Wnt/Shh interactions regulate ectodermal boundary formation during mammalian tooth development

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Interactions between the Wnt (wingless) and hedgehog signaling pathways were first described as playing a role in establishing boundaries between ectodermal cells in *Drosophila* segmentation. During the initiation of mammalian tooth development, boundaries that distinguish oral from dental ectoderm must be formed to correctly position the sites of tooth formation. We describe a reciprocal relationship between the expression of *Wnt-7b* in presumptive oral ectoderm and *Shh* in presumptive dental ectoderm in mouse embryos that mark boundaries between these cells with different developmental fates. By using a murine retrovirus to ectopically express Wnt-7b in presumptive dental ectoderm in mandibular arch explants, we show that *Shh* expression in the ectoderm and *Ptc* expression in the underlying ectomesenchyme are down-regulated, and tooth development is subsequently arrested. This suggests that Wnt-7b acts to repress *Shh* expression in oral ectoderm, thus maintaining the boundaries between oral and dental ectodermal cells. Implantation of beads soaked in Shh protein into Wnt-7b-infected explants resulted in complete rescue of tooth development, confirming that the repressive action of Wnt-7b specifically affects Shh signaling.

The tooth is an example of an organ that arises from ectodermal and mesenchymal tissue components, the result of a series of interactions between oral ectoderm and the underlying neural crest-derived mesenchyme (1). Teeth are essential for survival in many vertebrates, and missing or misplaced teeth can have fatal consequences, causing some species to be unable to make use of available food supplies. It is critical, therefore, that the dentition develops correctly, with the required number and type of teeth developing in specific positions in the jaws. In mammals, teeth are formed from the first branchial arch derivatives, the mandibular and maxillary processes and the frontonasal process. The first morphological signs of tooth development occur at embryonic day (E)11.5 in mice with a thickening of the oral ectoderm that demarcates the sites of tooth formation as being distinctive from the neighboring oral ectoderm. This thickening invaginates further into the underlying ectomesenchyme during the next 2 days of development, with mesenchyme condensing around the resulting epithelial tooth buds. The nonthickened oral ectoderm develops into the epithelial cell lining of the mouth. From the bud stage of tooth development, the tooth germs progress to the cap stage, during which a putative signaling center, the enamel knot, forms (2, 3). From this stage, tooth germs progress through a well-characterized path of cytodifferentiation in which the condensing mesenchyme, termed the dental papilla, gives rise to the tooth pulp and the dentine-producing odontoblasts. The epithelium differentiates into enamel-secreting ameloblasts that are a cell type unique to teeth.

Sonic hedgehog (Shh) is a member of the family of hedgehog-secreted peptides, of which *Drosophila* hedgehog (hh) was the first to be identified (4–6). Genes involved in the hedgehog signaling pathway, namely *Patched (Ptc), Smoothened (Smo), Gli* genes and protein kinase A have been identified in a number of species, suggestive of evolutionary conservation of this pathway. Ptc and Smo are transmembrane proteins thought to form a receptor complex for the Hh ligand (7, 8), and the Gli zinc-finger transcription factors have been demonstrated to have both activating and inhibitory roles in the Hh pathway (9–13). A second *Ptc* gene has been isolated, *Ptch-2,* which encodes a putative receptor for Shh (14, 15). Shh has been demonstrated to be essential for embryonic development because Shh-deficient mice have numerous developmental abnormalities, including defects in the neural tube, central nervous system, limbs, and, most particularly, severe craniofacial defects (16). Shh has a role in the dorsoventral patterning of the neural tube (17, 18), anteroposterior patterning of the limb (17, 19, 20), left–right asymmetry of the early embryo (21, 22), and patterning of the somites (23, 24).

Shh is expressed in the developing teeth (3, 25–28) and has recently been suggested to play a role in the development of the dentition (27). Shh has been shown to activate the transcription of *Ptc* and *Gli-1* in the developing tooth germ, and analysis of *Gli-2* and *Gli-3* mutant mice revealed a role for these transcription factors in mediating the Shh signal (27). Addition of exogenous Shh protein to tooth germs and areas adjacent to tooth germs can cause local changes in the morphology of the epithelium, suggestive of a role for the Shh pathway in epithelial cell proliferation (27). The highly restricted expression of *Shh* at sites of tooth formation is thus likely to be essential for specifying the sites where tooth buds will invaginate and teeth will form. The mechanisms that establish and maintain expression of *Shh* in localized domains of ectoderm and repress expression in the surrounding nondental (oral) ectoderm are thus an essential component of determining the sites of tooth formation.

