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DEVELOPMENT AND DISEASE

Rescue of cleft palate in *Msx1*-deficient mice by transgenic *Bmp4* reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis

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SUMMARY

Cleft palate, the most frequent congenital craniofacial birth defects in humans, arises from genetic or environmental perturbations in the multi-step process of palate development. Mutations in the *MSX1* homeobox gene are associated with non-syndromic cleft palate and tooth agenesis in humans. We have used *Msx1*-deficient mice as a model system that exhibits severe craniofacial abnormalities, including cleft secondary palate and lack of teeth, to study the genetic regulation of mammalian palatogenesis. We found that *Msx1* expression was restricted to the anterior of the first upper molar site in the palatal mesenchyme and that *Msx1* was required for the expression of *Bmp4* and *Bmp2* in the mesenchyme and *Shh* in the medial edge epithelium (MEE) in the same region of developing palate. In vivo and in vitro analyses indicated that the cleft palate seen in *Msx1* mutants resulted from a defect in cell proliferation in the anterior palatal mesenchyme rather than a failure in palatal fusion. Transgenic expression of human *Bmp4* driven by the mouse *Msx1* promoter in the *Msx1*$^{-/-}$ palatal mesenchyme rescued the cleft palate phenotype and neonatal lethality. Associated with the rescue of the cleft palate was a restoration of *Shh* and *Bmp2* expression, as well as a return of cell proliferation to the normal levels. Ectopic *Bmp4* appears to bypass the requirement for *Msx1* and functions upstream of *Shh* and *Bmp2* to support palatal development. Further in vitro assays indicated that Shh (normally expressed in the MEE) activates *Bmp2* expression in the palatal mesenchyme which in turn acts as a mitogen to stimulate cell division. *Msx1* thus controls a genetic hierarchy involving BMP and Shh signals that regulates the growth of the anterior region of palate during mammalian palatogenesis. Our findings provide insights into the cellular and molecular etiology of the non-syndromic clefting associated with *Msx1* mutations.

Key words: *Bmp*, *Shh*, *Msx1*, Mouse embryo, Cleft palate, Organogenesis

INTRODUCTION

The mammalian palate is formed by the union of three elements: the primary palate of the frontonasal process and the two lateral maxillary palatal shelves that will form the secondary palate. The formation of the mammalian secondary palate is a multi-step process that includes palatal shelf growth, elevation of the shelves, fusion between paired shelves and the disappearance of the midline epithelial seam (Ferguson, 1988). The closure of the palate separates the oropharynx and its various functions from the nasopharynx. In mice, the development of the secondary palate initiates at embryonic day 11.5 (E11.5) with the formation of tissue folds overlying the future palatal shelves within the oral cavity. Mesenchymal cell proliferation within the maxillary processes results in this appearance of the palatal shelf primordia (Burdett et al., 1988). At E12.5 and E13.5, palatal primordia grow vertically down beside the tongue. Between E13.5 and E14.0, a rapid elevation of the palatal shelves brings the two processes into horizontal apposition above the tongue. This movement is followed by the fusion of the medial edge epithelium (MEE) of the palatal shelves around E14.5, resulting in a continuous palate. Cleft palate, the most frequent congenital craniofacial birth defect in humans, arises from genetic or environmental perturbations in any step of the palatogenetic process (Johnson and Bronsky, 1995). Recent studies have indicated the importance of a growing number of genes in palate development. Mutations in genes encoding a variety of molecules, including transcription

factors, growth factors or their receptors, have been shown to induce cleft palate formation (Satokata and Maas, 1994; Kaartinen et al., 1995; Matzuk et al., 1995a; Matzuk et al., 1995b; Proetzel et al., 1995; Peters et al., 1998; Qiu et al., 1998; Lu et al., 1999; Miettinen et al., 1999; Szeto et al., 1999; Zhao et al., 1999).

The palatal shelves are derived from migratory cranial neural crest cells, which associate with the craniopharyngeal ectoderm [divided into the oral, nasal, and medial edge epithelium (MEE)]. Similar to many other vertebrate organs, the development of mouse secondary palate relies largely on sequential and reciprocal interactions between epithelial and mesenchymal tissue layers (Slavkin, 1984). Peptide growth factors are known to play crucial roles as inductive signals that mediate such epithelial-mesenchymal interactions during organogenesis (Thesleff et al., 1995). Several families of peptide growth factors have been implicated in vertebrate facial development (reviewed by Francis-West et al., 1998). These inductive factors include sonic hedgehog (Shh), a member of the hedgehog family, bone morphogenetic proteins (BMPs) and members of the transforming growth factor β (TGFβ) superfamily. Shh has been shown to be essential for the growth and development of the chick facial primordia (Hu and Helms, 1999). In the chick, a blockade of Shh signaling results in growth inhibition in the facial primordia, while overexpression of Shh induces growth in the facial primordia by increasing cell proliferation (Hu and Helms, 1999). Similarly, the ectopic application of exogenous BMP2, BMP4 or BMP7 also increases cell proliferation in the chick mandibular primordia (Barlow and Francis-West, 1997; Wang et al., 1999). Thus, these growth factors may control outgrowth of the facial primordia. In the developing palatal shelves in the mouse, *Shh* expression has been detected in the palatal epithelium (Bitgood and McMahon, 1995). However, the actual function of *Shh* in palate development is unclear, as *Shh*-deficient mice exhibited holoprosencephaly, with almost complete lack of facial skeletal structures (Chiang et al., 1996). The transcripts of both *Bmp2* and *Bmp4* have also been detected in developing mouse palatal shelves (Lyons et al., 1990; Bitgood and McMahon, 1995). However, targeted mutations of these two genes in mice have not been informative as to their roles in palatogenesis because of embryonic lethality before palate formation in these mutants (Winnier et al., 1995; Zhang and Bradley, 1996).

The *Msx1* homeobox gene is expressed in several developing organs in vertebrates, including the facial primordia, particular at the sites where epithelial-mesenchymal interactions occur during organogenesis (Davidson, 1995). *Msx1* is believed to participate in these interactions by regulating the expression of signaling molecules (Chen and Maas, 1998). Mice deficient for the *Msx1* gene exhibited neonatal lethality and severe craniofacial abnormalities, including cleft secondary palate, an absence of alveolar processes, and arrest of tooth development at the bud stage (Satokata and Maas, 1994; Houzelstein et al., 1997). In humans, mutations in the *MSX1* gene are associated with isolated non-syndromic cleft palate and tooth agenesis (Vastardis et al., 1996; Blanco et al., 1998; Lidral et al., 1998; Van den Boogaard et al., 2000), consistent with the phenotype observed in *Msx1* mutant mice. In *Msx1*-deficient mice, the bilateral primordial palatal shelves form and elevate normally, but failed to make contact and never fuse, which results in cleft palate (Satokata and Maas, 1994). Despite these findings, controversy remains over whether *Msx1* is expressed in developing palatal shelves or not (MacKenzie et al., 1991; Ferguson, 1994; Satokata and Maas, 1994). The role of *Msx1* in tooth development has been studied extensively (Chen et al., 1996; Bei and Maas, 1998; Bei et al., 2000; Zhang et al., 1999; Zhang et al., 2000a; Zhao et al., 2000), but the molecular mechanisms underlying the generation of cleft palate in *Msx1* mutants remain unknown.

To determine the molecular aetiology of the non-syndromic clefting associated with *Msx1* null mutations, we studied the genetic regulation of mammalian palatogenesis using *Msx1*-deficient mice as a model system. Our analyses demonstrated that *Msx1* expression was restricted to the anterior region of the mesenchyme of the developing secondary palatal shelves. *Msx1* was required for the expression of *Bmp4* and *Bmp2* in the palatal mesenchyme and *Shh* in the medial edge epithelium (MEE). We also showed that disruption of *Msx1* function caused impairment of in cell proliferation in the palatal mesenchyme, which leads to the formation of cleft secondary palate in mutant mice. Using a transgenic approach, *Bmp4* was ectopically expressed in the *Msx1* mutant palatal mesenchyme, and its expression rescued the cleft palate phenotype and neonatal lethality. Associated with this rescue of cleft palate was a restored pattern of *Shh* and *Bmp2* expression, as well as a recovery of normal cell proliferation in the palatal mesenchyme. Therefore BMP4 appears to bypass a requirement for *Msx1* and to function upstream of *Shh* and *Bmp2* to regulate palate development. We carried out further in vitro functional analyses to determine the position of these genes within the genetic pathway that regulates palatal development, and demonstrated that Shh derived from the MEE activated *Bmp2* expression in the mesenchyme that in turn stimulated cell proliferation. *Msx1* thus controls a genetic hierarchy involving BMP and Shh signals regulating the growth of anterior palate during mammalian palatogenesis.

