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# Analysis of Epithelial–Mesenchymal Interactions in the Initial Morphogenesis of the Mammalian Tooth

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**Epithelial–mesenchymal interactions govern the development of epidermal organs such as teeth. During the early stages of tooth development, a local ectodermal thickening which expresses several signaling molecules appears. It is believed that these in turn signal to the underlying mesenchyme triggering mesenchymal condensation and tooth development. For example, epithelially expressed Bmp4 induces *Msx1* and *Lef1* as well as itself in the underlying mesenchyme. In this paper we have investigated the role of four epithelial signaling molecules, Bmp2, Shh, Wnt10a, and Wnt10b, in the early inductive cascades that govern tooth development. We show that all four genes are specifically expressed in the epithelium between E11.0 and E12.0 when tooth morphogenesis is first apparent. Although Shh, Bmp2, and Wnt10b have similar, if not identical, expression patterns, each signal has a distinct molecular action on the jaw mesenchyme. Whereas Shh and Wnt10b can induce general Hedgehog and Wnt targets, *Ptc* and *Gli* for Shh and *Lef1* for Wnt10b, only Bmp2 is able to induce tooth-specific expression of *Msx1*. Thus, there are distinct targets for all three pathways. Interestingly, both Bmp and Wnt signaling activate *Lef1*, making it a candidate for integrating the two distinct signaling pathways.** 

*Key Words:* **epithelial–mesenchymal interactions; tooth development; Wnt signaling; Shh signaling; Bmp signaling.**

## INTRODUCTION

Epithelial–mesenchymal interactions govern the development of all epidermal organs, including teeth, whiskers, hair follicles, and mammary glands (Kollar, 1970; Kollar and Baird, 1970; Sakakura, 1987; Lumsden, 1988; Hardy, 1992; Jahoda, 1992). Interestingly, the initial morphological development of these organs is similar; the epithelium undergoes a local thickening followed by a local condensation of the mesenchyme beneath it. The epithelium then invaginates into the condensing mesenchyme until it has reached a characteristic bud structure. After this stage, the development of these organs diverges in order to give rise to specialized organs with vastly different morphologies, cell types, and functions (Lefkowitz *et al.*, 1953; Cohn, 1957; Sakakura, 1987; Hardy, 1992).

Despite practically indistinguishable early morphological events, different animal species develop dentitions that are species specific with respect both to the pattern of the tooth crown and to the pattern of tooth placement in the jaws. In mice, dentition of the lower jaw consists of a set of incisors at the tip of the jaws separated from three sets of molars by the diastema, a region devoid of teeth. Incisor and first molar development begins at E11.0 and E11.5, respectively, when the oral epithelium thickens above the neural-crest-derived mesenchyme. At E13.5, cells at the tip of the molar bud stop dividing and form the enamel knot, a morphologically distinct group of cells (Butler, 1956). It is believed that these cells function as a signaling center that stimulates the rest of the epithelium to continue to proliferate until the molar primordium has reached the cap stage at approximately E14.5 (Jernvall *et al.*, 1994, 1998; Vaahtokari *et al.*, 1996; Åberg *et al.*, 1997). After this stage, additional enamel knots appear and pattern the crown of the tooth, and adjacent epithelium and mesenchymal cell layers differentiate into enamel-secreting ameloblasts and dentin-secreting odontoblasts, respectively (Lefkowitz *et al.*, 1953; Cohn, 1957). The second and third molars develop later.

At each stage during tooth development, the epithelium and the mesenchyme signal to one another in order to tightly coordinate their development. Although this is achieved through multiple and reciprocal epithelial–mesenchymal interactions, at any one stage either the epithelium or the mesenchyme is instructive and is able to drive organ formation (for review, see Maas and Bei, 1997).

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For example, classical embryological experiments show that until the bud stage, the epithelium is instructive. If E11.0 oral ectoderm is recombined with nontooth neural-crest-derived mesenchyme and grafted in a neutral environment, a fully developed tooth is formed (Lumsden, 1988). Conversely, the mesenchyme does not have the ability to instruct the nontooth ectoderms tested to date. After bud stage, the epithelium loses its instructive property and the mesenchyme acquires it (Kollar and Baird, 1969, 1970; Lumsden, 1988).

Classical experiments have also demonstrated that tooth development can be studied *in vitro*. Whole jaws and even isolated tooth rudiments explanted prior to E11.0 support tooth development if they are grown in organ culture (Szabo, 1954; Yamada *et al.,* 1980). In the presence of ascorbic acid, fully formed teeth secreting the enamel and dentin matrices are formed (Thesleff, 1976). This technique has been adapted in order to study the molecular mechanisms underlying epithelial–mesenchymal interactions. Following expression pattern analysis, the effects of epithelial signaling molecules have been studied by coating beads with protein and culturing them with presumptive molar mesenchyme. For example, at E12.0, members of the bone morphogenetic protein signaling family, *Bmp2* and *Bmp4,* are expressed in the epithelium (Wozney *et al.,* 1988; Lyons *et al.,* 1989), while two closely related homeobox genes, *Msx1* and *Msx2* (Robert *et al.,* 1989; Hill *et al.,* 1989; Monaghan *et al.,* 1991), and *Bmp4* and *lympoid enhancer-binding factor 1* (*Lef1*) (a member of the HMG DNA-binding protein family) (Travis *et al.,* 1991) are expressed in the underlying mesenchyme (MacKenzie *et al.,* 1991, 1992; Vainio *et al.,* 1993; van Genderen *et al.,* 1994; Kratochwil *et al.,* 1996). Manipulations performed in organ culture showed that the isolated molar mesenchyme responds to Bmp2 or Bmp4 protein by ectopically expressing *Bmp4, Lef1, Msx1,* and *Msx2* (Jowett *et al.,* 1993; Vainio *et al.,* 1993; Chen *et al.,* 1996; Kratochwil *et al.,* 1996). These data were further refined by the analysis of the null alleles of *Msx1* and *Lef1* in which the teeth are arrested at bud stage. By combining marker analysis of the mutant teeth with manipulations performed in organ culture, a more precise genetic pathway was established. In the mesenchyme, *Msx1* induces *Bmp4* which in turn induces *Lef1* (Vainio *et al.,* 1993; Chen *et al.,* 1996; Kratochwil *et al.,* 1996). Expression pattern analysis has also shown that prior to E11.5, *Msx1* (Dr. Irma Thesleff, personal communication), *Bmp4,* and *Lef1* are expressed in the thickening epithelium (Vainio *et al.,* 1993; Kratochwil *et al.,* 1996).

We are interested in further delineating the role of epithelial signaling molecules during the initiation of tooth development. To this end, we have focused on the role of three signaling families, the Bmp, Hedgehog, and Wnt families. Interactions among these three signaling families have been shown to be essential for the development of some imaginal disc-derived structures in *Drosophila,* and individual members have been shown to regulate specific aspects of mammalian development. Previous studies demonstrated that *Sonic hedgehog* (*Shh*) (Echelard *et al.,* 1993) is expressed in the epithelium of the developing tooth throughout embryogenesis, but its function in this organ is unknown (Bitgood and McMahon, 1995; Iseki *et al.,* 1996; Vaahtokari *et al.,* 1996). Several components of the Hh/Shh pathways have also been described. Among these Ptc has been identified as a receptor for Shh (Marigo *et al.,* 1996a; Stone *et al.,* 1996) and Gli as a transcriptional effector of Shh signaling (reviewed in Hammerschmidt *et al.,* 1997; Tabin and McMahon *et al.,* 1997). Several Wnt members are expressed during tooth development (A. P. McMahon, unpublished data). We report here on two members, *Wnt10a* and *Wnt10b* (Adamson *et al.,* 1994; Christiansen *et al.,* 1995; Lee *et al.,* 1995), whose expression correlates with the initiation of tooth development. As some Wnt signaling has been shown to be mediated through the action of a Lef1/$\beta$-catenin transcription complex, the observed requirement for Lef1 in tooth development is consistent with a role for Wnt signaling (Brunner *et al.,* 1997; for review, see Cadigan and Nusse, 1997; Moon *et al.,* 1997).

## MATERIALS AND METHODS

### *Dissections and Cultures*

The mandibular portion of the first branchial arch was dissected from E11.0 Swiss Webster embryos in PBS using a dissecting microscope. Throughout the text the ages of specimens are given assuming mating at midnight; E12.5 represents noon on the 13th day of pregnancy. Beads or NIH3T3 pellets were inserted into the jaw mesenchyme through the opening created when the mandibles were dissected out of the embryo. They were then manipulated to lie close to the oral epithelium. The mandibles were cultured on 0.1-$\mu$m Nucleopore filters (Costar) resting on stainless steel grids at the surface of the culture medium (Yamada *et al.,* 1980). The culture medium was made up of Dulbecco's modified Eagle's medium (Sigma D5796) supplemented with 10% fetal calf serum (Hyclone), 1× penicillin/streptomycin (GibcoBRL 0511), and 2 mM glutamine (Gibco BRL 1273). After 30–36 h of culture at 37°C, 5% $CO_2$, the cultures were fixed in 4% paraformaldehyde in PBS overnight and then gradually transferred to methanol for storage at −20°C.