Comparisons of the expression of Wnt genes with *Shh* during early tooth development have identified *Wnt-7b* expression in oral ectoderm as reciprocal to *Shh*, being expressed in all oral ectoderm except for those cells that express *Shh*. To investigate the possible role of Wnt-7b in regulating *Shh* expression, we ectopically expressed *Wnt-7b* in ectoderm cells that normally express *Shh* by using a murine retrovirus. This resulted in down-regulation of *Shh* expression and was accompanied by prevention of tooth-bud formation and a failure of tooth development. The specificity of Wnt-7b repression of *Shh* expression was confirmed by the ability of exogenous Shh protein to rescue tooth development after ectopic expression of *Wnt-7b* and repression of endogenous *Shh* expression. In mammalian tooth development, Wnt-7b acts to maintain *Shh* expression in restricted areas of ectoderm that define dental ectoderm. Shh acts to locally stimulate epithelial cell proliferation to produce tooth buds, and these interactions thus ensure the correct positioning of the sites of tooth formation within the oral cavity.

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Materials and Methods

Explant Cultures. Freshly harvested embryos were put into a solution of DMEM (with glutamax) (GIBCO/BRL) containing 20 units/ml penicillin/streptomycin (GIBCO/BRL). The mandibles and/or tooth germs were dissected from the embryo by using watchmakers forceps (BDH) and 27-gauge sterile needles (Sherwood Medical Industries, Athy, Ireland). First branchial arches or tooth germs were then placed on a 0.1-μm Millipore filter and resuspended for 4 h in drop cultures of DMEM/10% FCS/30 μl viral supernatant added with polybrene (8 μg/ml). The mandibular explants were then transferred on their filters to a stainless steel wire mesh (0.25-mm diameter wire, Goodfellows, Cambridge, U.K.) in an organ culture dish (Marathon) and cultured for 3 days. Media consisted of DMEM, 10% FCS (GIBCO/BRL), and 20 units/ml penicillin/streptomycin. All cultures were carried out in a standard incubator at 37°C with an atmosphere of 5% CO_2 in air and 100% humidity. After the period of culture, explants were washed in ice-cold methanol for 2 min and then fixed in 4% paraformaldehyde for 2 h at room temperature. They were then embedded in paraffin for section *in situs*. For LacZ staining, cultures were fixed in 2% gluteraldehyde on ice for 1 h and then stained with 1 mg/ml X5-bromo-4-chloro-3-indolyl β-D-galactoside in β-galactosidase stain base at 37°C overnight. The explants were then embedded in paraffin, sectioned, and counterstained with eosin for histology.

Bead Preparation. Affi-Gel-blue beads (Bio-Rad) were washed and dried before being placed in Shh protein (1.25 μg/μl rat Shh; 19.6 kDa amino terminal fragment, a gift from Ontogeny, Boston, MA) at 37°C for 30 min. BSA control beads were prepared in a similar way by using 100 μg/μl BSA. Beads were stored at 4°C and used within 1 wk.

Retroviral Genome Construction and Production. The retroviral vector, polyPOZ, was derived from pLNPOZ, a bicistronic single transcription vector containing a *lacZ* reporter (provided by A. D. Miller, Fred Hutchinson Center, Seattle, WA) (29) by replacing the neo coding sequence with a polylinker. Murine Wnt-7b was cloned as a *Bam*HI/*Sal*I fragment into the *Bgl*II/*Sal*I site of this polylinker. Stable retroviral producer cells were generated by superinfection of the GP + E86 cell line (provided by H. Land, Imperial Cancer Research Fund) (30). Sequential fluorescein-di-β-galactopyronaside-based FACS sorting generated high titer producer lines. Harvested supernatants were concentrated up to 10-fold by size exclusion ultrafiltration by using the Amicon stirrer cell with a ZM500 membrane, then passed through a 0.45-μm filter. All stocks were determined free of replication-competent retroviruses by marker rescue and reverse transcriptase assay. Titers were determined on NIH 3TE cells by using 5-bromo-4-chloro-3-indolyl β-D-galactoside histochemistry and were typically 5 $\times$ 106 *lacZ* colony-forming units per milliliter. Wnt-7b activity was determined by assessing postconfluence proliferation of C3H 10T1/2 cells after transduction with Wnt-7b polyPOZ retrovirus (31).