MATERIALS AND METHODS

Transgenic and knockout mice

The generation of *Msx1*$^{-/-}$ mice and *Msx1-Bmp4* transgenic mice have been described previously (Satokata and Maas, 1994; Zhang et al., 2000a). The *Msx1* heterozygotes and *Msx1-Bmp4* transgenic mice were outbred onto a CD-1 background. Breeding was performed using *Msx1*$^{+/-}$ and *Msx1-Bmp4* mice to produce *Msx1* null mutants carrying two alleles of *Msx1-Bmp4* transgene (*Msx1*$^{-/-}$/Tg), as described previously (Zhao et al., 2000). For all embryos used in this study, the embryonic ages were determined by the day when the vaginal plug was discovered and designated as embryonic day 0.5 (E0.5). Wild-type mouse embryos used in this study were collected from mating of CD-1 mice. The genotype of *Msx1*$^{-/-}$, *Msx1-Bmp4* and *Msx1*$^{-/-}$/Tg adult mice and embryos were determined by a PCR-based method using genomic DNA extracted from tails or extra-embryonic membranes, as previously described (Satokata and Maas, 1994; Chen et al., 1996; Zhang et al., 2000a).

Palatal shelf organ cultures and bead implantation

Paired secondary palatal shelves from individual E13.5 embryos were microdissected and placed in Trowell type organ cultures with chemically defined medium according the method described previously (Taya et al., 1999). Each pair was orientated so that the MEE of each palatal shelf was in contact. Paired palatal shelves were initially cultured with Minimal Essential Medium supplemented with

300 μg/ml L-glutamate, 50 μg/ml glycine, 100 μg/ml ascorbate, 1% penicillin/streptomycin at 37°C in a 5% CO_2 air environment for 6 hours. In this initial culture period, a sufficiently low volume of medium was used to allow firm attachment of specimens onto the filters. After 6 hours the culture medium was replaced with DMEM/F12 (1:1) supplemented with 1% L-glutamine, 1% ascorbate and 1% penicillin/streptomycin. Samples were cultured for 3 days with one change of medium and were then harvested.

For bead implantation experiments, Affi-Gel blue agarose beads (BioRad, Hercules, CA) were soaked in proteins as previously described (Chen et al., 1996). Protein concentrations used were consistent throughout all experiments. BMP2 and BMP4 (Genetics Institute, Cambridge, MA) were used at 100 μg/ml. Shh N-terminal peptide [Shh-N; from R&D Systems, Minneapolis, MN (catalog number 461-SH-025)] was used at 1 mg/ml, and Noggin [R&D Systems (catalog number 719-NG-050)] (Zhang et al., 2000a) at 0.2 mg/ml. Anti-Shh antibodies (5E1) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) (Ericson et al., 1996) were used at 0.35 mg/ml. The palatal shelves of E12.5 embryos were further microdissected into an anterior region (all tissues anterior to the first molar) and a posterior region (all tissues posterior to the first molar) (see Fig. 1M), and each sample was pooled for analyses.

The palatal tissues were then incubated in 0.5% trypsin and 2.5% pancreatin in phosphate-buffered saline (PBS) on ice for 30 minutes before transfer to a stop solution consisting of 50% horse serum in PBS. The palatal mesenchyme was then separated from epithelium using a pair of fine forceps and placed on filters in Trowell type cultures. Protein-soaked beads were implanted onto the top of explants in DMEM culture medium supplemented with 10% fetal calf serum (Chen et al., 1996). Samples were harvested after 24 hours in culture, and processed for whole-mount in situ hybridization.

In situ hybridization

Samples used for whole-mount and section in situ hybridization were fixed in freshly made 4% paraformaldehyde/PBS. For section in situ hybridization, samples were dehydrated through graded ethanol series and embedded in paraffin wax. Serial paraffin sections were made at 10 μm. For whole-mount in situ hybridization, samples were bleached

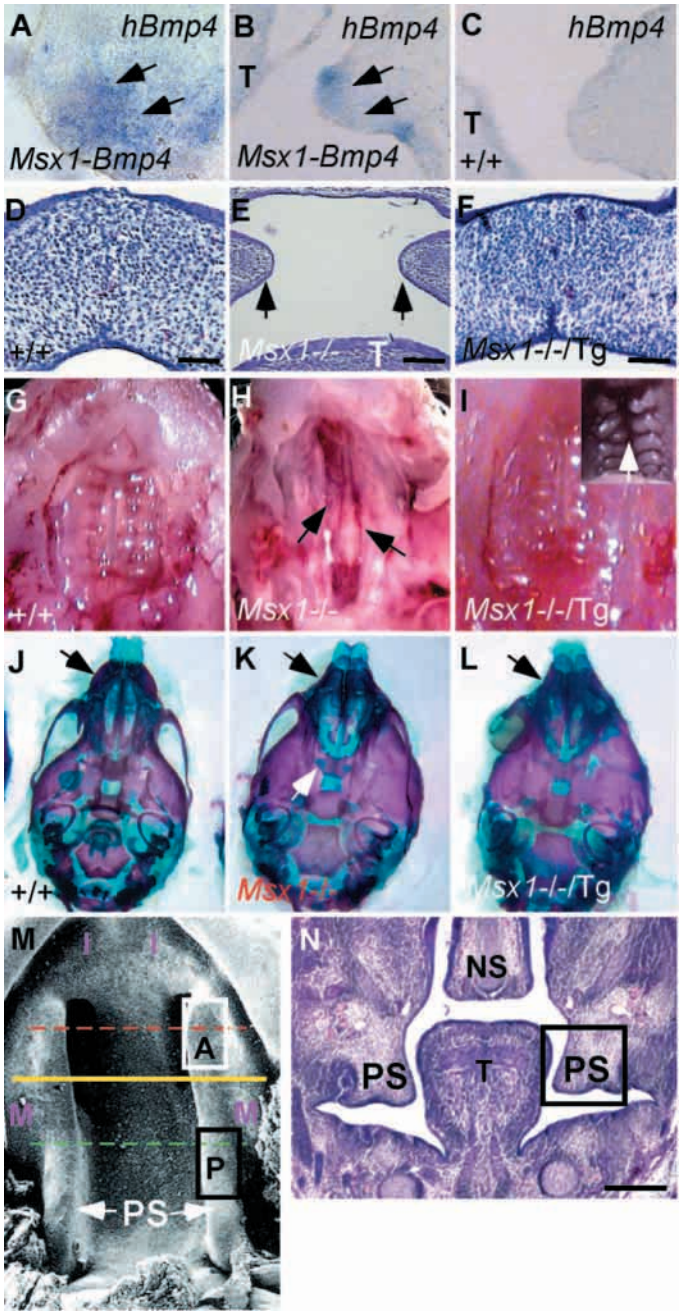


Fig. 1. Ectopic *Bmp4* expression in the palatal mesenchyme rescued the cleft palate in *Msx1*$^{-/-}$ mice. (A-C) Expression of the human *Bmp4* transgene (arrows) was detected in the anterior palatal mesenchyme of E12.5 (A) and E13.5 (B) *Msx1-Bmp4* transgenic embryos using a transgene-specific probe, as described previously (Zhao et al., 2000). No signals were detected in an E13.5 wild-type control palate using the same transgene-specific probe (C). (D) A coronal section through an E14.5 wild-type embryonic head showing fusion of the palatal shelves. (E) An E14.5 *Msx1*$^{-/-}$ embryo showing cleft secondary palate (arrows). (F) A coronal section of an E14.5 *Msx1*$^{-/-}$/Tg embryo showing fusion of the palatal shelves. (G,H) A wild-type newborn mouse displaying a closed palate (G) when compared with the cleft palate (arrows) shown in an *Msx1*$^{-/-}$ newborn (H). (I) Rescue of cleft palate in an *Msx1*$^{-/-}$/Tg newborn mouse. (Insert) Rugae did not fuse at the midline (arrow) of the palate in a three-month-old *Msx1*$^{-/-}$/Tg mouse. (J-L) Skeletal staining showing cleft palate in a newborn *Msx1*$^{-/-}$ mouse (K) and the rescue of cleft palate in a newborn *Msx1*$^{-/-}$/Tg mouse (L), when compared with a newborn wild type mouse (J). The sphenoid bone (white arrow in K), which could be directly viewed in the *Msx1* mutant, was not apparent in the *Msx1*$^{-/-}$/Tg mouse (L). The sharp appearance of the premaxilla (black arrow in K) found in *Msx1* mutant was also seen in a *Msx1*$^{-/-}$/Tg mouse (L), when compared with the normal rounded morphology of premaxilla in a wild-type mouse (black arrow, J), indicating that the rescue seen in *Msx1*$^{-/-}$/Tg mouse was specific to the cleft palate phenotype. (M) Scanning electron microscopic image of the oral view of an E13.5 wild-type embryonic head showing the overall shape of the developing palate, the planes of sections shown in Figs 2, 3, 6, and the regions excised for the explantation studies shown in Figs 4, 5. The yellow line indicates cuts made during dissection to separate the anterior region (top to the line) from the posterior region (bottom from the line) of the palate in this study. The red line indicates the anterior section plane, and green line indicates the posterior section plane used in the section in situ hybridization studies and BrdU labeling experiments shown in Figs 2, 3, 6. The white-lined box labeled 'A' represents the anterior region of palatal tissue, and the black-lined box labeled 'P' represents the posterior region of palatal tissue used for the explantation experiments shown in Figs 4, 5. (N) A coronal section through the oral region of an E13.5 embryo indicating the precise location (boxed) of palatal shelf shown in Fig. 1A-C, Figs 2, 3, Fig. 6D-F. I, incisor; M, molar; T, tongue; NS, nasal septum; PS, palatal shelf. Scale bars: in D,E, 100 μm; in N, 300 μm.

