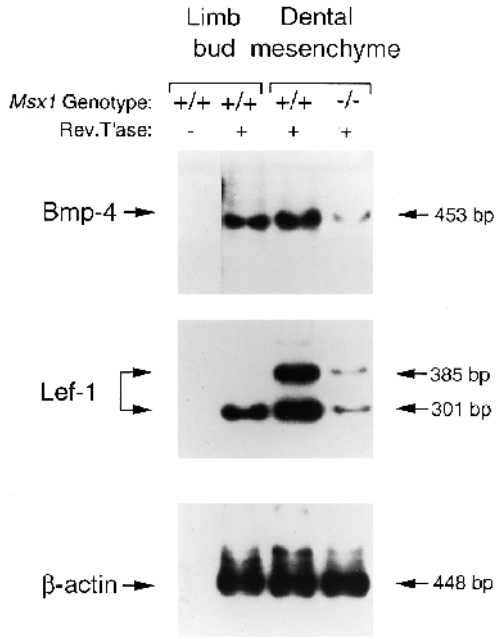

Fig. 2. *Bmp4* and *Lef1* transcripts are reduced in *Msx1* mutant dental mesenchyme. RT-PCR assay of *Bmp4*, *Lef1* and β-actin transcripts in wild-type and *Msx1* mutant E14.5 dental mesenchyme and wild-type E11.5 limb bud. The presence (+) or absence (–) of reverse transcriptase is indicated. Limb bud RNA was analyzed for positive and negative controls. The presence of a 385 bp *Lef-1* PCR product in the dental mesenchyme conforms to a known pattern of alternative splicing observed for the human *LEF1* gene (Zhou et al., 1995). The results, quantitated by phosphorimager, indicate a ~10-fold decrease in the levels of *Bmp4* and *Lef1* transcripts in the *Msx1* mutant dental mesenchyme relative to wild type.

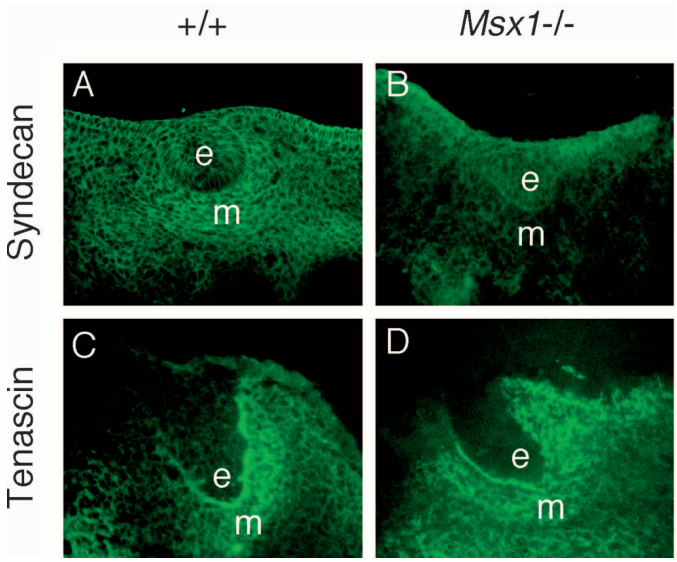

Fig. 3. Syndecan-1 but not tenascin is reduced in *Msx1* deficient dental mesenchyme. Immunohistochemical analysis of syndecan-1 (A,B) and tenascin (C,D) in wild-type (A,C) and *Msx1* deficient (B,D) E13.5 first lower molar tooth germs at the bud stage demonstrating reduced expression of syndecan but not tenascin in the mutant mesenchyme. e, dental epithelium; m, dental mesenchyme.

expression in *Msx1* mutant mesenchymes (Fig. 4 and Table 1). These results indicate that *Msx1* is required for BMP4 to induce its own expression in dental mesenchyme.