### *Bead Coating*

Five microliters of Affi-Gel blue beads (75–150 $\mu$m in diameter, Bio-Rad 153-7302) or heparin acrylic (Sigma H5263) was added to an equal volume of Shh protein (14 mg/ml) amino acids 25–198. The protein was purified as in Marti *et al.* (1995). The beads were then incubated on ice for at least 1 h. The excess protein was washed away with Dulbecco's PBS, and the beads were stored in PBS at 4°C for up to 2 weeks. The same procedure was used for coating Affi-Gel blue beads with Bmp2, and the excess protein was washed away with 0.02 M sodium acetate, pH 5.0, + 0.2% BSA. Recombinant Bmp2 protein was a gift from Genetics Institute.

### *Cell Lines*

NIH3T3 cell lines expressing either Wnt1 or LacZ were created by retroviral infection (Kispert *et al.,* 1998). Wnt10b was subcloned

into the expression vector pLNCX which utilizes a CMV promotor (Miller and Rosman, 1989) and then transfected directly into NIH3T3 cells and placed under G418 selection for several weeks. Cell pellets were made by hanging drops after dissociating the cultures to single cells by trypsinization.

### *In Situ Hybridization*

*In situ* hybridization was performed as described in Parr *et al.* (1993) and modified according to Knecht *et al.* (1995). Incubation times used for intact and cultured mandibles were 15 min in 6% $H_2O_2$ and 35 min in 10 $\mu$g/ml proteinase K. Incubation times for vibratome sections were 45 min in $H_2O_2$ and 12 min for 40-$\mu$m sections and 35 min for 100-$\mu$m sections in proteinase K. Digoxigenin and fluorescein probes were synthesized using the Boehringer-Mannheim RNA labeling kit. A double-labeling procedure was performed as described in Cygan *et al.* (1998). Whole mounts were developed using BM purple AP substrate (BM 1442074) and INT/BCIP (BM 1681460).

### *Probes*

Antisense RNA probes were made as described for Bmp2 (referred to as Bmp-2A) (Lyons *et al.*, 1989), Bmp4 (Jones *et al.*, 1991), Fgf8 (Crossley and Martin, 1995), Gli (Hui *et al.*, 1994), Lef1 (Kratochwil *et al.*, 1996), Ptc (Goodrich *et al.*, 1996), and Shh (Echelard *et al.*, 1993). The 840-bp Msx1 probe was generated from an *Eco*RI fragment cloned into pT219 (a gift from Dr. R. Hill), linearized with *Bss*HII, and transcribed with T7. The 240-bp Msx2 probe was made from the cDNA (a gift from Dr. R. Maxson), linearized with *Bsu*36I, and transcribed with T7. Wnt10a corresponds to the 1.2-kb *Nco*I–*Bsm*I cDNA fragment (Wang and Shackleford, 1996). Wnt10b was synthesized from a 400-bp PCR fragment cloned as described in Gavin *et al.* (1990), linearized with *Eco*RI, and transcribed with T3 RNA polymerase.

### *Vibratome Sections*

Tissue was fixed overnight in 4% paraformaldehyde and then dehydrated through a methanol series. After rehydration, tissue was embedded in 30% BSA, 0.49% gelatin, 20% sucrose in PBS and crosslinked with glutaraldehyde (Bober *et al.*, 1994). Forty- or 100-$\mu$m sections were collected in PBS and then dehydrated once again for storage in methanol. After the whole-mount procedure the sections were mounted in 80% glycerol and photographed using Nomarski optics.

## RESULTS

### *Expression of Shh, Bmp2, Wnt10a, and Wnt10b Coincides in the Tooth Epithelium*

We compared the expression patterns of *Bmp2, Shh, Wnt10a,* and *Wnt10b* in the developing mandible between E11.0 and E12.0 by whole-mount *in situ* hybridization (Fig. 1). In all cases, expression was symmetrical on either side of the median sulcus, the groove representing the site of fusion between the right and left mandibular processes. We find that along the mesial–distal axis (see Fig. 1, II for axis definitions), where incisors develop mesially and molars distally, *Bmp2, Shh, Wnt10a,* and *Wnt10b* show similar patterns of expression restricted to the epithelium in the developing tooth field.

At E11.0, a stage at which there is only a slight thickening of presumptive incisor epithelium, *Wnt10b, Bmp2,* and *Shh* are expressed at the mesial tip of the jaw in a short, thin strip of epithelial cells that extends distally (Figs. 1B–1D). At this time, *Shh* expression is robust, whereas both *Bmp2* and *Wnt10b* are only weakly expressed. *Wnt10a* is not expressed at this stage (Fig. 1A). *Shh* is also expressed further distally at the site of amalgamation, the origin of the first and second pharyngeal arches (Fig. 1D).

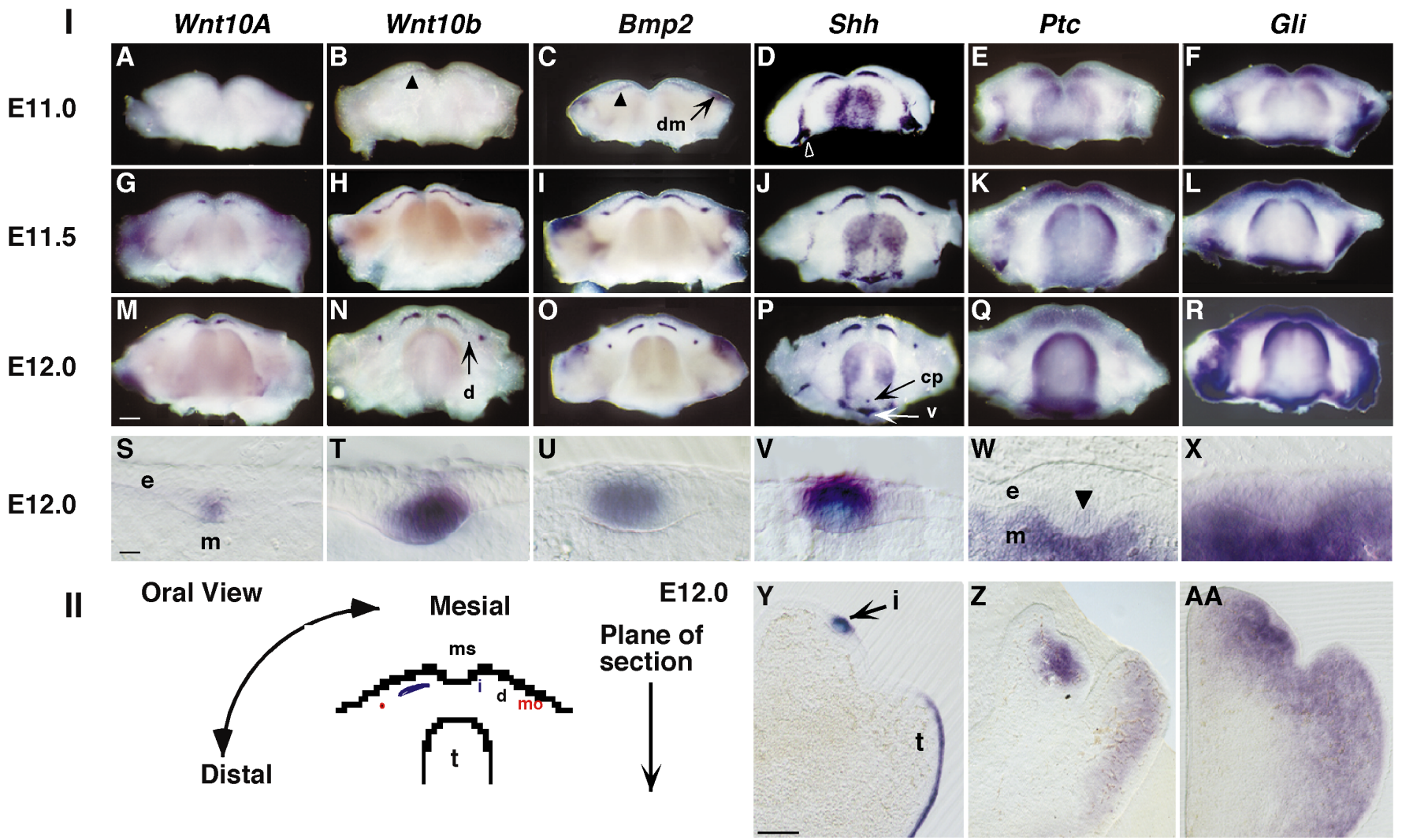
At E11.5, the midline expression of *Wnt10b, Bmp2,* and *Shh* extends further distally (Figs. 1H–1J). *Bmp2* and *Wnt10b* are upregulated relative to E11.0, and *Wnt10a* can now be detected at low levels in a similar domain (Fig. 1G). All four signals share a similar expression domain with highest levels of expression mesially and distally and an apparent downregulation in between.