***In Situ* Hybridization.** Whole-mount digoxygenin-labeled *in situ* hybridization was performed according to Wilkinson (32). Radioactive section *in situ* hybridization by using ^{35}S-UTP radiolabeled riboprobes was carried out as described by Angerer and Angerer (33).

Kidney Capsule Surgery. Male CD1 mice between the ages of 5 and 8 wk were anesthetized with Hypnorm (fentanyl citrate 0.315 mg/ml, Fluanisone 10 mg/ml; Janssen) and Hypnovel (Midazolam 5 mg/ml; Roche Diagnostics). Hypnorm and Hypnovel solution was prepared by adding 2 ml of Hypnorm to 8 ml dH_2O and then adding 2 ml of Hypnovel. Anaesthetic was administered into the peritoneal cavity by using a 25-gauge needle. After manipulation *in vitro*, isolated tooth germs were stored on ice and then transferred into the subcapsular space of the mouse kidney. Teeth were then allowed to develop for 10 days *in vivo* before harvest.

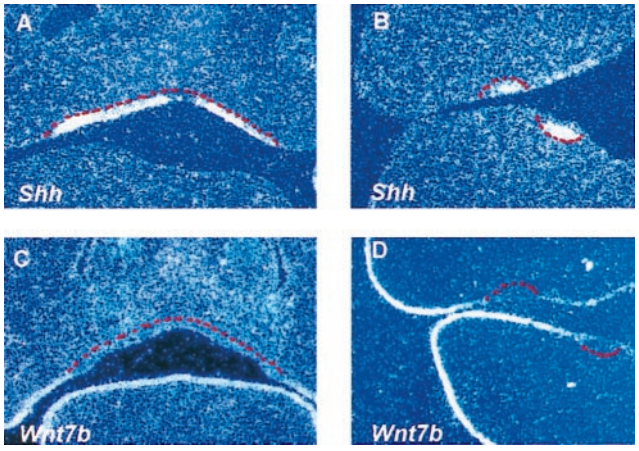

Fig. 1. Expression of *Shh* and *Wnt-7b* in presumptive oral and dental ectoderm. ^{35}Sα-UTP-labeled antisense *in situ* hybridization on frontal sections of embryo heads at E10.5. Maxillary incisor region (*A* and *C*) and maxillary and mandibular molar regions (*B* and *D*). *Wnt-7b* expression is excluded from presumptive dental ectoderm (*C* and *D*) which expresses *Shh* (*A* and *B*). Red dashes outline presumptive dental ectoderm.

Results and Discussion

Ectopic Expression of *Wnt-7b* Represses *Shh* and *Ptc* Expression and Prevents Tooth Bud Formation. The highly restricted expression of *Shh* ligand in presumptive dental ectoderm and its absence from the surrounding oral ectoderm suggest a role for this signaling pathway in the early development of tooth germs (25–27). To understand how *Shh* expression becomes restricted to presumptive dental ectoderm, we assayed the expression of other signaling protein genes during early development of oral and dental ectoderm and identified Wnt-7b as a possible candidate for restricting *Shh* expression. *Wnt-7b* expression was found to be widespread in developing oral ectoderm between E9.5 and E11.5 but was absent from ectoderm where teeth will develop and correspondingly from ectodermal cells that express *Shh* (Fig. 1). These reciprocal expression patterns suggested an interaction between these signaling pathways in ectoderm that may be involved in determining the boundaries between *Wnt-7b*- (oral) and *Shh*- (dental) expressing ectodermal cells.