with 6% H_2O_2 before dehydration with methanol. The following cDNAs were used to generate antisense riboprobes: an 800 bp mouse *Msx1* (Hill et al., 1989); a 1.2 kb mouse *Bmp2* (Blessing et al., 1993); a 1.0 kb mouse *Bmp4* (Jones et al., 1991); a 650 bp mouse *Shh* (Echelard et al., 1993); and a 2.25 kb mouse *Ptch1* cDNA (Goodrich et al., 1996). To examine the expression of the human *Bmp4* transgene in the *Msx1-Bmp4* transgenic mice, a 700 bp DNA fragment containing 500 bp SV40 intronic and poly(A) sequence and a 200 bp 3′-UTR sequence of the human *Bmp4* gene subcloned from the original transgene was used to generate probe. This probe does not crossreact with mouse *Bmp4*. Non-radioactive RNA probes were generated by in vitro transcription labeling with digoxigenin-UTP according to the manufacturer's protocol (Boehringer Mannheim, Indianapolis IN). Whole-mount and section in situ hybridization was performed as previously described (Zhang et al., 1999).

Histological and skeletal analyses and scanning electron microscopy (SEM)

Standard paraffin sectioning and Hematoxylin and Eosin staining was carried out for histological analysis. Skeletal structures were stained using Alcian Blue for non-mineralized cartilage and Alizarin Red for bone, as described previously (Zhang et al., 2000b). For SEM, samples were fixed with 2.5% glutaradehyde/0.1 M sodium cacodylate at 4°C for 12 hours. After samples were postfixed in 1% osmium tetroxide/0.1M sodium cacodylate, dehydrate through graded ethanol series, samples were trimmed and critical point dried in a Touisimis (Samdri-790) apparatus and gold coated with a Polaron E5100 sputter coater. Samples were examined with a JEOL JSM-35C scanning electron microscope.

BrdU labeling and TUNEL assay

For in vivo BrdU labeling, timed pregnant wild-type or *Msx1*$^{-/-}$ mice were injected intraperitoneally on E12.5 or E13.5 with BrdU labeling reagent (3 mg/100 g body weight). Two hours after injection, mice were sacrificed and embryos were harvested. Embryonic heads were fixed in Carnoy's fixative, dehydrated through a graded ethanol series, embedded in paraffin wax and sectioned at 10 μm. Immunodetection of BrdU was performed using a BrdU labeling and Detection kit (Beohringer Mannheim) according to the manufacturer's protocol. A fixed area of 22.5 mm^2 beginning at the MEE and extending laterally was selected using an ocular scale grid. BrdU-positive cells in the mesenchyme within the fixed area were counted. Three continuous sections from the anterior region (anterior to the first molar) and posterior region (posterior to the first molar), respectively, of each palatal shelf from each individual embryo were counted. For in vitro BrdU labeling, palatal shelves from E13.5 wild type and *Msx1* mutant embryos were dissected, and divided into anterior and posterior regions. The palatal epithelium was separated from the palatal mesenchyme after enzymatic treatment, as described above. The palatal mesenchyme samples were placed on filters in Trowell type organ cultures. Protein-soaked beads were implanted onto explants that were then cultured in DMEM supplemented with 10% fetal calf serum for 8 or 24 hours before transferring into the same medium containing 10 μmoles of BrdU. After 45 minutes of labeling, samples were fixed in Carnoy's fixative, embedded in paraffin wax, sectioned for processing and immunodetection of BrdU. TUNEL assay was performed using an in situ cell death detection kit (Boehringer Mannheim) as described previously (Zhang et al., 2000b).

RESULTS

Ectopic expression of *Bmp4* to the *Msx1*$^{-/-}$ palatal mesenchyme rescues cleft palate

Knockout studies have implicated the *Msx1* homeobox gene in mammalian craniofacial development (Satokata and Maas, 1994; Houzelstein et al., 1997). The phenotypic abnormalities exhibited in *Msx1*-deficient mice were mainly restricted to the first pharyngeal arch and its derivatives, including a complete cleft of the secondary palate, an absence of alveolar process and an arrest of tooth development at the bud stage (Satokata and Maas, 1994; Houzelstein et al., 1997). In the mutant, the initial development of bilateral palatal shelves appeared normal, and the palatal shelves of the *Msx1*$^{-/-}$ mice grew vertically and elevated properly. However, they failed to make contact and did not fuse at the midline, resulting in a wide open cleft secondary palate which apparently contributes to neonatal lethality (Fig. 1E,H,K) (Satokata and Maas, 1994).

We have previously reported the generation of the transgenic mice harboring the human *Bmp4* gene driven by the mouse *Msx1* promoter (Zhang et al., 2000a). The human and mouse BMP4 proteins are highly conserved and exhibit 95% identity at the amino acid level. In these transgenics, the mouse *Msx1* promoter directed transgene expression in the craniofacial region, including the dental mesenchyme, mimicking the endogenous *Msx1* expression pattern (Zhang et al., 2000a; Zhao et al., 2000). Transgene expression was also detected in the mesenchyme of the anterior palatal shelves at E12.5 and E13.5 (Fig. 1A,B). This expression pattern is consistent with the endogenous *Msx1* expression in developing palatal shelves (see below). No overt phenotype was detected in these *Msx1-Bmp4* transgenic mice (Zhang et al., 2000a).

BMP4 has been shown to function downstream of *Msx1*, and a downregulation of *Bmp4* expression in the dental mesenchyme of *Msx1* mutants might account for the arrest of tooth development at the bud stage (Chen et al., 1996). To test whether ectopically expressed *Bmp4* in dental mesenchyme could bypass *Msx1* function to rescue tooth development in *Msx1*$^{-/-}$ mice, we introduced the *Msx1-Bmp4* transgenic allele into a *Msx1*$^{-/-}$ background to generate *Msx1*$^{-/-}$ mice carrying the *Msx1-Bmp4* transgene (*Msx1*$^{-/-}$/Tg) (Zhang et al., 2000a; Zhao et al., 2000). Surprisingly, tooth development was only partially rescued, but some *Msx1*$^{-/-}$/Tg mice survived the neonatal lethality observed in *Msx1* null animals. These mice grew to adulthood, but completely lacked teeth (Zhao et al., 2000). A gross morphological examination of the surviving *Msx1*$^{-/-}$/Tg mice revealed an intact palate (Fig. 1I,L), although the rugae did not fuse at the midline (insert in Fig. 1I). Of 66 *Msx1*$^{-/-}$/Tg mice examined from six independent lines, 41 (62%) exhibited complete palatal closure. Histological analyses further demonstrated that the palatal shelves of *Msx1*$^{-/-}$/Tg mice could make contact and fuse, as evidenced by the disappearance of the midline seam at the site of contact (Fig. 1F). Skeletal staining showed that the abnormal appearance of the premaxilla seen in *Msx1*$^{-/-}$ mice (Fig. 1K) (Satokata and Maas, 1994) remained in *Msx1*$^{-/-}$/Tg mice (Fig. 1L), indicating that transgenic expression specifically rescued the cleft palate phenotype. These results demonstrate that the ectopic expression of *Bmp4* in the palatal mesenchyme can bypass the requirement for *Msx1* to support palate development.

Msx1 is expressed in the developing palate and is required for the expression of *Bmp2*, *Bmp4* and *Shh*

Because of a contradiction on *Msx1* expression in developing palate, it was speculated that the cleft palate observed in *Msx1* mutants was the result of a failure of tooth development which is known to require *Msx1* function (MacKenzie et al., 1991;

Ferguson, 1994; Satokata and Maas, 1994). However, the fact that *Msx1*$^{-/-}$/Tg mice displayed a closed palate, but lack teeth, rules out this possibility (Zhao et al., 2000) (this study). To establish a role for *Msx1* in mouse palatogenesis, *Msx1* expression was carefully examined in the developing palate at E12.5 and E13.5. Using section in situ hybridization, *Msx1* transcripts were detected in the mesenchyme of developing palatal shelves (Fig. 2A,B) at both E12.5 and E13.5, with expression restricted to the region anterior to the first molars. No *Msx1* transcripts were detected in the posterior portion of developing palatal shelves (Fig. 2C). This palatal *Msx1* expression appeared relatively weak when compared with the level of expression in the dental mesenchyme (Fig. 2A-C). These results clarify the previous contradictory results on the palatal *Msx1* expression (MacKenzie et al., 1991; Ferguson, 1994; Satokata and Maas, 1994) and suggest a role for *Msx1* in the development of mouse secondary palate.