By E12.0, *Wnt10a, Wnt10b, Bmp2,* and *Shh* have been completely downregulated in the diastema and are coexpressed at the midline in the incisor domains and further distally in the molar primordia (Figs. 1M–1P). Sections through the incisors show that *Wnt10a, Wnt10b, Bmp2,* and *Shh* are expressed at the tip of the tooth epithelium (Figs. 1S–1V). Once again, *Wnt10a* expression is considerably weaker than that of the other genes. Double-whole-mount *in situs* of *Wnt10a*/*Shh* and *Wnt10b*/*Shh* confirmed that these genes are expressed in either the same or intermixed populations of cells (data not shown).

As the tooth continues to develop, expression of *Wnt10a* and *Wnt10b* is maintained in the epithelium. At bud stage, E13.5, both genes are most strongly expressed at the tip of the invaginating epithelium (Figs. 2A and 2C). The distribution of *Wnt10b* appears to be asymmetric, although this might be an artifact of the plane of section. At cap stage, E14.5, both genes are strongly expressed in the enamel knot (Figs. 2B and 2D) along with *Bmp2* and *Shh* (Vainio *et al.*, 1993; Bitgood and McMahon, 1995; Vaahktokari *et al.* 1996). Thus, although there is some difference in the kinetics of *Wnt10a* activation, all four members show a pattern of ectodermal expression which is indistinguishable at this level of resolution. As the ectoderm is believed to govern tooth development until E12.0, these data suggest that the concerted action of Bmp, Hedgehog, and Wnt signaling may be a critical component of these interactions.

### *Expression of Ptc and Gli in the Mesenchyme along the Mesial–Distal Axis*

To address which cells might respond to these ectodermal signals, we looked at the distribution of *Ptc* and *Gli* which are both transducers and transcriptional targets of Shh signaling. Between E11.0 and E11.5, both genes are strongly expressed in the mesenchyme along the mesial–distal axis in patterns that are complementary to and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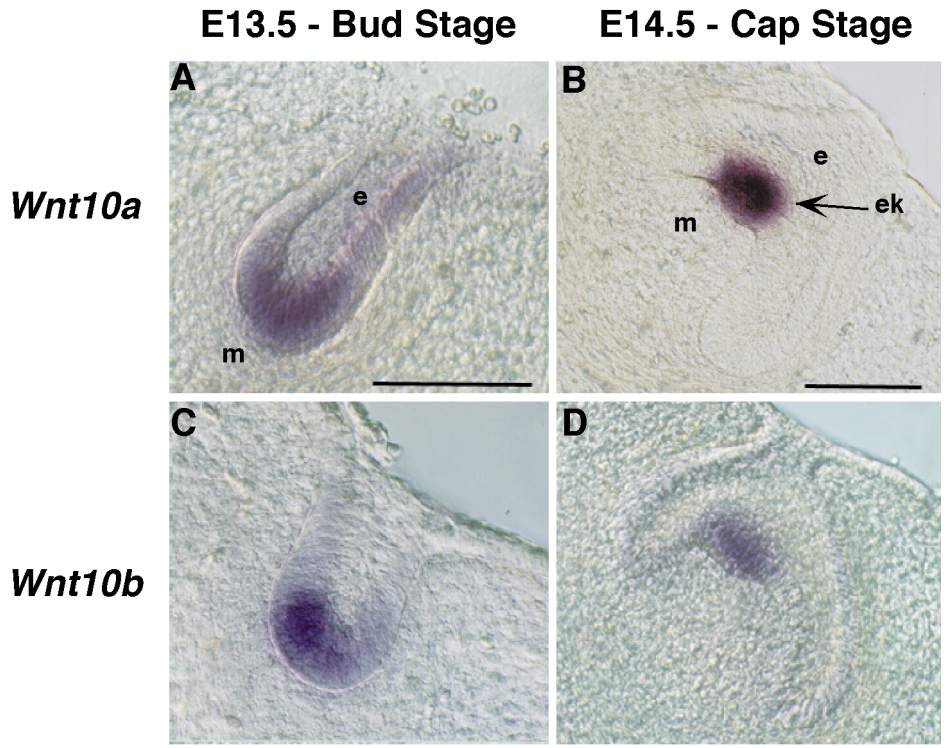


**FIG. 2.** Expression pattern of *Wnt10a* and *Wnt10b* at bud and cap stages. Frontal sections through E13.5 and E14.5 molars. *Wnt10a* (A) and *Wnt10b* (C) are expressed at the tip of the epithelial bud. *Wnt10a* (B) and *Wnt10b* (D) are expressed in the enamel knot. e, epithelium; ek, enamel knot; m, mesenchyme; Scale bars = 0.1 mm.

somewhat broader than *Shh*, suggesting that Shh signals from the epithelium to the underlying tooth mesenchyme (Figs. 1E, 1F, 1K, and 1L). Throughout these stages, weak expression of *Gli* is also detected in the epithelium. By E12.0, *Ptc* expression is completely downregulated in the diastema (Fig. 1Q), whereas *Gli* (Fig. 1R) expression is maintained at low levels. As earlier, *Ptc* is expressed at high levels immediately beneath the thickened involuted dental epithelium of the incisor (Goodrich *et al.*, 1996; Thomas *et al.*, 1997), but unlike earlier stages *Ptc* is also weakly expressed at the tip of the invaginating epithelial cells (Fig. 1W). *Gli* transcripts are also detected in both the mesenchyme and at lower levels in the incisor epithelium (Fig. 1X).

**FIG. 1.** Expression of *Wnt10a*, *Wnt10b*, *Bmp2*, *Shh*, *Ptc*, and *Gli* in the developing mandible between E11.0 and E12.0. (A–F) Gene expression at E11.0. *Wnt10a* is not expressed (A). *Wnt10b* (B), *Bmp2* (C), and *Shh* (D) are expressed along the mesial–distal axis in a tight strip of cells on either side of the midline (arrowheads). *Ptc* (E) and *Gli* (F) are expressed similarly, but more diffusely. *Shh*, *Ptc*, and *Gli* are also expressed in the presumptive tongue, and *Shh* is expressed at the site of amalgamation (open arrowhead). *Bmp2* is expressed in the most distal mesenchyme. (G–L) Gene expression at E11.5. *Wnt10a* (G), *Wnt10b* (H), *Bmp2* (I), *Shh* (J), *Ptc* (K), and *Gli* (L) expression extends distally from the mesial tip of the jaw to the molar anlagen. (M–R) Gene expression at E12.0. *Wnt10a* (M), *Wnt10b* (N), *Bmp2* (O), *Shh* (P), *Ptc* (Q), and *Gli* (R) are expressed in the incisor domains and further distally in the molar anlagen. There is no expression in the diastema. *Shh*, *Ptc*, and *Gli* continue to be expressed in the presumptive tongue. (S–X) Parasagittal sections at E12.0 through the incisors. *Wnt10a* (S), *Wnt10b* (T), *Bmp2* (U), and *Shh* (V) are expressed in the epithelium at the tip of the invaginating epithelial cells of the incisors. *Ptc* (W) is expressed in the mesenchyme underlying the thickened epithelium and weakly at the tip of the invaginating epithelium (arrowhead). (X) *Gli* is expressed in the mesenchyme. Parasagittal sections at E12.0 through the incisors and the tongue. *Shh* (Y) is expressed in the tooth and tongue epithelium. *Ptc* (Z) is expressed in the tooth and tongue mesenchyme underlying the epithelium. (AA) *Gli* expression throughout the oral mesenchyme. (II) Schematic depicting an oral view of a mandible at E12.0. i, incisor; cp, circumvallate papillae; d, diastema; dm, distal mesenchyme; e, epithelium; m, mesenchyme; mo, molar; ms, median sulcus; t, tongue; v, V-shaped junction of the left and right second pharyngeal arches. Scale bars: A–R = 0.5 mm; S–X = 0.01 mm; Y–AA = 0.1 mm.

### Other Areas of Expression in the Developing Jaw

In addition to expression along the mesial–distal axis, *Shh, Ptc,* and *Gli* are broadly expressed in the developing tongue as early as E11.0 (Figs. 1D–1F, 1J–1L, and 1P–1R). As in the tooth, in the tongue *Shh* is only expressed in the epithelium (Fig. 1Y). *Shh* transcripts are also found at the base of the tongue in a V-shaped notch that separates the medial ends of the left and right second pharyngeal arches and in the circumvallate papillae. By E12.5, *Shh* expression in other regions of the tongue is restricted to papillae associated with the taste buds (Bitgood and McMahon, 1995). *Ptc* and *Gli* expression is restricted to the mesenchyme, but whereas *Ptc*-expressing cells are only located immediately beneath *Shh*-expressing cells (Fig. 1Z), *Gli* is more broadly expressed in the jaw and tongue mesenchyme (Fig. 1AA). Unlike the tooth, *Bmp2, Wnt10a,* and *Wnt10b* are not expressed in the tongue at these stages, although *Bmp2* is also expressed in the most distal mesenchyme of the outgrowing jaw (Fig. 1O). Thus, it is likely that Bmp2 and Shh play roles beyond tooth development in the jaws, but that the tooth represents a unique site on the oral surface at which all three signals converge.