To investigate possible interactions between Wnt-7b and Shh, we constructed a recombinant MMLTV virus to misexpress *Wnt-7b* in mandibular arch explant cultures. Infection of mandibular arch explant cultures from E10.5 embryos with the virus produced infection of the entire ectoderm including ectopic expression of *Wnt-7b* in presumptive dental ectoderm after 3 days. The extent of Wnt-7b viral infection in the explants was followed by staining sections for LacZ expression (Fig. 2*A*). The ectopic expression of *Wnt-7b* was confirmed by *in situ* hybridization with a *Wnt-7b* probe (data not shown). Comparison of *Wnt-7b*-infected explants with controls infected with a virus containing a LacZ reporter gene showed that tooth buds formed normally in control virus explants (Fig. 2*B*). No buds were evident in Wnt-7b-infected cultures (Fig. 2*A*), suggesting that misexpression of *Wnt-7b* in presumptive dental ectoderm prevented tooth bud formation. The effect of *Wnt-7b* ectopic expression in presumptive dental ectoderm on *Shh* expression was assayed by *in situ* hybridization of infected explants. No

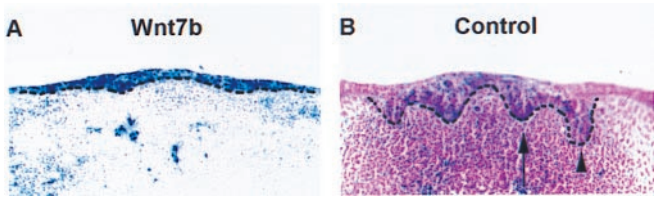

Fig. 2. Eosin-stained frontal sections through distal (incisor) regions of mandibular arch explants from E10.5 embryos cultured for 3 days with Wnt-7b or control virus supernatants. LacZ reporter expression in infected incisor ectoderm reveals extent of viral spread. LacZ staining is concentrated in the ectoderm. (*A*) Tooth germs have not been formed in the Wnt-7b-infected explants. (*B*) Incisor tooth development has reached the bud stage in explants infected with the control virus. Black dashes outline odontogenic epithelium. Arrow, incisor tooth bud; arrowhead, vestibular lamina.

expression of *Shh* was detectable in Wnt-7b-infected explants compared with normal expression in controls (Fig. 3 *A* and *B*).

Because Shh induces expression of *Ptc* in presumptive dental mesenchyme (27), expression of *Ptc* in Wnt-7b-infected cultures was assayed. No expression of *Ptc* was detected in Wnt-7b-infected cultures, whereas controls showed normal levels of localized expression (Fig. 3 *C* and *D*). The loss of *Shh* expression in presumptive dental ectoderm is thus reflected by loss of *Ptc* expression in both epithelium and mesenchyme. To determine whether *Wnt-7b* expression from the virus affected the expression of other early tooth marker genes expressed in mesenchyme and ectoderm, several were assayed by *in situ* hybridization. Expression of *Lef*-1 and *Bmp-4* was examined in Wnt-7b-infected and control explants (Fig. 3 *E–H*). These genes are all expressed in dental mesenchyme at this time in response to epithelial signals (34, 35). Expression of these genes was found to be present in Wnt-7b-infected cultures, indicating that the cultures were healthy and that the early epithelial–mesenchymal signaling interactions involved in odontogenesis had taken place, despite the absence of Shh. Expression of these mesenchymal genes is known not to be regulated by Shh (27). Expression of *Mfz6*, a Wnt receptor, was also examined in the ectoderm of Wnt-7b-infected explants and found to be normal (Fig. 3 *I* and *J*). This is consistent with Wnt-7b having a specific effect on the expression of *Shh*.

Ectopic expression of *Wnt-7b* in presumptive dental ectoderm, which normally expresses *Shh*, resulted in loss of *Shh* expression and, as a consequence, loss of *Ptc* expression with early arrest of tooth development before bud formation. Expression of genes in response to early epithelial–mesenchymal interactions was not affected, suggesting that initiation of the tooth development program was underway and that mesenchyme was responding to other ectodermal signals, but the formation of epithelial invaginations was prevented. Addition of Shh protein ectopically to tooth germs or nondental oral ectoderm has previously been shown to alter the morphology of tooth germs and to create epithelial thickenings/invaginations in the nondental ectoderm, suggesting that Shh may have a role in cell proliferation (27). Moreover, the fact that Shh does not induce expression of mesenchymal genes such as *Msx-1, Dlx-2,* and *Lhx-6/-7* that are induced by other ectodermal signals such as FGF-8 and BMP-4 indicated a role other than epithelial–mesenchymal signaling. The failure of tooth bud formation after Wnt-7b-induced loss of Shh signaling was thus consistent with this proposed role for Shh.