To establish a genetic hierarchy involving *Msx1* in palatogenesis, the expression of *Bmp2*, *Bmp4* and *Sonic hedgehog* (*Shh*), the known downstream genes of *Msx1* in the developing tooth germ (Chen et al., 1996; Zhang et al., 2000a), was analyzed in the developing palatal shelves of both wild type and *Msx1*$^{-/-}$ embryos at E12.5 and E13.5. In wild-type palatal shelves, *Bmp4* was found expressed in both the palatal epithelium and mesenchyme at E12.5 (Fig. 2D), and then was found to be restricted to the mesenchyme adjacent to the MEE at E13.5 (Fig. 2E). *Bmp2* expression was detected in both the palatal epithelium and mesenchyme (Fig. 2G,H) at both the stages examined, while *Shh* was only detected in the palatal epithelium, most prominent in the MEE (Fig. 2J,K). Similar to the pattern of *Msx1* expression, *Bmp2* and *Bmp4* expression was only detected in the anterior palate, but not in the posterior portion of the palate (Fig. 2F,I). In the posterior palatal shelves at E13.5, *Shh* transcripts were not detected in the MEE, but were detected in the oral side epithelium where the rugae would form (Fig. 2L), as reported previously (Bitgood and McMahon, 1995). In *Msx1*$^{-/-}$ embryos, *Bmp4* expression was downregulated in the mesenchyme, but remained unaltered in the epithelium of the palatal shelves at E12.5 (Fig. 3A), while the expression of *Shh* and *Bmp2* was not affected at this stage (Fig. 3D,G). By contrast, at E13.5 when *Bmp4* transcripts were detected exclusively in the palatal mesenchyme in wild-type

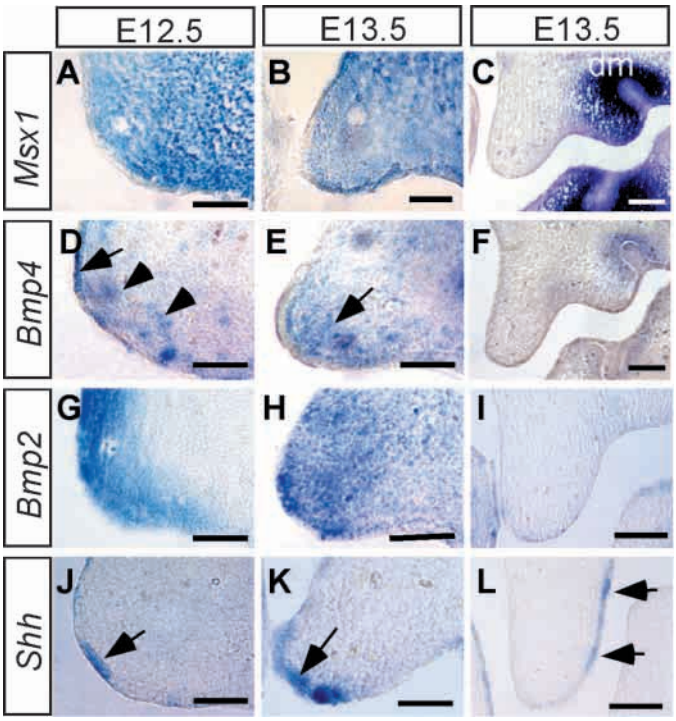


Fig. 2. Expression of *Msx1*, *Bmp2*, *Bmp4* and *Shh* in the developing palatal shelves. (A-C) *Msx1* transcripts were detected in the mesenchyme of the anterior region of developing palatal shelf at E12.5 (A) and E13.5 (B), but was not detected in the posterior region of palatal shelf (C). Strong *Msx1* expression was seen in the maxillary molar dental mesenchyme (dm) (C). (D-F) *Bmp4* expression was found in the palatal epithelium (arrow) and mesenchyme (arrowheads) at E12.5 (D), and was restricted to the mesenchyme (arrow) at E13.5 (E). *Bmp4* expression was absent in the posterior region of E13.5 palatal shelf (F). (G-I) *Bmp2* expression was detected in both the epithelium and mesenchyme of the anterior region of developing palatal shelves at E12.5 (G) and E13.5 (H), but was absent in the posterior palate (I). (J,K) *Shh* expression was detected in the MEE (arrows) of the anterior palatal shelves at E12.5 (J) and E13.5 (K), but was not detected in the MEE of the E13.5 posterior palatal shelf (L), where Shh expression in the rugae was seen (arrows). Scale bar, 100 μm.

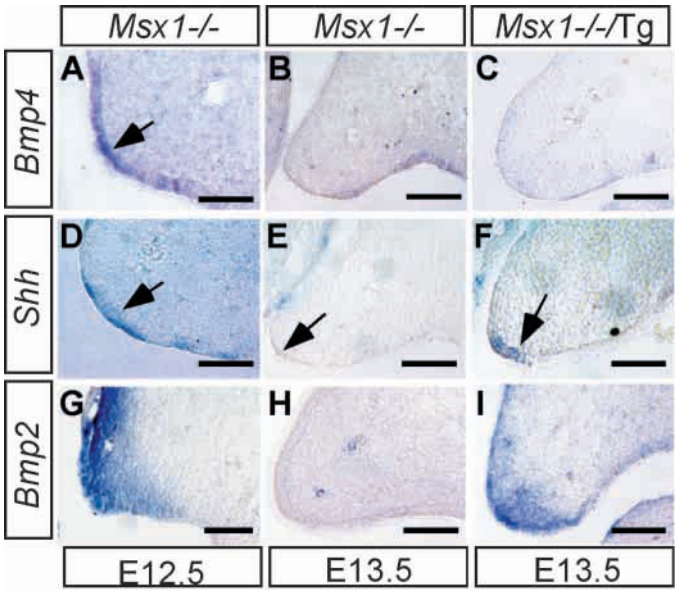


Fig. 3. Expression of *Bmp4*, *Shh* and *Bmp2* was downregulated in the anterior region of the *Msx1*$^{-/-}$ palatal shelves, but was restored in the *Msx1*$^{-/-}$/Tg palatal shelves. (A) *Bmp4* expression was downregulated in the *Msx1*$^{-/-}$ palatal mesenchyme, but remained in the epithelium (arrow) at E12.5. (B) At E13.5, *Bmp4* expression was not detected in the *Msx1*$^{-/-}$ palatal mesenchyme. (C) Endogenous *Bmp4* expression was not detected in the *Msx1*$^{-/-}$/Tg palatal mesenchyme at E13.5, indicating that transgenic *Bmp4* did not induce endogenous *Bmp4* expression in the absence of *Msx1*. (D-F) *Shh* expression remained unaltered in the MEE (arrow) of the E12.5 *Msx1*$^{-/-}$ palatal shelf (D), but was not detectable in the MEE (arrow) of the E13.5 palatal shelf (E). (F) However, *Shh* expression was restored in the MEE (arrow) of the E13.5 *Msx1*$^{-/-}$/Tg palatal shelf. (G-I) *Bmp2* expression was detected in the E12.5 *Msx1*$^{-/-}$ palatal shelf (G), but was downregulated in the palate at E13.5 (H). In the E13.5 *Msx1*$^{-/-}$/Tg palatal shelf, *Bmp2* expression was again detected (I). Scale bar, 100 μm.

embryo, significantly reduced expression of *Bmp4* was observed the palatal mesenchyme of *Msx1* mutants (Fig. 3B). Similarly, *Shh* expression in the MEE and *Bmp2* expression in both the epithelium and mesenchyme of *Msx1*$^{-/-}$ palate were simultaneously downregulated at this stage (Fig. 3E,H). It is concluded, therefore, that *Msx1* functions upstream of *Bmp4*, *Shh* and *Bmp2* in the developing palatal shelves. As *Msx1* expression is restricted to the palatal mesenchyme, the downregulation of *Shh* and *Bmp2* in the epithelium of *Msx1*$^{-/-}$ palatal shelves is apparently a secondary effect due to the absence of *Msx1*.