In contrast, *Lef1* expression as well as expression of the immediate early growth response gene, *Egr1*, were induced by BMP4 beads, and these inductions were equally strong in wild-type and *Msx1* deficient molar mesenchymes (Fig. 4E,F and Table 1). This indicates that BMP4 soaked beads, mimicking mesenchymal BMP4, can induce *Lef1* expression in the absence of *Msx1*.

Maximal induction of syndecan-1 expression in dental mesenchyme by FGFs requires *Msx1*

BMP4 cannot substitute for all the inductive functions of the dental epithelium, including the induction of cell proliferation and of syndecan-1 expression in the dental mesenchyme (Vainio et al., 1993). FGF4 can substitute for the dental epithelium in inducing cell proliferation (Jernvall et al., 1994). To determine whether FGFs can induce the expression of syndecan-1 and whether this induction requires *Msx1*, beads containing either FGF1, FGF2 or FGF4 were implanted into

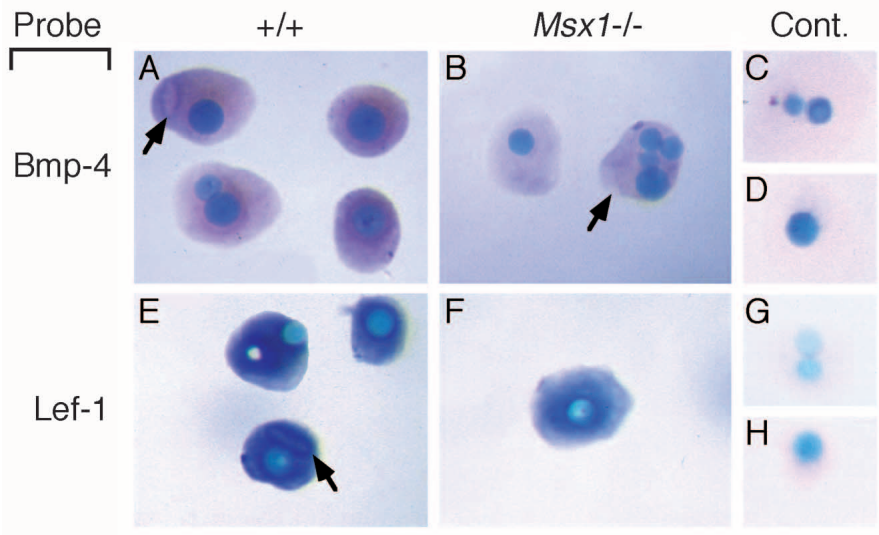

Fig. 4. BMP4 requires *Msx1* to induce its own expression, but not to induce *Lef1* in dental mesenchyme. BMP4 bead implantation experiments in wild-type (A,E) and *Msx1* deficient (B,F) molar mesenchymes. BMP4 agarose beads (soaked at 100 ng/μl) were implanted into microdissected E11.5 first lower molar tooth mesenchymes. After culture for 24 hours, the explants were analyzed by whole-mount in situ hybridization using a digoxygenin-labeled riboprobe for *Bmp4* or *Lef1*. The light blue is the bead, the purple or dark blue staining is the positive signal. A small piece of dental epithelium has been deliberately recombined with some mesenchymes at the edge of the rudiment (arrows). Neither BMP4 beads nor the epithelium induce *Bmp4* transcripts in the *Msx1* mutant mesenchymes (B), whereas *Lef1* is induced (F). Controls (Cont.) show that use of BSA soaked beads (C,G) or sense riboprobes (D,H) yields no staining.

