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Sonic hedgehog regulates growth and morphogenesis of the tooth

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SUMMARY

During mammalian tooth development, the oral ectoderm and mesenchyme coordinate their growth and differentiation to give rise to organs with precise shapes, sizes and functions. The initial ingrowth of the dental epithelium and its associated dental mesenchyme gives rise to the tooth bud. Next, the epithelial component folds to give the tooth its shape. Coincident with this process, adjacent epithelial and mesenchymal cells differentiate into enamel-secreting ameloblasts and dentin-secreting odontoblasts, respectively. Growth, morphogenesis and differentiation of the epithelium and mesenchyme are coordinated by secreted signaling proteins. Sonic hedgehog (*Shh*) encodes a signaling peptide which is present in the oral epithelium prior to invagination and in the tooth epithelium throughout its development. We have addressed the role of *Shh* in the developing tooth in mouse by using a conditional allele to remove *Shh* activity shortly after ingrowth of the dental epithelium. Reduction and then loss of Shh function results in a cap stage tooth rudiment in which the morphology is severely disrupted. The overall size of the tooth is reduced and both the lingual epithelial invagination and the dental cord are absent. However, the enamel knot, a putative organizer of crown formation, is present and expresses Fgf4, Wnt10b, Bmp2 and Lef1, as in the wild type. At birth, the size and the shape of the teeth are severely affected and the polarity and organization of the ameloblast and odontoblast layers is disrupted. However, both dentin- and enamel-specific markers are expressed and a large amount of tooth-specific extracellular matrix is produced. This observation was confirmed by grafting studies in which tooth rudiments were cultured for several days under kidney capsules. Under these conditions, both enamel and dentin were deposited even though the enamel and dentin layers remained disorganized. These studies demonstrate that *Shh* regulates growth and determines the shape of the tooth. However, Shh signaling is not essential for differentiation of ameloblasts or odontoblasts.

Key words: Sonic hedgehog, Tooth development, Morphogenesis, Growth, Mouse

INTRODUCTION

During organ development, neighboring cells coordinate their growth and differentiation in order to acquire form and function. In the case of teeth, cells from the oral ectoderm and mesenchyme interact to give rise to highly mineralized structures of specific shapes and sizes (Jernvall and Thesleff, 2000).

The early stages of development are common to all types of teeth. The oral ectoderm forms a tooth bud by invaginating into the underlying mesenchyme. In the mouse mandible first molar, initiation occurs at 11.0 days post coitus (11.0 d.p.c.) and the bud stage is reached at 13.5 d.p.c. (Cohn, 1957).

A great diversity in tooth shape exists and is thought to have played an important role in the evolution of mammalian species. Specific shape is acquired by the folding of the epithelium and by the growth of the dental papilla (Butler, 1956). Morphogenesis begins at the cap stage, 14.0 d.p.c., in the mandibular first molar (Cohn, 1957) when the tip of the epithelial bud splits into three populations of cells. The outer ones continue to divide and, as they do so, continue to invaginate into the oral mesenchyme, giving rise to the buccal (towards the cheek) and to the lingual (towards the tongue) cusps of the adult tooth (Cohn, 1957). The size of the two epithelial ingrowths varies between teeth. For example, in the mouse incisor, the buccal epithelial ingrowth is significantly deeper than the lingual one, whereas, in the first molar, the lingual epithelial ingrowth is slightly deeper (Hay, 1961; Jernvall and Thesleff, 2000).

The third population of cells, the enamel knot (Butler, 1956), lies in the center of the cap. These cells stop proliferating and express a variety of growth factors (Butler, 1956; Jernvall et al., 1998). It has been proposed that the enamel knot acts as an organizing center that patterns the tooth crown by controling cusp formation (Thesleff and Sharpe, 1997). In teeth such as the molars, in which multiple cusps are formed, secondary enamel knots develop at the tips of the future cusps (Jernvall et al., 1994). However, at present there are no functional data to support this hypothesis.

First molar cytodifferentiation begins during the bell stage

at 18.0 d.p.c. Mesenchyme cells immediately adjacent to the epithelium form a cell layer of polarized and elongated pre-odontoblasts that secrete pre-dentin. In turn, following the initiation of pre-dentin deposition, pre-ameloblasts form in the epithelium facing the pre-odontoblasts (the inner enamel epithelium). These cells elongate, polarize and secrete pre-enamel on top of the pre-dentin. With time the secreted matrices mineralize into enamel and dentin (Thesleff and Hurmerinta, 1981). Although cytodifferentiation is common to all teeth, there exist tooth specific variations. In the mouse, enamel is only deposited on the labial side of the incisor, whereas in the molar, the tip of each cusp remains free of enamel (Gaunt, 1956; Hay, 1961).

Although several genes have been identified that show interesting spatial and temporal regulation during the development of the mammalian tooth, the molecular framework underlying tooth development is poorly understood. Several homeobox containing transcriptional regulators, including *Dlx1*, *Dlx2*, *Lhx6*, *Lhx7*, *Msx1*, *Msx2* and *Pax9* are involved in the patterning of the jaw and development of the teeth (reviewed in Jernvall and Thesleff, 2000). In addition, secreted proteins such as bone morphogenetic proteins (Bmp), fibroblast growth factors (Fgf), Wnts and hedgehogs (Hh) have been proposed to regulate epithelial-mesenchymal interactions (for reviews see Jernvall and Thesleff, 2000; Peters and Balling, 1999). Their activities may in turn be regulated by matrix components, including proteoglycans and glycoproteins (Vainio and Thesleff, 1992).