Ectopic *Bmp4* restores expression of *Shh* and *Bmp2* in *Msx1*$^{-/-}$ palatal shelves

In the developing tooth germ, *Bmp4* functions both upstream and downstream of *Msx1*, but upstream of *Shh* and *Bmp2* (Chen et al., 1996; Zhang et al., 2000a). It is possible that, similar to the tooth germ, *Msx1* may control *Bmp4* expression in the palatal mesenchyme. If so, a downregulation of *Bmp4* expression in *Msx1*-deficient palatal mesenchyme could account for the absence of *Shh* and *Bmp2* expression in the mutant palatal shelves. To test whether such a genetic regulatory pathway exists in the developing palate, we examined the expression of *Shh* and *Bmp2* in *Msx1*$^{-/-}$/Tg palatal shelves where ectopic *Bmp4* was expressed in the absence of *Msx1*. These analyses indicated that the ectopic expression of the *Bmp4* transgene in the palatal mesenchyme of *Msx1*$^{-/-}$ mice indeed restored, at least partially if not completely, the expression of *Shh* and *Bmp2* in the E13.5 transgenic palatal shelves where the endogenous *Bmp4* expression was absent (Fig. 2C,F,I). These data unambiguously demonstrated that *Msx1* does not directly regulate the expression of *Shh* and *Bmp2* in the developing palate. Rather, *Msx1* controls *Bmp4* expression in the palate, which in turn functions upstream of *Shh* and *Bmp2*. These results also suggest that the expression of *Shh* and *Bmp2* was unaltered in the E12.5 *Msx1*$^{-/-}$ palatal shelves, most probably because endogenous *Bmp4* expression was preserved in the MEE at that stage. Consistent with this hypothesis is the fact that the expression of genes encoding for BMP receptors, including *Bmpr1a* and *Bmpr1b*, was detected in the epithelium of developing palate at E12.5 and E13.5, and remained unaltered in that of *Msx1* mutants (data not shown).

It was previously demonstrated that overexpression of *Bmp4* in developing mouse tooth germ leads to an inhibition of *Shh* expression in the dental epithelium (Zhang et al., 2000a). To test if the same regulatory mechanism is conserved in the developing palate, we examined *Shh* expression in the MEE of E12.5 and E13.5 *Msx1-Bmp4* transgenic embryos in which *Bmp4* transgene is ectopically expressed in the palatal mesenchyme (Fig. 1A,B). Unlike what was observed in the tooth germ of the transgenic embryo (Zhang et al., 2000a), *Shh* expression was not affected (data not shown). This observation suggests that different BMP regulatory mechanisms exist in tooth versus palate. Alternatively, it is possible that the level of the transgenic *Bmp4* expression is not high enough in the palatal mesenchyme to repress *Shh* expression in the MEE.

Msx1 is required for *Bmp4* induction by BMP4, but not for *Bmp2* induction by SHH in palatal mesenchyme

To dissect out a genetic pathway involving *Msx1, Bmp2, Bmp4* and *Shh* in developing mouse palate, bead implantation experiments were performed using the anterior region of the E12.5 wild-type and *Msx1*$^{-/-}$ palatal shelves. To determine whether palatal mesenchyme exhibits heterogeneity in response to growth factor signals, the posterior region of palate was also assayed. Anterior and posterior palatal tissues were collected by microdissection (see Fig. 1M for position cuts made to obtain anterior versus posterior palatal tissues), and their epithelia were removed following enzymatic treatment. Protein-soaked agarose beads were implanted into the explants of palatal mesenchyme. Explants were cultured for 24 hours, and then harvested for the examination of gene expression by whole-mount in situ hybridization.

In experiments using anterior palatal mesenchyme, BMP4-soaked beads induced the expression of *Msx1* (12/14) and *Bmp4* (14/16) in the wild type mesenchyme (Fig. 4A-C), but failed to induce *Bmp4* (0/4) in the *Msx1*$^{-/-}$ mesenchyme (Fig. 4D). BMP4-soaked beads failed to induce *Bmp2* in wild type mesenchyme (0/11; Fig. 4J) and *Msx1*$^{-/-}$ mesenchyme (0/6; Fig. 4I). These results indicate that *Msx1* is required for the induction of mesenchymal *Bmp4* by BMP4 itself in the anterior palatal mesenchyme. These assays also indicated that transgenic *Bmp4* expression is not directly responsible for the restoration of *Bmp2* expression in the *Msx1*$^{-/-}$/Tg palatal mesenchyme. In other assays, Shh-soaked beads failed to induce *Bmp4* expression in the wild-type mesenchyme (0/11; Fig. 4F). This finding is further supported by the fact that beads soaked with an anti-Shh antibody did not affect endogenous *Bmp4* expression when implanted into E12.5 wild-type palatal tissue containing both the epithelium and mesenchyme (8/8) (Fig. 4G). By contrast, Shh-soaked beads induced *Bmp2* expression in both wild-type (10/12) and *Msx1*$^{-/-}$ mesenchyme (7/7), respectively (Fig. 4L,M). These observations suggest that *Bmp2* expression in the mesenchyme is regulated by epithelially derived Shh during palate development and that *Msx1* is not necessary for the induction of *Bmp2* expression by Shh.

In parallel experiments using E12.5 posterior palatal mesenchyme, BMP4-soaked beads induced neither *Msx1* nor *Bmp4* in wild type palatal mesenchyme (0/18 for *Msx1*; 0/12 for *Bmp4*; Fig. 5A,B). Similarly, Shh-soaked beads failed to induce *Bmp2* expression in this tissue (0/9; Fig. 5C). By contrast, FGF8, a factor known to induce *Pax9* expression in dental mesenchyme (Neubüser et al., 1997), induced *Pax9* expression in the posterior palatal mesenchyme, but not in the anterior palatal mesenchyme (7/8 for the posterior; 0/8 for the anterior; Fig. 5D-F). These results indicate that heterogeneity exists along the anteroposterior axis of the mesenchyme in the developing palate in response to growth factor induction, and further demonstrated that a genetic cascade involving *Msx1, Bmp4, Bmp2* and *Shh* operates in a restricted manner in the anterior palate to regulate palate development.

Impairment of palatal growth causes cleft palate in *Msx1*$^{-/-}$ mice

An interruption of palate development at any step, for example, during initiation, growth, elevation or fusion, causes cleft palate. Unlike *Tgfb*3 deficient mice in which the palatal shelves were shown to make contact at the midline, but failed to fuse (Kaartinen et al., 1995; Proetzel et al., 1995), *Msx1*$^{-/-}$ palatal shelves initiated and elevated normally, but never made contact (Satokata and Maas, 1994). This phenotype suggests an

impairment in either growth or fusion in the palatal shelves. To differentiate these two possibilities, we used an in vitro organ culture system and chemically defined medium as described previously (Taya et al., 1999). Pairs of palatal shelves from E13.5 wild-type, *Msx1*$^{-/-}$ and *Msx1*$^{-/-}$/Tg embryos were isolated and placed in contact with the MEE of each facing the other and the cultured for 3 days before histological analyses. Palatal shelves from all three genotypes underwent fusion, which was indicated by the disappearance of the midline seam and the establishment of the mesenchymal continuity (4/4 for each genotype; Fig. 6A-C). These results clearly indicate that failure of fusion is not the cause of cleft palate in *Msx1*$^{-/-}$ embryos. Cell proliferation was also examined in all three genotypes and this in vivo BrdU labeling demonstrated that a significantly reduced level of cell proliferation occurred in the *Msx1*$^{-/-}$ palatal mesenchyme at E13.5 but not E12.5, when compared with the wild-type controls (Fig. 6D,E,G, and data not shown). Consistent with the restricted pattern of *Msx1* expression in the palatal shelves, a significantly reduced number of BrdU-labeled cells were observed in the anterior region (Fig. 6E), but not in the posterior region (Fig. 6G; data not shown) of the *Msx1*$^{-/-}$ palatal shelves, as confirmed by the Student's *t*-test analysis ($P<0.01$; Fig. 6G). TUNEL assays further revealed that the level of apoptosis was unaltered in *Msx1*$^{-/-}$ palatal shelves along the anteroposterior axis (data not shown). These results indicate that the formation of a cleft palate in *Msx1*$^{-/-}$ mice results from defective cell proliferation in the anterior palatal mesenchyme, a mechanism that is consistent with the previously proposed role of *Msx1* in facilitating cell proliferation (Song et al., 1992). In addition, ectopic *Bmp4* expression in the *Msx1*$^{-/-}$ palatal mesenchyme returned cell proliferation to normal levels (Fig. 6F,G), providing a cellular basis for the observed rescue of cleft palate in *Msx1*$^{-/-}$/Tg mice.