### Mandible Cultures

The striking similarities between the expression patterns of *Bmp2, Shh, Wnt10a,* and *Wnt10b* in the developing tooth together with their known genetic interactions (and that of closely related family members) in other systems suggested that they might interact during tooth development (for review, see Perrimon, 1996; Hammerschmidt *et al.*, 1997).

To assay the effect of Bmp2, Shh, and Wnt10b protein on gene expression in both the epithelium and the mesenchyme, we isolated whole mandibles and applied an external source of protein in the mesenchyme close to the oral epithelium. The source of protein, either purified recombinant protein attached to beads or cell pellets producing particular proteins, was placed just outside the tooth domain, and controls, buffer-soaked beads or LacZ-transfected cells, were placed in a similar position on the contralateral side. The dissections were performed on E11.0 jaws and cultured for 30–36 h according to Yamada *et al.* (1980). By performing a whole-mount *in situ* analysis after culture and by assessing the distribution of markers on the control side in comparison with normal jaws, we were able to evaluate the extent of mandible development and to determine how the expression on the contralateral side may have been modified by the various experimental grafts. Based on an analysis of 444 cultures, we have determined that 84% remained healthy. Of the 114 assessed for expression of jaw markers, 77% developed to stages E11.5–E12.0 and showed epithelial thickenings characteristic of this stage of tooth development (data not shown). Only those cultures developing to this stage are included in our analysis.

### Bmp2 Induces Msx1 Expression in the Oral Epithelium

Previous reports have demonstrated that Bmp2 and Bmp4 regulate gene expression in the tooth mesenchyme (Vainio *et al.*, 1993; Jowett *et al.*, 1993; Kratochwil *et al.*, 1996). Consistent with this view, Bmp2 coated beads inserted into whole arches induce the mesenchymal tooth markers *Msx1, Bmp4, Lef1* (Figs. 3A, 3D and 3G), and *Msx2* (data not shown) in a tight ring around the beads. *Msx1* induction is very robust in 91% of cultures ($n = 47$; Fig. 3A). Sectioning reveals that *Msx1* is induced in the mesenchyme, but also in the oral epithelium surrounding the Bmp2 bead (Fig. 3C). Bmp2 also induces *Bmp4* expression in 86% of cultures ($n = 21$) and *Lef1* in 58% of cultures ($n = 17$; Figs. 3D and 3G). In contrast to *Msx1,* induction of *Bmp4* and *Lef1* was only observed in the mesenchyme (Figs. 3F and 3I). Interestingly, induction of *Msx1, Bmp4,* and *Lef1* is polarized in the grafts. Mesenchymal expression is only observed in the mesenchyme between the oral epithelium and the bead (Figs. 3C, 3F, and 3I). To address whether Bmp2 might activate other signals in the ectoderm, we examined expression of *Shh, Bmp2, Wnt10b, Ptc,* and *Gli.* None of these were induced by Bmp2 ($n = 9$ to 18; Figs. 3J–3N).

### Shh Induces Ptc and Gli, but Represses Wnt10b

We next assayed the activity of Shh by inserting beads coated with the N-terminus of Shh, the active subunit of the protein, into the mandibles (Bumcrot *et al.*, 1995; Marti *et al.*, 1995; Porter *et al.*, 1995). As *Ptc* and *Gli* are transcriptional targets of Shh, we assayed for their induction in cultured mandibles by whole-mount *in situ* hybridization. *Ptc* and *Gli* are broadly ectopically induced in the oral tissue surrounding the Shh coated bead in 68% ($n = 34$) and 60% ($n = 15$) of cases, respectively (Figs. 4A and 4E). Sections reveal that on the oral surface induction of *Ptc* and *Gli* occurs only in the mesenchyme, suggesting that only cells in the mesenchyme are competent to respond to Shh during the culture period (Figs. 4C and 4G). Moreover, as with the Bmp2 implants, only the first few cell layers below the epithelium respond to Shh by ectopically expressing *Ptc* and *Gli.* However, on the aboral surface many cell layers from the bead, *Ptc,* is strongly induced in the epithelium (Figs. 4D and 4I). We did not observe a similar induction of *Gli* on the aboral surface. Finally, although several lines of evidence suggest that *Bmp4* is a target of Shh signaling (Roberts *et al.*, 1995), we did not observe ectopic activation of *Bmp4 Msx1,* or *Lef1,* which is a target of Bmp signaling in the tooth mesenchyme ($n = 5$–14; Figs. 4N–4P). Thus, Shh signaling appears to occur through a separate pathway from Bmp2 and Bmp4, and as observed in many other circumstances the induction of *Gli* and *Ptc* is a specific response.

Surprisingly, we find that Shh represses expression of *Wnt10b* in the molar epithelium and on the aboral surface in the presumptive whisker epithelium, in 82% of cases

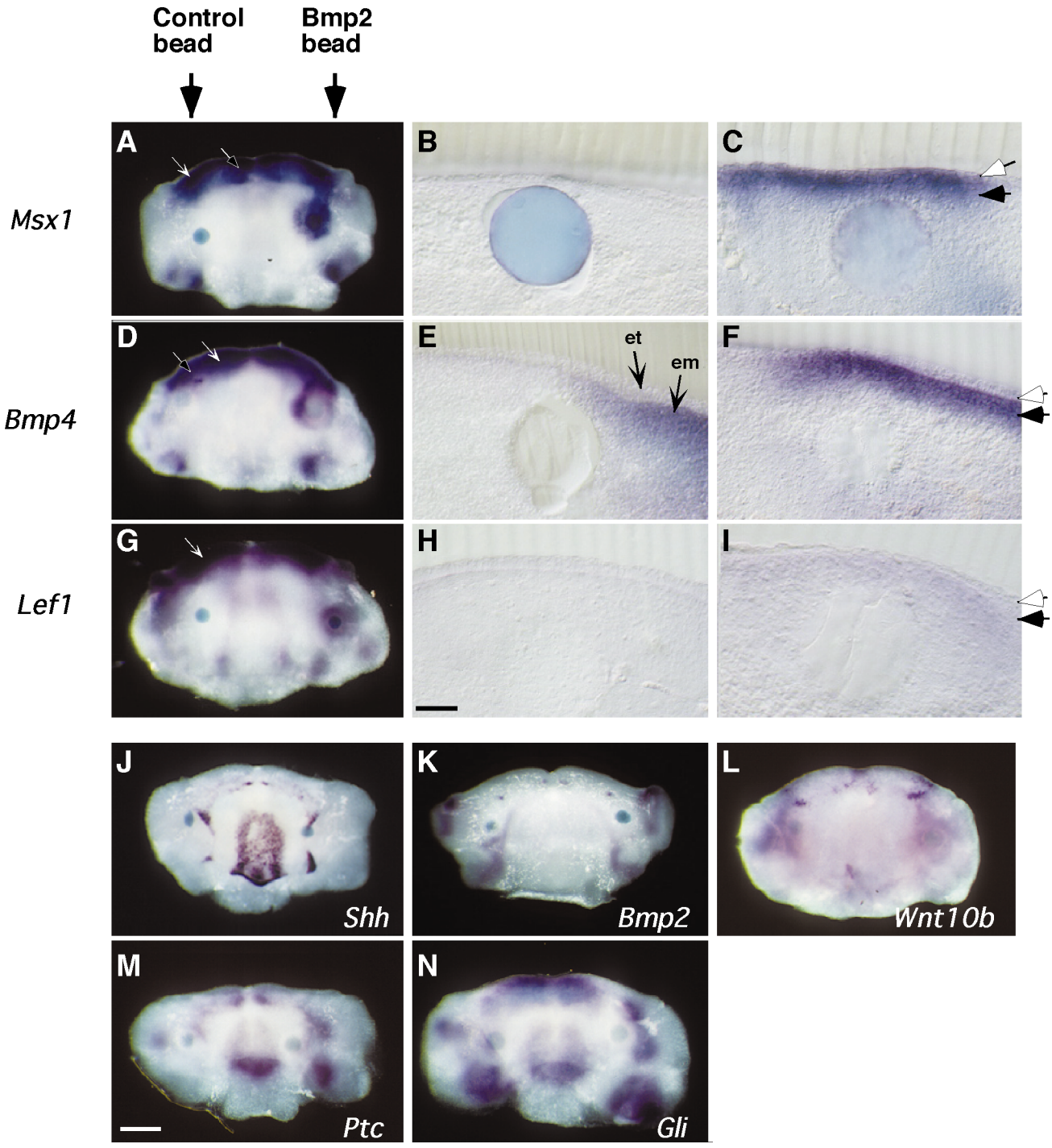


**FIG. 3.** Bmp2 induces *Msx1*, *Bmp2*, and *Lef1*, but not *Shh*, *Wnt10b*, *Bmp2*, *Gli*, or *Ptc*. Bmp2 beads and control beads were inserted into E11.0 mandibles on either side of the tongue. After culture they were analyzed by whole-mount *in situs*. *Msx1* (A), *Bmp4* (D), and *Lef1* (G) are ectopically induced around the Bmp2 beads, but not around the control beads. Endogenous expression in the outgrowing jaw (arrows) and in the teeth is conserved (open arrowheads). Parasagittal sections show that control beads do not induce *Msx1* (B), *Bmp4* (E), and *Lef1* (H). Endogenous *Bmp4* expression (em) underlying the tooth thickening (et) is visible in E. Sections through the Bmp2 beads show that *Msx1* (C) is ectopically induced in the oral epithelium (open arrow) and in the underlying mesenchyme (filled arrow); *Bmp4* (F) and *Lef1* (I) are induced in the mesenchyme (filled arrow) underlying the oral epithelium (open arrow). *Shh* (J), *Bmp2* (K), *Wnt10b* (L), *Ptc* (M), and *Gli* (N) were not induced by either Bmp2 or control beads, but endogenous patterns were conserved (compare with Fig. 1). Scale bars: A, D, G, and J–N = 0.6 mm; B, C, E, F, H, and I = 0.05 mm.