Rescue of Tooth Development after Ectopic *Wnt-7b* Expression by Exogenous Shh. Misexpression of *Wnt-7b* in presumptive dental ectoderm was thus found to repress *Shh* expression and prevent tooth bud formation, consistent with the role of Shh in epithelial

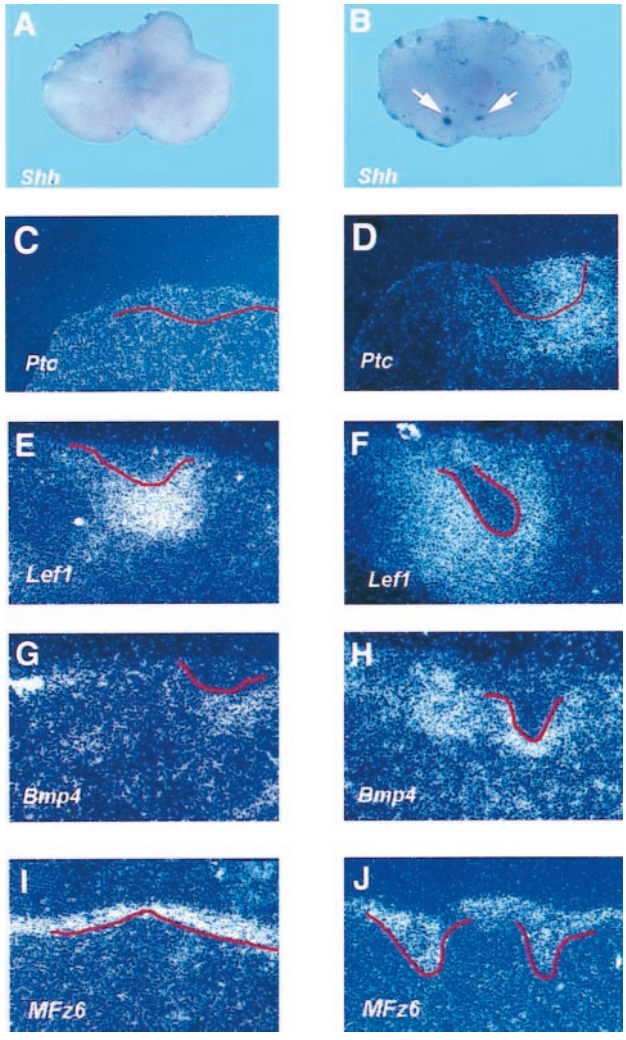

Fig. 3. Expression of early tooth germ marker genes after Wnt-7b misexpression (*A, C, E, G,* and *I*) and explants infected with control virus (*B, D, F, H,* and *J*). Red lines outline odontogenic epithelium. (*A*) *Shh* expression is down-regulated in Wnt-7b-infected tooth germs compared with those infected with control virus (*B*) (digoxygenin-labeled whole mount *in situ* hybridization). (*C*) *Ptc* expression is down-regulated in Wnt-7b-infected tooth germs compared with the control (*D*) (frontal section, $^{35}S\alpha$-UTP-*in situ* hybridization). (*E, G,* and *I*). *Lef-1, Bmp-4,* and *Mfz6* expression are present in Wnt-7b-infected tooth germs as compared with the controls (*F, H,* and *J*), (frontal section, $^{35}S\alpha$-UTP-*in situ* hybridization).