Table 1. Induction of *Bmp4, Lef1, Egr1, Syndecan-1* and *Msx* expression in wild-type and *Msx-1* deficient dental mesenchymes*

	Inducer					
	BMP4 beads		FGF4 beads		+/+ Epithelium	
Probe	+/+	–/–	+/+	–/–	+/+	–/–
Bmp4	15/19	0/8	0/5	-†	8/8	0/4
Lef1	13/16	6/8	0/4	-†	5/5	4/4‡
Egr1	7/7	8/11	0/4	-†	exp.§	-†
Msx1	5/5	-¶	2/2	-¶	2/2	-¶
Msx2	7/8	4/4	4/5	9/9	exp.§	-†
Syndecan-1	not exp.§	-†	8/8	5(wk)/10	5/5	1/3
Syndecan-1 (antibody)	-†	-†	10/10	4(wk)/4	5/6	0/3

*Additional data not shown: FGF1 beads, syndecan-1 probe, 2/2 wild-type mesenchymes strongly positive; FGF2 beads, syndecan-1 probe, 1/3 wild-type mesenchymes weakly positive. Control experiments employing BMP4 beads and sense probes, or BSA beads and antisense probes were negative in all cases (see Fig. 4).

†Not performed.

‡The induction of *Lef1* expression in mutant mesenchyme by dental epithelium may indicate that under the recombination conditions, epithelial BMP4 can make a partial contribution to mesenchymal *Lef1* expression.

§Previously performed by Vainio et al., 1993 (syndecan-1, *Egr1*); Karavanova et al., 1992 (*Egr1*); Jowett et al., 1993 (*Msx2*). exp., expressed; not exp., not expressed; wk, weak.

¶Presence of aberrant transcripts from the mutant allele precludes interpretation.

Table 2. Partial rescue of *Msx1* deficient tooth germs by BMP4

	Defined medium		Defined medium + BMP4	
	+/+	–/–	+/+	–/–
Bud stage	1	11*	0	10†
Cap stage	1	0	2	6
Bell stage	9	0	10	0

*10 buds noted to be small and immature relative to E13.5 wild-type buds.

†6 buds noted to be elongated.

E13-13.5 tooth germs were cultured in chemically defined medium in the absence or presence of recombinant BMP4 (100 ng/ml) (Fig. 7). In some cases, addition of BMP4 to wild-type tooth germs produced advanced but slightly deformed structures. For details, see Materials and Methods.

isolated E11.5 dental mesenchyme. Experiments employing wild-type molar mesenchymes invariably resulted in a strong induction of syndecan-1, *Msx1* and *Msx2* expression, as assayed both by whole-mount in situ hybridization and, in the case of syndecan-1, also by immunostaining (Fig. 5 and Table 1). *Msx1* mutant mesenchymes, treated in parallel, revealed markedly lower levels of syndecan-1 expression, whether analyzed by immunostaining or by whole-mount in situ hybridization (Fig. 5). Similarly, when wild-type dental epithelium was recombined with *Msx1* mutant dental mesenchyme, syndecan-1 expression was again markedly reduced compared to recombinants employing wild-type dental mesenchyme (Fig. 5 and Table 1). These experiments indicate that FGFs are capable of inducing syndecan-1 expression in dental mesenchyme, and that *Msx1* is required for maximal induction.

Similar to dental epithelium, FGF4 beads strongly induced the expression of both *Msx1* and *2* in wild-type dental mesenchyme (Fig. 6A-C, Table 1). In contrast to dental epithelium, however, FGF4 was unable to induce the expression of *Bmp4* in explanted wild-type dental mesenchymes (Fig. 6D, Table 1). Since FGF4 induced strong *Msx2* expression in *Msx1* mutant dental mesenchymes, it is possible that the weak induction of syndecan-1 expression by FGF4 in the *Msx1* mutant is mediated by *Msx2*.

BMP4 can partially rescue the *Msx1* mutant tooth phenotype

Since BMP4 can function as an inducing signal in early mammalian tooth development and its expression is markedly reduced in *Msx1* deficient dental mesenchyme, the bud stage arrest of *Msx1* deficient tooth germs could be caused by the absence of BMP4 in the dental mesenchyme. To test this hypothesis, we examined whether recombinant BMP4 could rescue progression of the *Msx1* mutant tooth germ beyond the bud stage of tooth morphogenesis. Molar tooth germs from E13.5 wild-type and *Msx1* mutant embryos were isolated and cultured for 6 days in a chemically-defined medium in vitro (Yamada et al., 1980) with or without the addition of BMP4 at a concentration of 100 ng/ml. About 80% (19/23) of wild-type tooth buds exhibited development to the bell stage in the defined medium with or without BMP4 (Fig. 7A, Table 2). In contrast, in all 11 cases, explanted *Msx1* deficient tooth germs remained at the bud stage in the defined medium without BMP4, and in most cases the buds appeared small (Fig. 7B, Table 2).