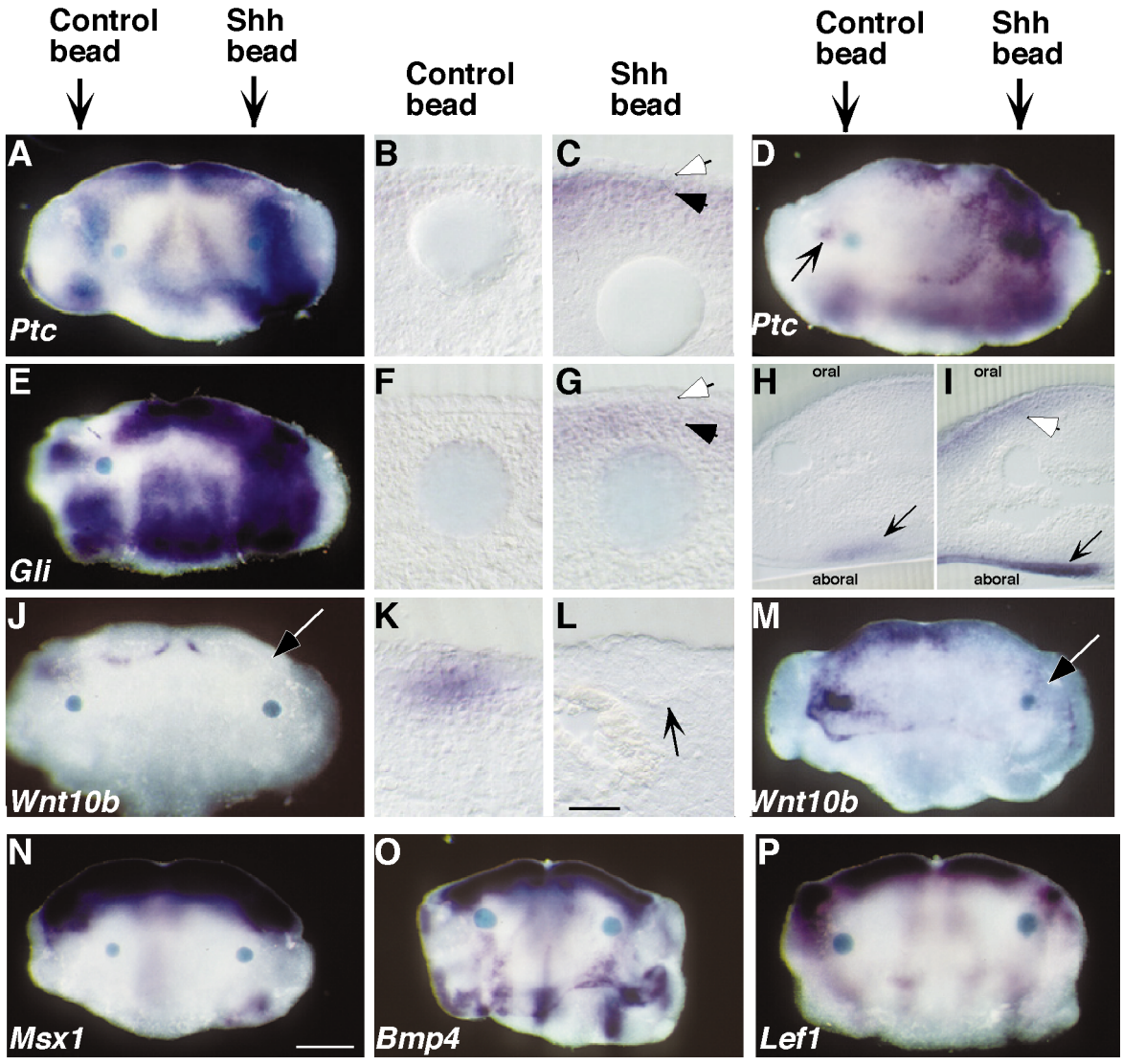


**FIG. 4.** Shh beads induce ectopic expression of *Ptc* and *Gli* and repress expression of *Wnt10b*. (A–I) Shh beads induce *Ptc* and *Gli* expression in the mandible. Shh coated beads induce *Ptc* (A) and *Gli* (E) on the oral surface, but control beads do not. Parasagittal sections through control bead and the surrounding tissue show that neither *Ptc* (B) nor *Gli* (F) is induced. Sections through Shh coated bead and the surrounding tissue show that *Ptc* (C) and *Gli* (G) are induced in the oral mesenchyme (filled arrowhead), but not in the epithelium (open arrowhead). (D) *Ptc* is also induced on the aboral side by a Shh bead. (H) Parasaggital section showing no *Ptc* expression on the oral surface of the jaw and weak mesenchymal expression on the aboral surface corresponding to the presumptive whisker anlage (arrow) associated with the control bead implant. (I) Section through the Shh bead showing ectopic expression of *Ptc* in the mesenchyme on the oral surface and in the epithelium on the aboral surface. (J–M) Shh beads repress *Wnt10b*. (J) *Wnt10b* is repressed by Shh beads, but not by control beads. (K) Section through molar epithelial thickening near the control bead. *Wnt10b* is expressed in the epithelium. (L) Section through molar epithelial thickening near the *Shh* coated bead. *Wnt10b* expression is repressed but the epithelial tooth thickening (arrow) is still present. (M) *Wnt10b* is repressed by the Shh bead (arrow) but not the control bead on the aboral surface. (N–P) *Msx1*, *Bmp4*, and *Lef1* are not induced by Shh. Endogenous expression of *Msx1* (N), *Bmp4* (O), and *Lef1* (P) in the outgrowing jaw and in the teeth is conserved; neither Shh beads nor control beads induce their expression. Scale bars: B, C, F, G, K, L, H, and I = 0.05 mm; A, D, E, J, and M–P = 0.5 mm.

($n$ = 23; Figs. 4J and 4M). Despite the absence of *Wnt10b*, normal thickening of the dental lamina occurs (Fig 4L) and normal buds develop after 3 days of culture (data not shown). We were not able to conclusively determine if *Wnt10a* is similarly repressed by Shh because at E11.5–E12.0 *Wnt10a* expression is weak and cannot be accurately scored in cultures. In the presence of Shh bead implants, no repression of other epithelial markers including *Bmp2*,