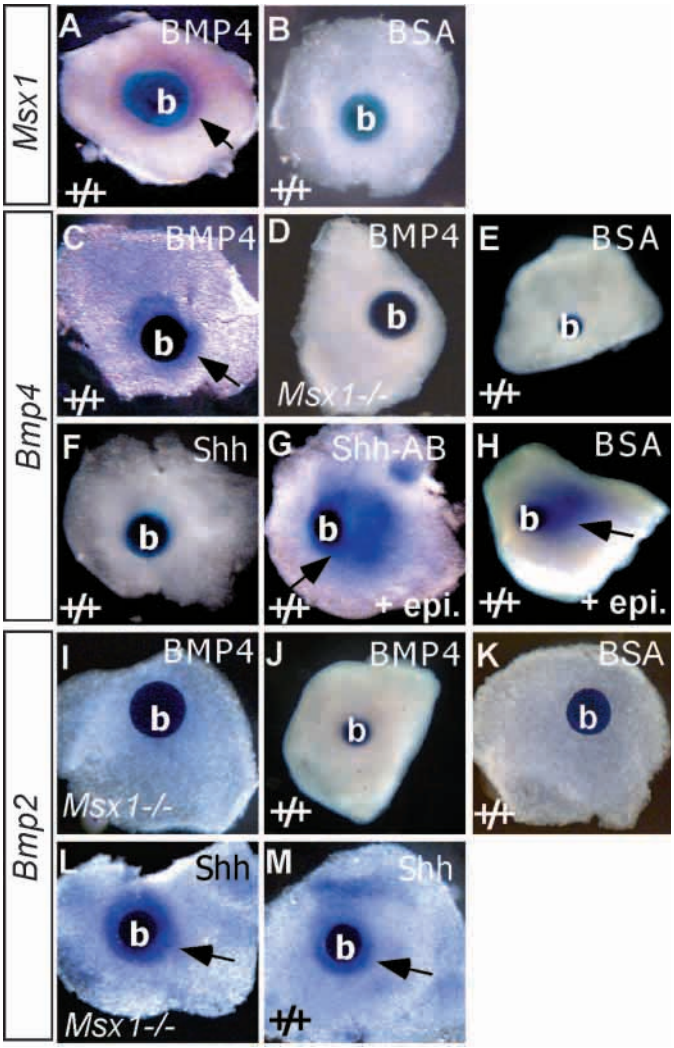


Fig. 4. Gene expression induced by protein soaked beads in the anterior palatal mesenchyme. (A-E) BMP4-soaked beads induced the expression of *Msx1* (A) and *Bmp4* (C) in E12.5 wild-type palatal mesenchyme, but failed to induce *Bmp4* expression in the *Msx1*$^{-/-}$ palatal mesenchyme (D). Control BSA beads failed to induce *Msx1* (B) and *Bmp4* (E). (F) Shh-soaked beads failed to induce *Bmp4* expression in wild-type palatal mesenchyme. (G,H) Endogenous *Bmp4* expression was not affected by an anti-Shh antibody bead implanted in the palatal explant containing both the epithelium and mesenchyme (G), when compared with the BSA control (H). (I-K) Similar to the BSA-soaked control beads (K), BMP4-soaked bead failed to induce *Bmp2* expression in the *Msx1*$^{-/-}$ palatal mesenchyme (I) and wild-type palatal mesenchyme (J). (L,M) Shh-soaked beads induced *Bmp2* expression in the palatal mesenchyme of both *Msx1* mutant (L) and wild-type (M) palatal mesenchyme. (See Fig. 1M for indication of cuts made during dissection to isolate the regions of palatal tissue used in this figure.) b, bead.

BMP2 mediates the effect of Shh signal on cell proliferation in palatal mesenchyme

It has been shown that both Shh and BMP2 stimulate cell proliferation in chick facial primordia (Barlow and Francis-West, 1997; Hu and Helms, 1999). In our studies, a reduced level of cell proliferation in *Msx1*$^{-/-}$ palatal shelves at E13.5 was accompanied by a downregulation of *Shh* and *Bmp2* expression, suggesting that Shh and BMP2 regulate cell proliferation in

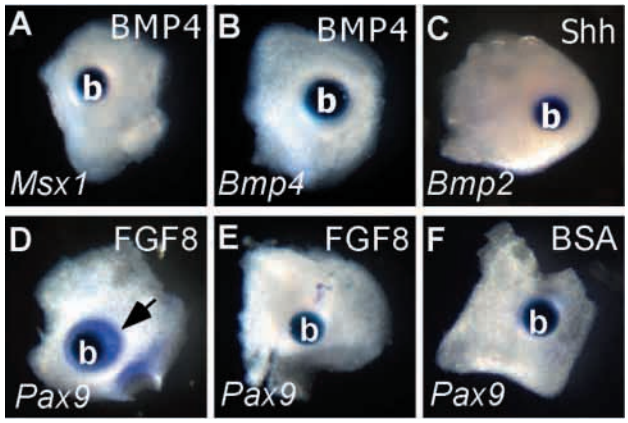
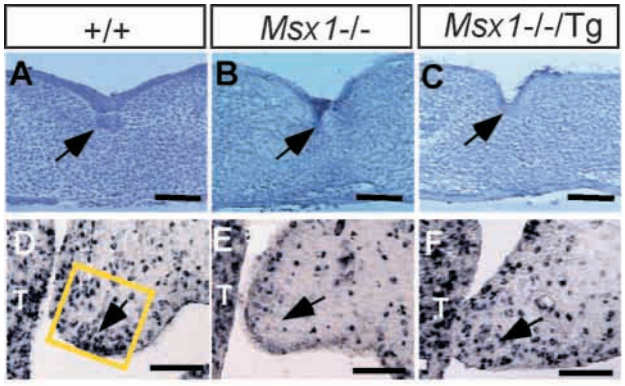


Fig. 5. Gene expression induced by protein-soaked beads in the posterior palatal mesenchyme of wild-type embryos. (A,B) BMP4-soaked beads failed to induce the expression of *Msx1* (A) and *Bmp4* (B) itself in the posterior palatal mesenchyme. (C) Shh-soaked beads also failed to induce *Bmp2* expression in the posterior palatal mesenchyme. (D-F) FGF8-soaked beads induced *Pax9* expression in the posterior palatal mesenchyme (D), but failed to do so in the anterior palatal mesenchyme (E), when compared with controls in which BSA-soaked beads were implanted into the posterior palatal mesenchyme (F).

developing palate. To test this hypothesis, BMP2-soaked beads were implanted onto explanted anterior palatal mesenchyme isolated from E13.5 wild-type and *Msx1*$^{-/-}$ embryos. A marked increase in cell proliferation was observed in mesenchyme from both genotypes after 8 hours in culture (5/5 for wild type and 4/5 for *Msx1*$^{-/-}$; Fig. 7A-D), with a biased induction of cell proliferation in the medial aspect where rapid cell division normally occurs. By contrast, Shh-soaked beads failed to induce cell proliferation in both wild type (data not shown) and *Msx1*$^{-/-}$ palatal mesenchyme (Fig. 6H) after 8 hours in culture. However, Shh-soaked beads were able to induce cell proliferation around the beads in both wild type (7/9) and *Msx1*$^{-/-}$ palatal mesenchyme (4/5) after 24 hours in culture (Fig. 7E,F), indicating that Shh does induce cell proliferation in this tissue but does so via an indirect mechanism. Furthermore, beads soaked with antibody specific for Shh repressed cell proliferation (8/8) in the anterior region of E13.5 wild-type palatal shelves containing both the epithelium (endogenous Shh source) and the mesenchyme (Fig. 7I,J), indicating that Shh derived from the MEE is critical for maintaining cell proliferation in the palatal mesenchyme. Strikingly, the repression of cell proliferation induced by the anti-Shh antibody was reversed when a single bead soaked with both anti-Shh antibody and BMP2 was implanted into wild type palatal



G

position	genotype	BrdU-labelled cells/unit*	No. of mouse	P
anterior palate	+/+	29.3 ± 8.2	6	
	Msx1-/-	10.2 ± 2.8	9	<0.01
	Msx1-/-/Tg	32.6 ± 5.9	5	>0.5
posterior palate	+/+	30.3 ± 1.5	3	
	Msx1-/-	30.6 ± 4.5	3	>0.5
	Msx1-/-/Tg	32.5 ± 5.0	4	>0.5

Fig. 6. Cleft palate in *Msx1* mutants is caused by defective cell proliferation in the anterior portion of palatal shelves. (A-C) Palatal shelves from E13.5 *Msx1*$^{-/-}$ embryo (B), like those from wild type (A) and *Msx1*$^{-/-}$/Tg embryos (C), fused and exhibited a disruption of the midline seam when placed in contact in vitro. Arrows indicate the remainder of the midline seam. (D-F) Cell proliferation, indicated by BrdU labeling, was significantly reduced in the E13.5 *Msx1*$^{-/-}$ palatal mesenchyme (E), particularly in the mesenchyme immediately beneath the MEE (arrow), when compared with mesenchyme in wild-type palates (D). Cell proliferation was restored to normal levels in *Msx1*$^{-/-}$/Tg palatal shelves (F). (G) A summary of BrdU-labeling studies in the anterior and posterior portions of the palatal shelves of the E13.5 wild type, *Msx1*$^{-/-}$ and *Msx1*$^{-/-}$/Tg embryos. The box in D represents the position and size of counting area (22.5 mm^2), as determined by use of an ocular grid. Using Student's *t*-test, *P* values were obtained by comparing numbers from *Msx1*$^{-/-}$ or *Msx1*$^{-/-}$/Tg with those from wild type. T, tongue. Scale bars: 100 μm.