However, when cultured in defined medium supplemented with recombinant BMP4, 60% (6/10) of the *Msx1* mutant tooth germs which remained at bud stage after the addition of BMP4 exhibited a marked elongation of the epithelial bud (Fig. 7C, Table 2). Moreover, about 40% (6/16) of the *Msx1* mutant tooth germs were able to progress morphologically beyond bud stage to the cap stage (Fig. 7D, Table 2). Thus, exogenous BMP4 is able to partially rescue the tooth phenotype of *Msx1* mutant mice. These results demonstrate that the absence of BMP4 in dental mesenchyme in the *Msx1* mutant is sufficient to account for the arrest in tooth development at the bud stage, and suggest that expression of BMP4 in the dental mesenchyme is required for progression from the bud to the cap stage of tooth morphogenesis. Moreover, the results further support the conclusion that *Bmp4* function in dental mesenchyme resides genetically downstream of *Msx1*.

DISCUSSION

In *Msx1* mutant embryos, the initiation of dental epithelial invagination is unaffected, but tooth development subsequently arrests at the bud stage. Since *Msx1* is only expressed in the dental mesenchyme and not the dental epithelium from E11.5 to E14.5, *Msx1* function is required in the dental mesenchyme for progression of molar tooth development beyond the bud stage. Here we show that *Msx1* is required in early tooth development for the transfer of *Bmp4* expression from dental epithelium to dental mesenchyme. Moreover, exogenous BMP4 is able to partly rescue tooth development in *Msx1* mutant tooth germs, confirming the molecular epistasis and demonstrating that *Msx1* is required in the mesenchyme for reciprocal signaling to the dental epithelium. Thus, while

Msx gene expression can be induced by BMPs and FGFs, *Msx* genes can also function upstream of signaling molecules. We propose that a major developmental function of *Msx* genes during organogenesis is to permit reciprocal inductive signaling to occur back and forth between tissue layers.

A regulatory hierarchy involving *Msx1*, *Bmp4* and *Lef1*

Based on the results presented above, we propose a model ordering *Msx1*, *Bmp4* and *Lef1* in a genetic pathway in early murine molar tooth development (Fig. 8). At both the E11.5 initiation and E12.5-13.5 bud stages, our model places *Msx1* upstream of mesenchymal *Bmp4* and *Lef1* expression, because their expression is specifically reduced in *Msx1* deficient tooth mesenchyme. In addition, at the initiation stage, the model places *Msx1* downstream of epithelial *Bmp4*, because *Bmp4* is expressed in dental epithelium as early or earlier than expression of *Msx1* in the subjacent mesenchyme (Turecková et al., 1995), because BMP4 can induce *Msx1* expression in explanted dental mesenchyme (Vainio et al., 1993; this paper) and because, in contrast to mesenchymal *Bmp4* expression, epithelial *Bmp4* expression is preserved in *Msx1* mutant tooth germs (this paper). The model is also supported by our bead experiments in mesenchymal explants indicating that *Msx1* is required for BMP4 mediated induction of mesenchymal *Bmp4* expression, but not for BMP4 mediated induction of mesenchymal *Lef1* expression. Lastly, expression of both *Msx1* and *Bmp4* is maintained in *Lef1* mutant dental mesenchyme (Kratochwil et al., 1996), consistent with the proposed model.