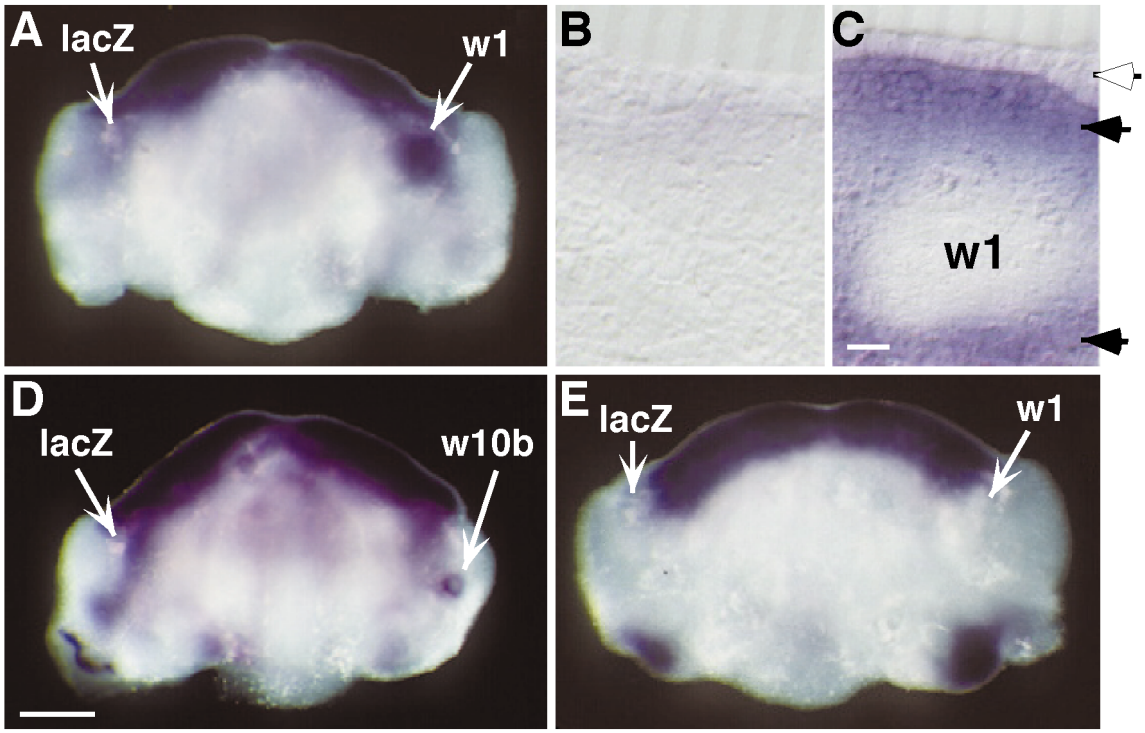


**FIG. 5.** Wnt-expressing cells induce ectopic *Lef1* expression. (A) Wnt1-expressing cells induce *Lef1* in the cells surrounding the pellet, whereas LacZ-expressing cells do not. (B) Parasaggital section through the control bead and surrounding tissue; *Lef1* is not induced. (C) *Lef1* is induced in the mesenchyme surrounding the pellet of Wnt1-expressing cells (filled arrow) but not in the epithelium (open arrow). Wnt1-expressing cells do not express *Lef1*. (D) *Lef1* is induced by Wnt10b-expressing cells. (E) *Msx1* is not induced by Wnt1-expressing cells. lacZ, lacZ-expressing cells; w1, Wnt1-expressing cells; w10b, Wnt10b-expressing cells. Scale bars: A, D, and E = 0.5 mm; B and C = 0.05 mm.

*Fgf8*, and *Shh* is observed compared to control bead implants (data not shown). Thus, the inhibitory effect of Shh on *Wnt10b* appears to be specific.

### *Wnt-Expressing Cells Induce Lef1*

Finally, we addressed the response of the mesenchyme and epithelium to two Wnts, Wnt1 and Wnt10b, both of which were identified in the context of mammary transformation (Nusse and Varmus, 1982; van Ooyen and Nusse, 1984; Lee *et al.*, 1995). As recombinant Wnt proteins are not available, we compared the response of cells to cell implants expressing these Wnts. Pellets of Wnt-expressing cells were inserted into one side of the mandible and lacZ-expressing cells into the contralateral side. Both Wnt1-expressing cells (Fig. 5A) and Wnt10b-expressing cells (Fig. 5D) are able to induce *Lef1*, although the response to Wnt1 is broader and more consistent than to Wnt10b, 64% of grafts ($n$ = 14) versus 42% ($n$ = 38). Wnt10b is most likely a member of the Wnt1 class of Wnt signals, and the differences in activity between the Wnt1- and Wnt10b-expressing cells can be accounted for by the fact that a much larger percentage of cells express Wnt1 than Wnt10b in each respective cell line. As Wnt1 and Wnt10b are similar, but Wnt1 cells are more active, we decided to focus our study on the Wnt1-expressing cells. Sections through mandibles show that Wnt1 induction of *Lef1* is specific to the mesenchyme even in cases where the pellet is in contact with the epithelium. Interestingly, unlike the Bmp2 bead implants, *Lef1* is induced all around the cell implant, not just in the mesenchyme under the epithelium (Fig. 5C). Further, Wnt cell implants did not induce *Msx1* ($n$ = 8) (Fig. 5E), suggesting that Wnt10b signaling is distinct from Bmp signaling which induces both *Msx1* and *Lef1*.

## DISCUSSION

We have investigated the role of epithelial signaling in the early inductive cascades that govern tooth development. We show that although Shh, Bmp2, and Wnt10b have very similar, if not identical, expression patterns in the dental lamina from E11.0, each signal has a distinct molecular action on the jaw mesenchyme. Whereas Shh and Wnt10b can induce general Hedgehog and Wnt targets, *Ptc* and *Gli* for Shh and *Lef1* for Wnt10b, only Bmp2 is able to

induce tooth-specific expression of *Msx1*. Our evidence suggests that all three pathways are separable. Thus, even though both Wnt10b and Bmp2 induce *Lef1,* this is most likely through distinct pathways as the analysis of the response of *Msx1* and *Lef1* mutant mesenchymes indicates that the Bmp2/4 response is dependent upon *Msx1* activity (Kratochwil *et al.,* 1996; Chen *et al.,* 1996), whereas no *Msx1* induction is observed in response to Wnt10b. However, the observation that *Lef1* is a target of Bmp and Wnt signaling suggests that Lef1 may act *in vivo* to integrate both signaling pathways. These data further contribute to the understanding of the coordinate interactions between epithelial and mesenchymal cell types and to the understanding of the pathways that govern morphogenesis of a complex organ, the tooth.

### *Shh Signals to the Mesenchyme*

To address whether *Ptc* and *Gli* expression in the mesenchyme indicates that the mesenchyme transduces Shh signaling, we analyzed the gene expression of *Ptc, Gli,* and *Bmp4* after culturing mandibles in the presence of ectopic Shh. All three genes are expressed in the mesenchyme of the tooth and can be induced by Shh in other vertebrate systems (Roberts *et al.,* 1995; Epstein *et al.,* 1996; Goodrich *et al.,* 1996; Marigo *et al.,* 1996b). For example, beads, soaked in Shh, inserted into the anterior limb mesenchyme or next to the dorsal neural tube of higher vertebrates, induce ectopic expression of *Ptc* and *Gli.* Bmp2, which is 86% identical to Bmp4, can also be induced by Shh coated beads inserted into the anterior limb mesenchyme, in the presence of the limb ectoderm (Laufer *et al.,* 1994; Yang *et al.,* 1997), and *Bmp4* is ectopically induced in the gut mesenchyme by Shh (Roberts *et al.,* 1995). However, in the mandible, in the presence of the epithelium, only *Ptc* and *Gli* are induced by Shh in the oral mesenchyme in a broad area around the bead. Neither *Bmp4* nor *Lef1,* which is downstream of *Bmp4* in the tooth mesenchyme, is induced. Thus, this suggests that *Bmp4* is not a target of Shh signaling in this system. Bellusci *et al.* (1997) have also observed that in the lung, where Shh and Bmp4 are coexpressed (Bitgood and McMahon, 1996), overexpression of Shh throughout the distal epithelium leads to upregulation of *Ptc,* but not of the *Bmps* in the adjacent mesenchyme. This would suggest that in contrast to the morphogenesis of imaginal disc structures in the fly where Hedgehog action is mediated by two secondary signals, Decapentaplegic (Bmp2/4 orthologue) and Wingless (Wnt1 orthologue) (Basler and Struhl, 1994; Diaz-Benjumea *et al.,* 1994), in several vertebrate organs, including the tooth, Shh may play a more direct role. Although these data point toward independent Shh and Bmp4 pathways, we cannot exclude the possibility that as in the limb Shh requires another factor in order to induce *Bmp4* and that this factor is restricted to the tooth-forming areas and is not itself inducible by Shh.

### *Wnt10b Signals to the Mesenchyme*

The epithelial expression of *Wnt10a* and *Wnt10b* and the adjacent mesenchymal expression of *Lef1* at E12.0 suggest that Wnt10a and Wnt10b might signal to the mesenchyme. This hypothesis is supported by the ectopic induction of *Lef1* by Wnt-expressing cell grafts. Interestingly, Lef1 is believed to directly regulate Wnt targets when associated with β-catenin (Behrens *et al.,* 1996; Huber *et al.,* 1996) and thus transcriptional upregulation of *Lef1* might reinforce a cell's response to Wnt signaling. Further, the observation that both Wnts and Bmps can induce *Lef1* suggests that Bmp signaling may be required in order to establish a zone which will be competent to respond to Wnt10b or that Lef1 is a key integrator of distinct signaling pathways.

### *Mesenchymal Competence to Respond to Bmp2, Shh, and Wnts Varies*

We observe that beads soaked in either Bmp2 or Shh induce mesenchymal gene expression in a strip of cells a few cell layers beneath the epithelium, whereas cells in the core of the mandible which also contact the beads do not. Two different explanations can account for this observation. (1) During early mandible development, the oral epithelium renders the underlying mesenchyme competent to respond to signals from the epithelium. These signals must occur very early since if the epithelium is removed from the mesenchyme at E11.0 and the oral mesenchyme alone is cultured in the presence of Bmp4, induction still occurs (Vainio *et al.,* 1993). Similar experiments to address Shh response in the absence of the epithelium have not been attempted. (2) There is evidence that the neural-crest-derived cells that migrate into the branchial arches remain adjacent to the epithelium and that the core mesenchyme is populated by the paraxial mesoderm (Noden, 1988; Trainor and Tam, 1995). These data raise the possibility that different cell lineages could explain different responses to the ectopic sources of protein. However, Imai *et al.* (1996) show that the neural crest colonizes the entire mandible.