cell invagination and the requirement of Shh for normal tooth bud formation (ref. 27 and unpublished observations). The normal expression of early tooth germ marker genes not regulated by Shh indicates that misexpression of *Wnt-7b* does not indiscriminately affect early epithelial–mesenchymal signaling interactions in tooth development, but rather specifically represses *Shh* expression. If this is correct, addition of exogenous Shh protein might be expected to rescue tooth development. To test this, beads soaked in recombinant Shh protein were implanted into Wnt-7b-infected explants at E10.5 close to the locations of dental ectoderm. These explants were then cultured for an additional 3 days and transferred to renal capsules of adult mice. Renal transfer of mandibular arch explants provides suitable conditions for development of complete teeth and thus provides a stringent assay for any rescue (35, 36). Explants infected with either nonrecombinant MMLTV virus or a recombinant Wnt-5b virus produced normal teeth (9/12 and 5/6

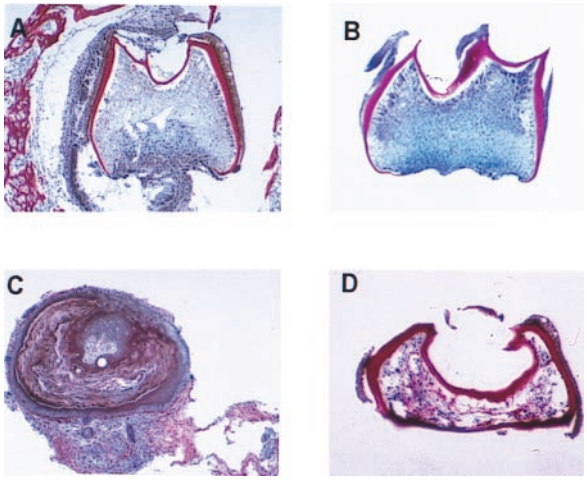

Fig. 4. Sections of teeth recovered from E10.5 mandible explants infected with control, Wnt-5b, and Wnt-7b viruses after transfer into renal capsules for 10 days. (*A*) Molar tooth from explant infected with control virus implanted with a PBS bead. (*B*) Molar tooth from explant infected with *Wnt-5b* virus and a PBS bead. (*C*) A fibrous cyst retrieved from explant infected with *Wnt-7b* virus and a PBS bead. (*D*) Molar tooth from explants infected with *Wnt-7b* virus with a Shh bead implanted. The teeth in *A* and *B* are phenotypically normal, whereas the rescued tooth in *D* has an abnormal pulp chamber and cuspal morphology. Note that beads were placed on the surface of developing tooth germs, which approximates the superior surface of the resultant tooth.

respectively) (Fig. 4 *A* and *B*). Wnt-7b-infected explants produced no teeth after renal transfer (0/16) (Fig. 4*C*). The implantation of Shh beads into Wnt-7b-infected cultures produced 3 teeth from 16 explants, showing that addition of Shh protein could rescue tooth development (Fig. 4*D*). The low efficiency of tooth rescue was predictable because timing, positioning, and concentration of the Shh protein bead implantation are likely to be important, and these factors were difficult to control. However, the structures that formed were easily identifiable as teeth by their size, shape, and the presence of dentine. The rescued teeth were, however, abnormal, most noticeably having little or no pulp tissue.

The ability of exogenous Shh protein to rescue tooth development after repression of endogenous *Shh* by misexpression of *Wnt-7b* in presumptive dental ectoderm is strong evidence that Wnt-7b acts specifically to repress *Shh* expression in dental ectoderm. *Wnt-7b* expression could first be detected at E8.5, slightly ahead of *Shh* in stomodeal ectoderm (data not shown). Because of the small size of the first branchial arch at this early stage, it is not possible to culture successfully as an explant and infect with viruses. Thus the role of Wnt-7b in establishing the domains of *Shh* expression and the boundaries between oral and dental ectoderm cannot be investigated with this approach. Wnt-7b clearly has a role in maintaining *Shh* expression in presumptive dental ectoderm by its ability to repress expression. The mechanism of this action of Wnt-7b remains obscure because frizzled receptors such as Mfz6 are expressed throughout the oral ectoderm and are not excluded from presumptive dental ectoderm cells (37). The possibility that a reciprocal interaction exists between Shh and Wnt-7b has not been established, although *Wnt-7b* expression is detectable in the oral ectoderm of Shh$-/-$ embryos (data not shown). However, the greatly reduced size of the branchial arch in Shh$-/-$ embryos and the lack of any distinctive dental epithelial thickenings make it difficult to draw definitive conclusions whether Shh can repress *Wnt-7b* expression in dental ectoderm. Addition of Shh beads close to *Wnt-7b*-expressing ectoderm in explants has no discernible effect on expression (data not shown).