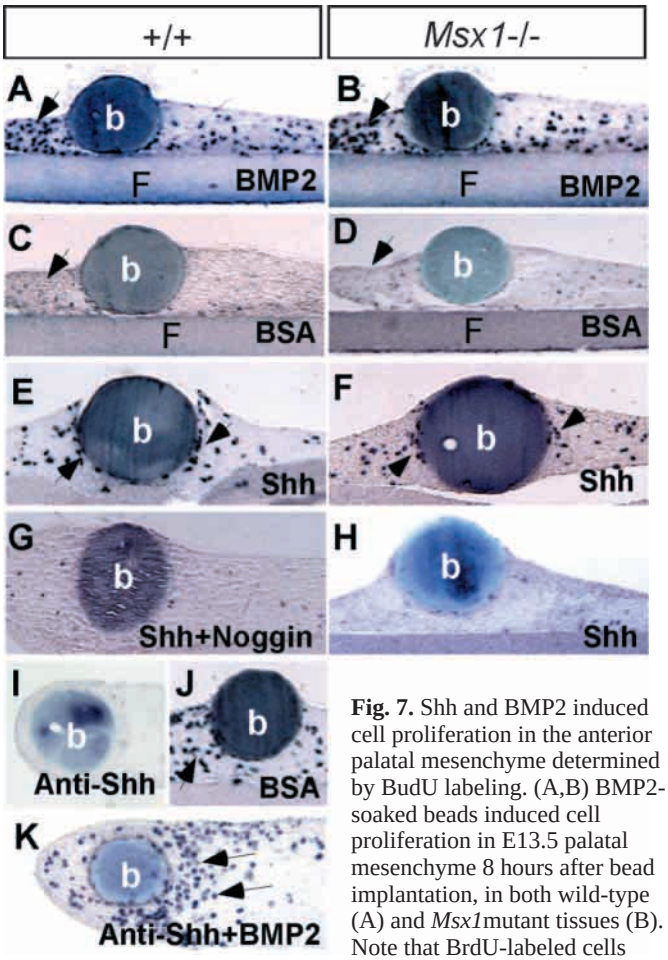


Fig. 7. Shh and BMP2 induced cell proliferation in the anterior palatal mesenchyme determined by BudU labeling. (A,B) BMP2-soaked beads induced cell proliferation in E13.5 palatal mesenchyme 8 hours after bead implantation, in both wild-type (A) and *Msx1*mutant tissues (B). Note that BrdU-labeled cells asymmetrically localized closer to the MEE (arrows). (C,D) BSA-soaked control beads failed to induce cell proliferation in the palatal mesenchyme of E13.5 wild-type (*C*) and *Msx1* mutant embryos (D). (E,F,H) Shh-soaked beads induced cell proliferation (arrows) around the beads in E13.5 wild-type (E) and *Msx1*$^{-/-}$ palatal mesenchyme (F) 24 hours after bead implantation. However, Shh-soaked beads failed to induce cell proliferation in both wild-type (data not shown) and *Msx1*$^{-/-}$ palatal mesenchyme (H) 8 hours after implantation. (G) A bead loaded with both Shh and Noggin proteins failed to induce cell proliferation 24 hours after bead implantation. (I) Beads soaked with an anti-Shh antibody inhibited cell proliferation in palatal tissue explants containing both the epithelium and mesenchyme of E13.5 wild-type embryo. (J) A BSA-soaked control bead did not affect cell proliferation (arrow) when implanted into E13.5 wild-type palatal tissue explants containing both the epithelium and mesenchyme. (K) Cell proliferation (arrows) was induced in E13.5 wild-type palatal tissue explants containing both the epithelium and mesenchyme 24 hours after implantation of a bead soaked with both an anti-Shh antibody and BMP2 protein. In all panels, the MEE aspect is towards the left. All palatal tissues used in this figure were from the anterior region of palatal shelves. b, bead.

explants containing both the epithelium and mesenchyme (Fig. 7K). As *Shh* is known to act upstream of *Bmp2* in various developing organs (Laufer et al., 1994; Yang et al., 1997; Drossopoulou et al., 2000; Zhang et al., 2000a), including palate (as shown in this study), we asked whether the cell proliferation induced by Shh is mediated through the induction of *Bmp2* expression. In these assays, Noggin-soaked beads were implanted into wild-type palatal explants containing both the epithelium and mesenchyme, and cultured the explants in vitro for 24 hours before BrdU labeling. Noggin, a known antagonist of BMP function (Smith and Harland, 1992; Zimmerman et al., 1996) was found to significantly repress cell proliferation in the samples (data not shown). Moreover, implantation of a single bead soaked with both Shh and Noggin into wild-type palatal mesenchyme failed to induce proliferation after 24 hours in culture (0/9; Fig. 7G). These data support the hypothesis that BMP2 acts downstream of Shh and mediates the mitogenic activity of Shh in the induction of cell proliferation in the developing palatal mesenchyme. BMP activity is required for cell proliferation in palatal mesenchyme. However, the role of BMP2 in inducing cell proliferation appears limited to the anterior palatal mesenchyme (where *Bmp2* is normally expressed) as evidenced by the fact that BMP2 was unable to induce cell proliferation in the explants of posterior palatal mesenchyme (data not shown). These observations further support the idea that a number of different genetic pathways act along the anterior-posterior axis of palatal shelves and are involved in the regulation of palate development.

DISCUSSION

Mammalian palatogenesis is a complex process that involves the participation of many genes that encode growth factors, their receptors and transcription factors. Mutations in a number of genes are known to cause cleft palate, and the production of this phenotype in each mutant may differ mechanistically. In this study, we have analyzed the cellular and molecular basis of the non-syndromic clefting associated with the *Msx1* null mutation. Our data indicate that defective cell proliferation in the anterior palatal mesenchyme, where *Msx1* is normally expressed, apparently induced the formation of cleft palate in *Msx1* mutant mice. Associated with this impaired cell proliferation was the downregulation of expression of a number of growth factors, including BMP2, BMP4 and Shh, in the anterior region of the *Msx1*$^{-/-}$ palatal shelves. These growth factors form a network that mediates epithelial-mesenchymal interactions leading to the growth of anterior palate. We showed that ectopic expression of *Bmp4*, a downstream gene of *Msx1*, in the *Msx1*$^{-/-}$ palatal mesenchyme was able to restore a normal level of cell proliferation and rescue the cleft palate phenotype. Our results unambiguously demonstrate for the first time a role for BMPs and Shh in the development of the mammalian secondary palate.

Msx1 controls a network of growth factors mediating epithelial-mesenchymal interactions in mammalian palatogenesis

Based on the results presented here, we propose that *Msx1* controls a genetic hierarchy, involving BMP and Shh signals, that regulates the development of the mammalian palate, as shown in Fig. 8. *Msx1*, which is expressed in the anterior palatal mesenchyme, is required for the maintenance of *Bmp4* expression in this mesenchyme. This part of model is supported by data showing that *Bmp4* expression was repressed in the *Msx1*$^{-/-}$ palatal mesenchyme, and that BMP4 induced *Msx1* and *Bmp4* expression in wild-type palatal mesenchyme, but failed to induce *Bmp4* in the *Msx1*$^{-/-}$ palatal mesenchyme. BMP4 activity is then required for *Shh* expression in the MEE, as evidenced by the fact that *Shh* expression was restored in the MEE of *Msx1* mutant expressing transgenic *Bmp4*. The MEE-derived Shh then induces the expression of *Bmp2* in the palatal mesenchyme, where it regulates cell proliferation necessary in palatal growth. Although we can not exclude the possibility that mesenchymally expressed BMP4 may regulate cell proliferation directly, this seems unlikely because application of an anti-Shh antibody to the palatal shelves repressed cell proliferation, but did not alter endogenous *Bmp4* expression (Fig. 4G, Fig. 7I).