Interestingly, we do not see a similar restriction of gene induction to the mesenchyme just underlying the epithelium in the case of *Lef1* induction by Wnt-expressing cells. This suggests that all of the mesenchyme may be competent to respond to Wnts and that the localization of *Lef1* during tooth development below the epithelium is the result of the localization of the inductive epithelial signals. Alternatively, the grafted cells may supply additional factors which mimic ectodermal signaling and broaden the response to Wnt10b.

### *Epithelial Response to Shh, Wnt, and Bmp2*

As *Shh, Wnt10b,* and *Bmp2* are all expressed in the epithelium, we expected that there may be a regulatory hierarchy among these signals which could be discerned from grafts. However, in no case were grafts able to induce the expression of signaling factors within the epithelium,

despite the evidence of some responses. Bmp2 is able to signal to the oral epithelium and ectopically induce *Msx1*, which is normally expressed at low levels in the epithelium of the developing tooth (Dr. Irma Thesleff, personal communication). Thus, Bmp2 may signal to the epithelium as well as the mesenchyme during tooth development. As *Bmp4* expression in the epithelium is not downregulated in the *Msx1* mutants and as Bmp4 is not induced in the epithelium in response to Bmp2, the response of the epithelium to Bmp signals may be regulated differently from the response of the mesenchyme.

Our data indicate that although the dental epithelium, between E11.0 and E12.0, expresses *Gli* suggesting that it is capable of transducing the Shh signal, it does not express *Ptc*, the Shh receptor, and therefore would not be expected to respond to Shh. Yet, surprisingly, the molar epithelium clearly responds to Shh by repressing *Wnt10b* expression. This repression is specific to *Wnt10b* since *Bmp2* and *Shh* (which are coexpressed with *Wnt10b*) and *Fgf8* (which is a more general epithelial marker) are not affected by the presence of the Shh bead. This is even more intriguing, as on the aboral side the repression of *Wnt10b* expression is accompanied by a marked increase in *Ptc* expression in the epithelium. We do not rule out the possibility that in the oral tissue, in response to Shh, the mesenchyme expresses a signaling protein that acts upon the epithelium to repress *Wnt10b*. It is also possible that Shh is signaling through very low levels of Ptc or that Ptc2 is expressed in the early epithelium and can mediate Shh signaling in the oral epithelium (Motoyama *et al.*, 1998). Whatever the explanation, this observation is interesting given that Shh and Wnt10b are apparently expressed in intermixed cell populations, on both the oral and the aboral surface.

### *Bmp2, Shh, Wnt10a, and Wnt10b Are Expressed throughout a Putative Epidermal Organ Formation Domain*

Finally, the expression of *Bmp2*, *Shh*, *Wnt10a*, and *Wnt10b* in a strip along the mesial–distal axis of the mandible at E11.5 maps to a region of the epithelium from which the oral epidermal organs arise, the incisors and the molars. Surprisingly, these expression domains also include the diastema, an area which does not give rise to any epidermal organs and which is present in the adult mouse. Expression is transient in the diastema and by E12.0 it is restricted to the molar and the incisor regions. It therefore seems unlikely that these genes specify the position at which the epidermal organs develop. Rather, they are likely to define a strip of ectodermal cells that are competent to drive epidermal organ development and that other genes fine-tune their pattern of expression. A recent report indicates that *Pax9* in the mesenchyme is precisely restricted to the tooth domains by E10.5 (Neübuser *et al.*, 1997). This suggests a model in which the ectoderm independently acquires the ability to drive epidermal organ development and that teeth only arise from regions where the underlying mesenchyme expresses *Pax9*. It is however unlikely that Pax9 regulates the mesenchymal signals acting on the epithelium as *Pax9* mutants develop dental laminas in the expected pattern (Neübuser *et al.*, 1997). At present, the mechanism by which epithelial refinement of the expression patterns occurs is unknown. Although our experiments do not directly address the issue of competence to form teeth, it is likely that at E11.5 *Bmp2, Shh, Wnt10a,* and *Wnt10b* define a region in which the ectodermal cells are competent to thicken and undergo initial tooth morphogenesis.

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## REFERENCES

Åberg, T., Wozney, J., and Thesleff, I. (1997). Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Dev. Dyn.* **210,** 383–396.

Adamson, M. C., Dennis, C., Delaney, S., Christiansen, J., Monkley, S., Kozak, C. A., and Wainwright, B. (1994). Isolation and genetic mapping of two novel members of the murine Wnt gene family, Wnt11 and Wnt12, and the mapping of Wnt5a and Wnt7a. *Genomics* **24,** 9–13.

Basler, K., and Struhl, G. (1994). Compartment boundaries and the control of *Drosophila* limb pattern by hedgehog protein. *Nature* **368,** 208–214.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382,** 638–642.

Bellusci, S., Furuta, Y., Rush, M. G., Henderson, R., Winnier, G., and Hogan, B. L. (1997). Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development* **124,** 53–63.

Bitgood, M. J., and McMahon, A. P. (1995). *Hedgehog* and *Bmp* genes are coexpressed at many diverse sites of cell–cell interaction in the mouse embryo. *Dev. Biol.* **172,** 126–139.

Bober, E., Franz, T., Arnold, H. H., Gruss, P., and Tremblay, P. (1994). Pax-3 is required for the development of limb muscles: A possible role for the migration of dermomyotomal muscle progenitor cells. *Development* **120,** 603–612.

Brunner, E., Peter, O., Schweizer, L. and Basler, K. (1997). Pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in *Drosophila*. *Nature* **385,** 829–833.

Bumcrot, D. A., Takada, R., and McMahon, A. P. (1995). Proteolytic processing yields two secreted forms of sonic hedgehog. *Mol. Cell. Biol.* **15,** 2294–2303.

Butler, P. M. (1956). The ontogeny of molar pattern. *Biol. Rev.* **31,** 30–70.

Cadigan, K. M., and Nusse, R. (1997). Wnt signaling: A common theme in animal development. *Genes Dev.* **11,** 3286–3305.

Chen, Y., Bei, M., Woo, I., Satokata, I., and Maas, R. (1996). Msx1 controls inductive signaling in mammalian tooth morphogenesis. *Development* **122,** 3035–3044.

Christiansen, J. H., Dennis, C. L., Wicking, C. A., Monkley, S. J., Wilkinson, D. G., and Wainwright, B. J. (1995). Murine Wnt-11 and Wnt-12 have temporally and spatially restricted expression patterns during embryonic development. *Mech. Dev.* **51,** 341–350.

Cohn, S. A. (1957). Development of the molar teeth in the albino mouse. *Am. J. Anat.*, 295–320.

Crossley, P. H., and Martin, G. R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121,** 439–451.

Cygan, J. A., Johnson, R. L., and McMahon, A. P. (1997). Novel regulatory interactions revealed by studies of murine limb pattern in Wnt-7a and En-1 mutants. *Development* **124,** 5021–5032.

Diaz-Benjumea, F. J., Cohen, B., and Cohen, S. M. (1994). Cell interaction between compartments establishes the proximal–distal axis of *Drosophila* legs. *Nature* **372,** 175–179.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75,** 1417–1430.

Epstein, D., Marti, Scott, M., and McMahon, A. (1996). Antagonizing cAMP-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway. *Dev. Suppl.* **122,** 2885–2894.

Gavin, B. J., McMahon, J. A., and McMahon, A. P. (1990). Expression of multiple novel Wnt-1/int-1-related genes during fetal and adult mouse development. *Genes Dev.* **4,** 2319–2332.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A., and Scott, M. P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: Induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10,** 301–312.

Hammerschmidt, M., Brook, A., and McMahon, A. P. (1997). The world according to hedgehog. *Trends Genet.* **13,** 14–21.

Hardy, M. H. (1992). The secret life of the hair follicle. *Trends Genet.* **8,** 55–61.

Hill, R. E., Jones, P. F., Rees, A. R., Sime, C. M., Justice, M. J., Copeland, N. G., Jenkins, N. A., Graham, E., and Davidson, D. R. (1989). A new family of mouse homeo box-containing genes: Molecular structure, chromosomal location, and developmental expression of Hox-7.1. *Genes Dev.* **3,** 26–37.

Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G., and Kemler, R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech. Dev.* **59,** 3–10.

Hui, C. C., Slusarski, D., Platt, K. A., Holmgren, R., and Joyner, A. L. (1994). Expression of three mouse homologs of the *Drosophila* segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev. Biol.* **162,** 402–413.

Imai, H., Osumi-Yamashita, N., Ninomiya, Y., and Eto, K. (1996). Contribution of early-emigrating midbrain crest cells to the dental mesenchyme of mandibular molar teeth in rat embryos. *Dev. Biol.* **176,** 151–165.

Iseki, S., Araga, A., Ohuchi, H., Nohno, T., Yoshioka, H., Hayashi, F., and Noji, S. (1996). Sonic hedgehog is expressed in epithelial cells during development of whisker, hair, and tooth. *Biochem. Biophys. Res. Commun.* **218,** 688–693.

Jahoda, C. A. (1992). Induction of follicle formation and hair growth by vibrissa dermal papillae implanted into rat ear wounds: Vibrissa-type fibres are specified. *Development* **115,** 1103–1109.