At the bud stage, *Bmp4* is no longer expressed in the dental epithelium, and two additional features of the model warrant consideration. First, since the BMP4 bead implantation experiments are performed at E11.5 but culture continues for an additional 24 hours, the induction of *Msx1* expression by BMP4 could mimic the effects of endogenous mesenchymal BMP4, normally expressed by E12.5. Our experiments are thus compatible with the idea that mesenchymal BMP4 facilitates the re-induction of *Msx1* expression throughout the dental mesenchyme by a positive feedback loop. Since the dental mesenchyme expression of *Msx1* precedes that of *Bmp4*, mesenchymal BMP4 cannot be required for the initial activation of *Msx1* expression in the dental mesenchyme, but only for its maintenance and propagation. Second, exogenous BMP4 can rescue epithelial development in the *Msx1* mutant tooth germ. Both for simplicity and for reasons described later, we have chosen to show this as a direct functional effect of mesenchymal BMP4 upon the epithelium.

While our data show that *Msx1* is required for *Bmp4* and *Lef1* expression in dental mesenchyme, *Msx1* alone is not sufficient to induce their expression. For example, FGFs, which potently induce *Msx1* and *Msx2* expression in wild-type dental mesenchyme, are unable to induce expression of either *Bmp4* or *Lef1*, suggesting that other factors are required in order for *Msx1* to regulate their expression. Lef1 is thought to execute its molecular function by acting as a DNA architectural protein rather than as a classical transcription factor (Grosschedl et al., 1994). After binding in the minor groove to an AT-rich recognition sequence, Lef1 induces a large bend in the surrounding DNA which cooperatively increases the ability of other factors to activate transcription (Giese et al., 1995). Thus, although our data demonstrate that *Msx1* is required for *Lef1* expression in dental mesenchyme, a functional cooperation between Msx1 and Lef1 in co-regulating other downstream targets remains possible.

Recent studies have shown that Msx1 is capable of functioning as a transcriptional repressor with a variety of templates in a manner that does not require DNA binding by the homeodomain (Catron et al., 1995). Nonetheless, naturally occurring missense mutations in the MSX1 and MSX2 homeodomains cause dominantly inherited oligodontia and Boston type craniosynostosis, respectively, and the latter mutation increases the DNA binding affinity of the homeodomain by more than 6-fold (Vastardis et al., 1996; Jabs et al., 1993; L. Ma et al., personal communication). Given our finding that *Msx1* is necessary for *Bmp4* expression in molar tooth mesenchyme, the repressor model for *Msx* gene function would require that the Msx1 gene product repress a repressor of *Bmp4* gene expression. While possible, this model is less parsimonious than one in which Msx1 binds DNA via its homeodomain and functions as either a transcriptional activator or as an accessory factor for a transcriptional activator of *Bmp4*. Although the Msx1 homeodomain identifies several distinctive clusters of TAAT binding sites in the *Bmp4* promoter and upstream region by binding site selection (L. Ma and R. Maas, unpublished data), whether *Bmp4* is a direct target for regulation by the *Msx1* gene product remains for future investigation.

Msx1 controls inductive signaling in tooth development

Since the BMP4 expressed in dental epithelium can potentially diffuse into the surrounding mesenchyme, what would be the function of a regulatory circuit leading to the re-induction of BMP4 in the dental mesenchyme? Previously, BMP4 was proposed to function as a signal mediating epithelial-mesenchymal interactions during tooth development, a result interpreted primarily in terms of a diffusion mechanism, although a cell-cell contact (homeogenetic) mechanism was not excluded (Vainio et al., 1993). With the consideration that the diffusion distance of growth factors within tissue is likely limited (Jessell and Melton, 1992), *Msx1* might act as an 'amplifier' to permit the stronger and more rapid propagation of the BMP4 inductive signal throughout the dental mesenchyme than would occur in the case of simple diffusion alone.