The repression of *Shh* expression by Wnt-7b would appear to involve planar signaling within the ectoderm and would not require the mesenchyme, because misexpression of *Wnt-7b* by virus infection was limited to ectodermal cells after infection at E10.5. Similar planar signaling pathways in oral ectoderm have recently been identified involving BMP-4 regulation of *Dlx-2* expression (38). Although less likely, it is still possible that signaling may be occurring between ectoderm and mesenchyme by using other yet-to-be-explored Wnt and frizzled molecules within the mesenchyme. In addition, early mesenchyme–mesenchyme cell signaling involving activin has been identified showing that essential signaling within ectoderm and mesenchyme compartments occurs at the early initiation stages of tooth development (39). Understanding the relative timing of these events will be required to understand these complex interactions.

The mechanism that restricts *Wnt-7b* expression to oral and not dental ectoderm remains to be explained. Expression of other signaling molecules such as *Fgf-8* and *Bmp-4* is also highly spatially restricted in oral ectoderm before E10.5 in domains different from those of *Wnt-7b* and *Shh,* and these signals play independent roles in the earliest epithelial–mesenchymal interactions that act to define the proximodistal axis (incisor–molar) of the mandibular arch (36). Significantly, broad overlapping domains of expression of several homeobox genes can be identified in oral ectoderm, and it is possible that localized expression of signaling molecules is regulated by these areas of overlap. Although these relationships between Wnt-7b/Shh that act to maintain a boundary between oral and dental ectodermal cells show some similarity with *wingless* and *hedgehog* in *Drosophila,* it is probably too early to conclude that these interactions are evolutionarily conserved between vertebrates and invertebrates. It is intriguing, however, that one process in which these molecules do interact in the fly is in boundary formation during segmentation of the larval cuticle (ref. 40; reviewed in ref. 41).

Conclusions

In vertebrates, little is known about how different ectodermal territories are specified during organogenesis. Some progress has been made in limb development, where dorsoventral patterning involves the establishment of territories of gene expression in the apical ectodermal ridge, modulated by interactions between Wnt-7a, Bmp-4, and En-1 (42–44). We describe here an interactive relationship between a mammalian wingless family member, Wnt-7b, and a hedgehog family member, Shh, that acts to maintain cell boundaries in stomodeal ectoderm. In mice, these boundaries are required to distinguish oral from dental ectoderm and thus determine the sites of formation of vertebrate-specific organs, the teeth, in the developing oral cavity.

We show that *Wnt-7b* and *Shh* have reciprocal and exclusive expression patterns in early stomodeal ectoderm, with *Shh* being expressed exclusively in presumptive dental ectoderm and *Wnt-7b* expressed exclusively in oral (nondental) ectoderm. The boundaries between *Wnt-7b*- and *Shh*-expressing cells correspond to the distinction between ectodermal cells that will form the lining of the mouth and those that will contribute to tooth formation.

We demonstrate that misexpression of *Wnt-7b* in ectoderm cells that normally express *Shh* results in repression of *Shh* and *Ptc* expression and prevention of tooth bud formation. Thus, we propose that the boundaries between *Wnt-7b*- and *Shh*-expressing cells are maintained by the repressive action of a Wnt-7b-stimulated pathway on *Shh* transcription. Frizzled receptors such as Mfz6 are expressed in stomodeal ectoderm, so the repressive action of Wnt-7b on *Shh* expression in presump-

tive dental ectoderm cells may be by planar signaling within the ectoderm.

Although tooth development is arrested before bud formation after Wnt-7b misexpression, the presumptive dental mesenchyme cells do respond to other epithelial signals and express genes known to be activated early in tooth development. Thus tooth development is primed to take place, but the ectodermal cells do not invaginate to form a bud. The specificity of Wnt-7b action on *Shh* was confirmed by rescue experiments where the addition of exogenous Shh to replace that lost after Wnt-7b misexpression resulted in restoration of tooth development.

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