Jernvall, J., Aring, Berg, T., Kettunen, P., Ker, Auml, Nen, S., and Thesleff, I. (1998). The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot. *Dev. Suppl.* **125,** 161–169.

Jernvall, J., Kettunen, P., Karavanova, I., Martin, L. B., and Thesleff, I. (1994). Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: Non-dividing cells express growth stimulating Fgf-4 gene. *Int. J. Dev. Biol.* **38,** 463–469.

Jones, C. M., Lyons, K. M., and Hogan, B. L. (1991). Involvement of bone morphogenetic protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. *Development* **111,** 531–542.

Jowett, A. K., Vainio, S., Ferguson, M. W., Sharpe, P. T., and Thesleff, I. (1993). Epithelial–mesenchymal interactions are required for msx 1 and msx 2 gene expression in the developing murine molar tooth. *Development* **117,** 461–470.

Kispert, A., Vainio, S., Shen, L., Rowitch, D. H., and McMahon, A. P. (1998). Proteoglycans are required for maintenance of Wnt-11 expression in the ureter tips. *Development* **122,** 3627–3637.

Knecht, A. K., Good, P. J., Dawid, I. B., and Harland, R. M. (1995). Dorsal–ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm. *Development* **121,** 1927–1935.

Kollar, E. J. (1970). The induction of hair follicles by embryonic dermal papillae. *J. Invest. Dermatol.* **55,** 374–378.

Kollar, E. J., and Baird, G. R. (1969). The influence of the dental papilla on the development of tooth shape in embryonic mouse tooth germs. *J. Embryol. Exp. Morphol.* **21,** 131–148.

Kollar, E. J., and Baird, G. R. (1970). Tissue interactions in embryonic mouse tooth germs. *J. Embryol. Exp. Morphol.* **24,** 159–186.

Kratochwil, K., Dull, M., Farinas, I., Galceran, J., and Grosschedl, R. (1996). Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* **10,** 1382–1394.

Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A., and Tabin, C. (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79,** 993–1003.

Lee, F. S., Lane, T. F., Kuo, A., Shackleford, G. M., and Leder, P. (1995). Insertional mutagenesis identifies a member of the Wnt gene family as a candidate oncogene in the mammary epithelium of int-2/Fgf-3 transgenic mice. *Proc. Natl. Acad. Sci. USA* **92,** 2268–2272.

Lefkowitz, W., Bodecker, C. F., and Mardfin, D. F. (1953). Odontogenesis of the rat molar. *J. Den. Res.* **32,** 749–771.

Lumsden, A. G. S. (1988). Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. *Development* **103,** 155–169.

Lyons, K. M., Pelton, R. W., and Hogan, B. L. (1989). Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor-beta-like genes coordinately regulate aspects of embryonic development. *Genes Dev.* **3,** 1657–1668.

Maas, R., and Bei, M. (1997). The genetic control of early tooth development. *Crit. Rev. Oral Biol. Med.* **8,** 4–39.

MacKenzie, A., Ferguson, M. W., and Sharpe, P. T. (1991). The homeobox gene Hox 7.1 has specific regional and temporal expression patterns during early murine craniofacial embryogenesis, especially tooth development *in vivo* and *in vitro*. *Development* **111,** 269–285.

MacKenzie, A., Ferguson, M. W., and Sharpe, P. T. (1992). Expression patterns of the homeobox gene, Hox-8, in the mouse embryo suggest a role in specifying tooth initiation and shape. *Development* **115,** 403–420.

Marigo, V., Davey, R. A., Cunningham, J. M., and Tabin, C. J. (1996a). Biochemical evidence that patched is the Hedgehog receptor. *Nature* **384,** 176–179.

Marigo, V., Johnson, R. L., Vortkamp, A., and Tabin, C. J. (1996b). Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. *Dev. Biol.* **180,** 273–283.

Marti, E., Bumcrot, D. A., Takada, R., and McMahon, A. P. (1995). Requirement of 19k form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375,** 322–325.

Miller, A. D., and Rosman, G. J. (1989). Improved retroviral vectors for gene transfer and expression. *Biotechniques* **7,** 980–990.

Monaghan, A. P., Davidson, D. R., Sime, C., Graham, E., Baldock, R., Bhattacharya, S. S., and Hill, R. E. (1991). The Msh-like homeobox genes define domains in the developing vertebrate eye. *Development* **112,** 1053–1061.

Moon, R. T., Brown, J. D., and Torres, M. (1997). WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet.* **13,** 157–162.

Motoyama, J., Takabatake, T., Takeshima, K., and Hui, C. (1998). *Ptch2,* a second mouse Patched gene, is co-expressed with Sonic hedgehog. *Nature Genet.* **18,** 104–106.

Neübuser, A., Peters, H., Balling, R., and Martin, G. R. (1997). Antagonistic interactions between FGF and BMP signaling pathways: A mechanism for positioning the sites of tooth formation. *Cell* **90,** 247–255.

Noden, D. M. (1988). Interactions and fates of avian craniofacial mesenchyme. *Development* **103**(Suppl.), 121–140.

Nusse, R., and Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31,** 99–109.

Parr, B. A., Shea, M. J., Vassileva, G., and McMahon, A. P. (1993). Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* **119,** 247–261.

Perrimon, N. (1996). Serpentine proteins slither into the wingless and hedgehog fields. *Cell* **86,** 513–516.

Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K., and Beachy, P. A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. *Nature* **374,** 363–366.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75,** 1401–1416.

Robert, B., Sassoon, D., Jacq, B., Gehring, W., and Buckingham, M. (1989). Hox-7, a mouse homeobox gene with a novel pattern of expression during embryogenesis. *EMBO J.* **8,** 91–100.

Roberts, D. J., Johnson, R. L., Burke, A. C., Nelson, C. E., Morgan, B. A., and Tabin, C. (1995). Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development* **121,** 3163–3174.

Sakakura, T. (1987). Mammary embryogenesis. *In* "The Mammary Gland: Development, Regulation and Function" (M. N. and C. W. Daniel, Eds.), pp. 37–66. Plenum Press, New York.

Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F., and Rosenthal, A. (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* **384,** 129–134.

Szabo, G. (1954). Studies on the cultivation of teeth *in vitro. J. Anat.* **88,** 31–44.

Tabata, T., and Kornberg, T. B. (1994). Hedgehog is a signaling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* **76,** 89–102.

Tabin, C. J., and McMahon, A. P. (1997). Recent advances in Hedgehog signalling. *Trends Cell Biol.* **7,** 442–446.

Thesleff, I. (1976). Differentiation of odontogenic tissues in organ culture. *J. Dent. Res.* **84,** 353–356.

Thomas, B. L., Tucker, A. S., Qui, M., Ferguson, C. A., Hardcastle, Z., Rubenstein, J. L., and Sharpe, P. T. (1997). Role of Dlx-1 and Dlx-2 genes in patterning of the murine dentition. *Development* **124,** 4811–4818.

Trainor, P. A., and Tam, P. P. (1995). Cranial paraxial mesoderm and neural crest cells of the mouse embryo: Co-distribution in the craniofacial mesenchyme but distinct segregation in branchial arches. *Development* **121,** 2569–2582.

Travis, A., Amsterdam, A., Belanger, C., and Grosschedl, R. (1991). LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function. *Genes Dev.* **5,** 880–894. [Erratum appears in *Genes Dev.* **5**(6), 1113 (1991).]

Vaahtokari, A., Aberg, T., Jernvall, J., Keranen, S., and Thesleff, I. (1996). The enamel knot as a signaling center in the developing mouse tooth. *Mech. Dev.* **54,** 39–43.

Vainio, S., Karavanova, I., Jowett, A., and Thesleff, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* **75,** 45–58.

van Genderen, C., Okamura, R. M., Farinas, I., Quo, R. G., Parslow, T. G., Bruhn, L., and Grosschedl, R. (1994). Development of several organs that require inductive epithelial–mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* **8,** 2691–2703.

van Ooyen, A., and Nusse, R. (1984). Structure and nucleotide sequence of the putative mammary oncogene int-1: Proviral insertions leave the protein-encoding domain intact. *Cell* **39,** 233–240.

Wang, J., and Shackleford, G. M. (1996). Murine *Wnt10a* and *Wnt10b*: Cloning and expression in the developing limbs, face and skin of embryos and in adults. *Oncogene* **13,** 1537–1544.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988). Novel regulators of bone formation: Molecular clones and activities. *Science* **242,** 1528–1534.

Yamada, M., Bringas, P., Grodin, M., MacDougas, M., Cummings, E., Grimmett, J., Weliky, B., and Slavkin, H. (1980). Chemically-defined organ culture of embryonic mouse tooth organs: Morphogenesis, dentinogenesis and amelogenesis. *J. Biol. Buccale* **8,** 127–139.

Yang, Y., Drossopoulou, G., Chuang, P. T., Duprez, D., Marti, E., Bumcrot, D., Vargesson, N., Clarke, J., Niswander, L., McMahon, A., and Tickle, C. (1997). Relationship between dose, distance and time in Sonic Hedgehog-mediated regulation of anteroposterior polarity in the chick limb. *Development* **124,** 4393–4404.

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