In addition to its possible functions within the dental mesenchyme, we show here that exogenous BMP4 can bypass the requirement for mesenchymal *Msx1* function and permit the progression of epithelial development in *Msx1* mutant tooth germs from the bud stage to the cap stage. These results further establish that mesenchymal BMP4 functions downstream of *Msx1*, and support the idea that BMP4 synthesized in the dental mesenchyme acts upon the dental epithelium to support bud and cap stage development. In support of a reciprocal action of mesenchymal BMP4 upon the dental epithelium is the fact that a Type I BMP-receptor serine-threonine kinase which preferentially binds BMP4, ALK-3, is expressed at E12.5 in the dental epithelium (Dewulf et al., 1995; ten Dijke et al., 1994). Thus, following the shift in tooth inductive potential from dental epithelium to dental mesenchyme, *Msx1* controls the subsequent reciprocal inductive step in tooth development by regulating *Bmp4* expression in the dental mesenchyme. We conclude that the shift in *Bmp4* expression from dental epithe-

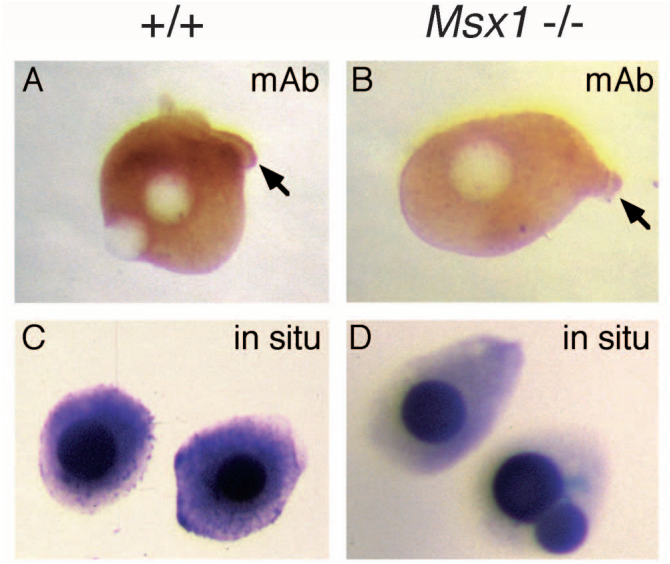

Fig. 5. Msx1 is required for maximal induction of syndecan-1 by FGF in the dental mesenchyme. Detection of syndecan-1 expression induced by FGF4 soaked beads by whole-mount immunostaining (A and B) and whole-mount in situ hybridization (C and D) in the first lower molar mesenchymes from E11.5 wild-type (A and C) and *Msx1* mutant embryos (B and D). Strong expression of syndecan-1 was induced by either FGF4 soaked beads or wild-type dental epithelium (arrow in A) in the wild-type dental mesenchymes (A and C), while only weak induction of syndecan-1 by FGF4 beads or wild-type epithelium (arrow in B) was observed in *Msx1* mutant dental mesenchyme (B and D). BSA-bead controls for both in situ and immunostaining and an in situ sense control probe gave no signal (data not shown).

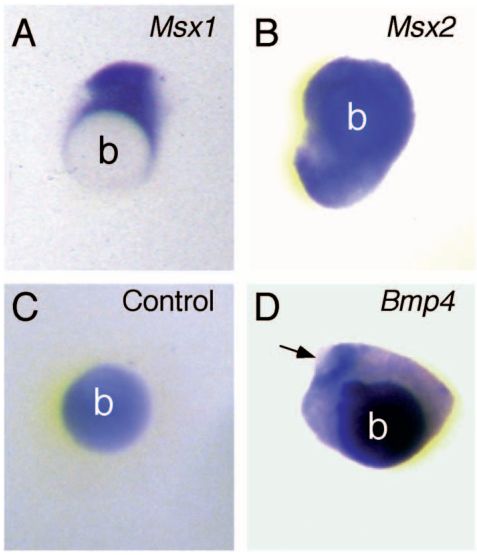

Fig. 6. FGF induces *Msx1* and *Msx2* in dental mesenchyme, but not *Bmp4*. (A and B) The respective induction of *Msx1* and *Msx2* gene expression by FGF4 beads (b) in wild-type dental mesenchyme. (C) A BSA soaked bead (b) does not induce *Msx2* expression. The arrow in D points to a piece of dental epithelium which has been recombined with wild-type dental mesenchyme to show that while the dental epithelium can induce *Bmp4* expression in the dental mesenchyme, the FGF4 bead cannot.