During organ development, interactions between neighboring tissue layers are crucial for growth and differentiation. These sequential and reciprocal tissue interactions are mediated by diffusible growth factors (Jessell and Melton, 1992; Thesleff et al., 1995). During mammalian palatogenesis, the interactions between the cranial neural crest-derived mesenchyme and the craniopharyngeal ectoderm regulate palate morphogenesis and differentiation (Slavkin, 1984; Ferguson and Honig, 1984). Our data demonstrate that *Msx1* controls a network of growth factors, including BMPs and Shh that function in the developing palate. The co-expression of *Shh* and *Bmp* observed in many developing vertebrate organs suggests a closely regulated relationship between Shh and BMPs (Bitgood and McMahon, 1995). The protein of each gene regulates the expression of the other, within the same or different tissue layers, either positively or negatively, depending on different organs (Laufer et al., 1994; Roberts et al., 1995; Duprez et al., 1996; Arkell and Beddington, 1997; Watanabe et al., 1998; Zhang et al., 2000a; Zhao et al., 2000). For example, Shh has been shown to induce the expression of *Bmp2* in the developing limb (Laufer et al.,

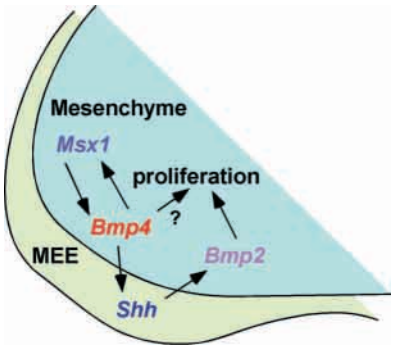


Fig. 8. A model for a genetic pathway integrating *Msx1*, *Bmp4*, *Shh* and *Bmp2* in the epithelial-mesenchymal interactions that regulate mammalian palatogenesis. In this model, it is proposed that in the anterior palatal shelves, mesenchymally expressed *Msx1*, which can be induced by *Bmp4*, is required for *Bmp4* expression in the palatal mesenchyme. Mesenchymally expressed BMP4 maintains *Shh* expression in the MEE and Shh in turn induces *Bmp2* expression in the mesenchyme. BMP2 functions to induce cell proliferation in the palatal mesenchyme, which leads to palatal growth.

1994; Duprez et al., 1996) and *Bmp4* in the gut mesenchyme (Roberts et al., 1995), but to repress *Bmp4* in the dorsal neural tube (Watanabe et al., 1998). In addition, the data presented here show that in the developing palatal shelves, BMP4 activity in the mesenchyme is required for the maintenance of *Shh* expression in the MEE, while the epithelially derived Shh in turn activates *Bmp2* in the palatal mesenchyme. These growth factors expressed in different tissue layers appear to act as signals mediating such epithelial-mesenchymal interactions leading to palatal growth.

In the developing mouse tooth germ, there exists a similar genetic hierarchy in which *Msx1* also controls the expression of *Bmp4* in dental mesenchyme. The mesenchymally expressed BMP4 in turn acts on the dental epithelium to regulate *Shh* expression. (Chen et al., 1996; Zhang et al., 2000a). This *Msx1* controlled signaling pathway is required for the induction of enamel knot and the progression of dental epithelial development from the bud stage to the cap stage (Chen et al., 1996; Jernvall et al., 1998; Zhao et al., 2000). However, in the developing palatal shelves, the *Msx1* controlled signaling network functions to facilitate cell proliferation within the mesenchyme. Therefore, although the similar *Msx1* governed genetic pathway is conserved in the mandible and palate, it is used to regulate the different developmental processes that bring about organ formation in these sites.

Msx1 and cleft palate formation

Mutations in the *Msx1* gene cause clefting of the secondary palate and tooth agenesis in both humans and mice (Stokata and Maas, 1994; Vastardis et al., 1996; Houzelstein et al., 1997; Van den Boogaard et al., 2000). However, whether *Msx1* is expressed in developing palate or not has been controversial, which led to the speculation that cleft palate formation in *Msx1*-deficient mice may be an intermediate effect that arises because of the primary failure of tooth development (MacKenzie et al., 1991; Satokata and Maas, 1994; Ferguson, 1994). In this study, we present evidence that *Msx1* is indeed expressed, although weakly, in the mesenchyme of the developing palatal shelves, and that expression is restricted to the anterior-most region of the palatal shelves. It is possible that in earlier studies, the high level of *Msx1* expression in the mesenchyme of the maxillary molar may have caused expression in the palate to be overlooked. We therefore conclude that the cleft palate seen in *Msx1* mutants is caused by an intrinsic defect in palatogenesis. This idea is further supported by the fact that a decrease in cell proliferation was observed in the $Msx1^{-/-}$ palatal mesenchyme. The rescue of cleft palate in *Msx1* mutants by transgenically expressed *Bmp4* also prevented neonatal death, indicating that lethality in *Msx1*-deficient mice resulted from cleft palate formation. It is interesting to note that abnormally low levels of cell proliferation were only seen in the anterior region of the palatal shelves where *Msx1* is normally expressed. The palatal growth appeared normal in the posterior portion of the $Msx1^{-/-}$ palatal shelves. Palatal closure is known to occur in an anterior-to-posterior sequence, and we have demonstrated a growth defect at the anterior region of the $Msx1^{-/-}$ palatal shelves that leads to complete palatal clefting. Taken together, these results suggest a zipper-like mechanism for palatal closure. The differential responsiveness of anterior versus posterior palatal mesenchyme to the induction of growth factors such as BMP and FGF provides the first evidence for the existence of heterogneity along the anteroposterior axis of developing palate. The development of the posterior region of mammalian palatal shelves seems to be regulated by a distinct set of genes, which warrant future investigation.

Msx1 may control cell proliferation by regulating the expression of growth factors

In the developing mouse limb, *Msx1* is strongly expressed in the progress zone where rapid cell proliferation occurs (Hill et al., 1989; Robert et al., 1989), implicating a role for *Msx1* in cell proliferation. Forced expression of *Msx1* in myoblasts or even myotubes promoted cell proliferation in vitro and concurrently inhibited terminal differentiation by repressing the expression of muscle-specific proteins including *MyoD* (*Myod1* – Mouse Genome Informatics) (Song et al., 1992; Woloshin et al., 1995; Odelberg et al., 2000). However, the precise role of *Msx1* in facilitating cell proliferation was unclear. Our results demonstrate that in the developing mouse palatal shelves, *Msx1* is indeed required for proper cell proliferation. In the absence of *Msx1*, a reduced level of cell proliferation occurs in the anterior palatal mesenchyme where *Msx1* is normally expressed. However, *Msx1* gene product appears not to be directly involved in regulating cell proliferation, at least in developing palatal shelves. Instead it controls a BMP4-mediated signaling pathway that functions to promote cell division. This finding is supported by our demonstrations that ectopically expressed *Bmp4* restored cell proliferation in the absence of *Msx1*, and that Shh and BMP2 induced cell proliferation in the $Msx1^{-/-}$ palatal mesenchyme in vitro. Both BMPs and Shh were able to bypass the requirement for *Msx1* and acted as mitogenic signals that stimulated cell proliferation in the developing palate.

BMP2 mediates the mitogenic activity of Shh

Several studies have demonstrated that Shh can function as a mitogen in many vertebrate organs, including presomitic mesoderm and somite (Fan et al., 1995; Marcelle et al., 1999), developing lung (Bellusci et al., 1997), limb bud (Duprez et al., 1998), cerebellum and retina (Jensen and Wallace, 1997; Wechsler-Reya and Scott, 1999). Of particular, relevance to our study, Shh has been shown to have mitogenic activity in the craniofacial tissues that include chick facial primordia, craniofacial neural crest cells and mouse tooth germ (Hardcastle et al., 1998; Ahlgren and Bronner-Fraser, 1999; Hu and Helms, 1999). Ectopic application of Shh induced a significant increase in the level of cell proliferation in the chick facial primordia (Hu and Helms, 1999) and stimulated abnormal levels of epithelial cell proliferation in the mouse oral cavity (Hardcastle et al., 1998). We provide evidence that Shh does not function directly as a mitogen in the palate. The mitogenic activity that Shh executed on mouse palatal mesenchyme apparently occurs through an intermediate mechanism that involves inducing and/or maintaining *Bmp2* expression. Our data indicate that it is BMP2 that functions to mediate the mitogenic signal from Shh during mammalian palatogenesis. As a downstream gene of Shh, *Bmp2* has also been shown to mediate partial polarizing activities of Shh function in developing limb (Laufer et al., 1994; Duprez et al., 1996; Drossopoulou et al., 2000). Evidence presented in this study establishes a new function for BMP2 in mediating the

mitogenic activity of Shh in the regulation of palatal growth in mammalian embryo.

BMPs, including BMP2, BMP4 and BMP7, have been shown to function as mitogens (Barlow and Francis-West, 1997; Wang et al., 1999), although an opposing role, that of repressing cell proliferation, has been reported for BMP4 in lung development (Bellusci et al., 1996). Similar to the mouse palatal mesenchyme studies presented here, the ectopic application of BMPs has been shown to enhance cell proliferation in the chick mandibular primordia (Barlow and Francis-West, 1997; Wang et al., 1999). As both mandibular mesenchyme and palatal mesenchyme are derived from cranial neural crest cells, they are likely to respond similarly to BMP signaling in terms of cell proliferation. However, at present it still remains unclear whether or not BMP acts directly or through secondary factors, to induce mitogenesis.

In summary, the results presented in this study provide evidence for the cellular and molecular aetiology of the non-syndromic clefting caused by the mutations in the *Msx1* gene, and identify a role for BMPs and Shh in mammalian palate development. Most importantly, ectopic expression of *Bmp4* in the $Msx1^{-/-}$ palatal mesenchyme was able to bypass the requirement for *Msx1* in palatogenesis and rescued the cleft palate phenotype. This finding suggests potential therapeutic strategies to prevent and cure embryonic cleft palate in the future.

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