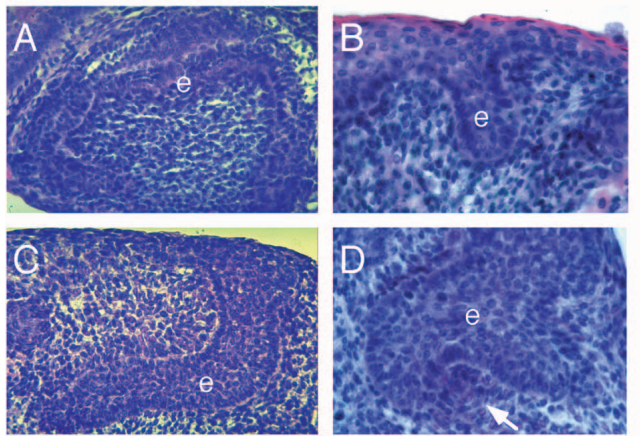

Fig. 7. BMP4 permits partial rescue of the *Msx1* deficient tooth phenotype and allows progression from bud to cap stage. (A) Histological analysis of a wild-type tooth germ after 6 days of in vitro culture in chemically defined medium in the absence of BMP4, showing progression to a bell stage. (B-D) Histological analysis of *Msx1* mutant tooth germs after 6 days of in vitro culture in chemically defined medium in the absence (B) or presence (C,D) of BMP4. (B) *Msx1* mutant tooth germs remained at an immature bud stage after 6 days of culture in defined medium. (C) In some cases in which *Msx1* mutant tooth germs were cultured in defined medium supplemented with BMP4 at 100 ng/ml, an elongated epithelial bud formed. (D) In other cases in which BMP4 was added to *Msx1* mutant tooth germs, morphologic cap structures were observed. Arrow points to a mass of condensed mesenchymal cells resembling dental papilla mesenchyme. e, dental epithelium. See also Table 2.

lium to mesenchyme accounts for the transfer in inductive potential from dental epithelium to the mesenchyme.

Apart from the bud stage arrest of the dental epithelium, the molar tooth phenotype in *Msx1* mutants is also associated with reduced amounts of mesenchymal condensation. This fact suggests a requirement for *Msx1* within the dental mesenchyme. Two molecules that have been suggested to play a role in the condensation of dental mesenchyme are the cell-surface heparan sulfate proteoglycan syndecan-1 and the extracellular matrix molecule tenascin. Both molecules are specifically localized in the mesenchymal condensates of the developing tooth germ (reviewed by Thesleff et al., 1995), and syndecan-1 has been shown to act as a receptor for several extracellular matrix molecules including tenascin (Bernfield et al., 1992). Our results indicate that *Msx1* is required for the expression of syndecan-1 but not tenascin in the dental mesenchyme. The reduction of syndecan-1 expression may explain the failure of mesenchymal condensation in *Msx1* mutant tooth germs.

BMP4 alone is not sufficient to recapitulate all of the endogenous signaling functions of the dental epithelium during tooth morphogenesis, including the induction of syndecan-1 expression and the stimulation of mesenchymal cell proliferation (Vainio et al., 1993). Our results clearly show that FGFs can substitute for the dental epithelium in the induction of syndecan-1 in the dental mesenchyme, that FGFs are potent inducers of *Msx* expression in dental mesenchyme and that the induction of syndecan-1 by FGFs is mediated at least in part by *Msx* genes. Similarly, it has been shown that FGF4 can stimulate the proliferation of dental epithelium and mes-

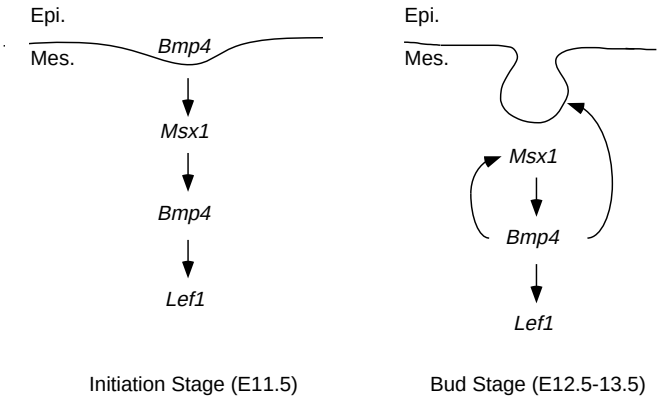

Fig. 8. Model integrating *Msx1*, *Bmp4* and *Lef1* into a genetic pathway in early tooth formation. The arrows are not necessarily intended to indicate a direct interaction, only an epistasis relationship. Other feedback loops and inter-relationships are possible. See text for details.

enchyme (Jernvall et al., 1994). However, since FGF4 is not expressed in the tooth germ until the E14 cap stage, it may not be an endogenous regulator of syndecan-1, instead mimicking the activity of another FGF. *Fgf*s 3, 7 and 8 are known to be expressed in the developing tooth germ, and *Fgf8* expression is localized to the dental epithelium at the time of tooth initiation and prior to the induction of syndecan-1 expression in the mesenchyme (Wilkinson et al., 1989; Finch et al., 1995; Heikinheimo et al., 1994; reviewed by Thesleff and Sahlberg, 1996). Although several FGFs were active in our experiments, we propose that FGF8 is the natural inducer of syndecan-1 expression in dental mesenchyme, and that this expression is mediated in part by *Msx1*. *Fgf8* expression is preserved in *Msx1* mutant dental epithelium at E11.5, consistent with a function upstream of *Msx1* (M. Bei and R. Maas, unpublished data).

General implications for organogenesis

We propose that *Msx* genes execute a general function during organogenesis by responding to and regulating the reciprocal expression of inductive signaling molecules in interacting tissues. Like peptide growth factors, *Msx1* and *Msx2* are frequently expressed in alternating and repeating patterns in interacting tissues during organogenesis. It is attractive to explain the *Msx* gene expression patterns in terms of a functional requirement for *Msx* in inductive signaling, since this too is an alternating and repeated event in organ formation. The striking correlation of *Msx* gene expression with that of members of peptide growth factor superfamilies suggests that *Msx* genes may participate in the regulation of a large and diverse set of peptide growth factors in organogenesis.

A number of signaling molecules can induce *Msx* gene expression, and *Msx* is necessary but not sufficient for the expression of its downstream targets. These facts suggest that specific combinations of inductive signals and transcription factors are required for the progressive specification of organ fates. This model places *Msx* genes in a central position in the inductive events that occur recurrently throughout vertebrate organogenesis. Additional support for this hypothesis comes from analyses of *Msx1-Msx2* double mutants, in which the potential for functional redundancy between *Msx1* and *Msx2* has been eliminated. These mutants exhibit a large number of organogenic defects, including inductive failures in mammary gland and hair follicle, organs which like the tooth form via epithelial-mesenchymal interactions, express *Bmp4* and *Lef1* and are defective in *Lef1* mutants (R. Maas et al., unpublished data; van Genderen et al., 1994). Moreover, misexpression of *Lef1* produces ectopic tooth structures (Zhou et al., 1995), while misexpression of *Bmp4* perturbs hair follicle development (Blessing et al., 1993). Thus, the inductive pathway described here for early tooth development may be conserved in other developing organs that form via sequential epithelial-mesenchymal interactions.

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