We have studied the activity of one secreted signaling factor, Sonic hedgehog (Shh). Shh is involved in growth and patterning of a number of organs, including the hair, which also develops from ectoderm and mesenchyme interactions (Chiang et al., 1999; Echelard et al., 1993; Ruiz i Altaba, 1999a; St-Jacques et al., 1998). In the developing tooth, the dynamic expression pattern of Shh is suggestive of multiple functions for the protein (Bitgood and McMahon, 1995; Dassule and McMahon, 1998; Hardcastle et al., 1998; Zhang et al., 1999). Prior to epithelial invagination, *Shh* is expressed in the epithelium of the presumptive tooth domain, suggesting a role for Shh in the initiation of tooth development. Consistent with this hypothesis, Shh-coated beads inserted into mandibles at 10.5 d.p.c. cause the initiation of ectopic epithelial thickenings (Hardcastle et al., 1998). Shh may also regulate ingrowth because, as the epithelium thickens and invaginates, *Shh* is expressed at the tip of the invaginating cells. Between bud and cap stage, *Shh* is upregulated in the cells that form the enamel knot, implicating Shh in patterning the tooth cap. Prior to cytodifferentiation, the *Shh* expression domain broadens to encompass the inner enamel epithelium and it is maintained in differentiating ameloblasts. This suggests a role for Shh in regulating cytodifferentiation of the inner enamel epithelium or the underlying odontoblast layer.

Hedgehog signaling is transduced by two multipass membrane proteins, patched (Ptch), the hedgehog receptor, and smoothened (Smoh) (for a review see McMahon, 2000). Gli family members serve as transcriptional effectors of the pathway (Ruiz i Altaba, 1999a). Ptch and a recently identified binding protein, hedgehog interacting protein (Hip), are transcriptional targets which negatively modulate hedgehog signals (Chen and Struhl, 1996; Chuang and McMahon, 1999). *Ptch* and *Gli1* gene expression in the epithelium and the mesenchyme of the tooth suggests that Shh may signal directly to both epithelial and mesenchymal components (Dassule and McMahon, 1998; Hardcastle et al., 1998).

In *Shh* null mutants, the first branchial arch, which gives rise to the jaw mandible and maxilla, fails to form (Chiang et al., 1996). This severe facial phenotype has precluded studying the potential roles of Shh in tooth development. To overcome this problem, we have generated a conditional allele of *Shh* (*Shh*^{c}) and removed Shh activity from the dental epithelium shortly after ingrowth of the tooth bud. We show that Shh is required for normal growth, morphogenesis and polarity within the tooth, but that it is not essential for cytodifferentiation of the ameloblast or odontoblast populations.

MATERIALS AND METHODS

Removal of Shh activity in the tooth

A conditional allele of *Shh* (*Shh*^{c}) was generated by flanking exon2, which encodes approximately half of the active N-terminal Shh signal, with loxP sites. This allele will be described in detail elsewhere (P. L. and A. P. M., unpublished). To remove Shh activity from the dental epithelium, we generated mice expressing Cre recombinase under the regulation of the enhancer element of an epithelial keratin, K14 (Turksen et al., 1992). Seven founder *K14-Cre* mice were identified by Southern blot analysis using a human growth hormone (*GH*) sequence which was present in the *K14-Cre* expression vector. Two lines (#199 and #200) were generated from mice that expressed the highest levels of *Cre* RNA in the skin. Both lines displayed high levels of Cre activity when crossed to *R26R* reporter mice which require a recombination event to permit *lacZ* expression (Soriano, 1999). Subsequent genotyping was performed by PCR using 5′TTCCTCAGGAGTGTCTTCGC3′ and 5′GTCCATGTCCTTCC-TGAAGC3′ primers to amplify 492 bp of the *GH* fragment in the K14 expression vector. Lines 199 and 200 were crossed to a *Shh* null allele (*Shh*^{n}) (St-Jacques et al., 1998) to generate mice that were heterozygous for both *K14-Cre* and *Shh*^{n}. Genotypes of *Shh*$^{+/n}$ mice were determined by PCR using primers 5′GGACACCATTC-TATGCAGGG3′ and 5′GAAGAGATCAAGGCAAGCTCTGGC3′ to amplify a 115 bp fragment of the null allele, and 5′GGACAC-CATTCTATGCAGGG3′ and 5′ATGCTGGCTCGCCTGGCTGTGG-AA3′ to amplify a 449 bp fragment of the wild-type allele. Mutant embryos were produced by crossing the *K14-Cre;Shh*$^{+/n}$ mice, maintained on a mixed background, to *Shh*$^{c/c}$ mice.

Histology, apoptosis and BrdU analysis

For histological analysis, embryos were fixed in either Bouin's fixative or 4% paraformaldehyde, then embedded in paraffin, sectioned at 6 μm and stained with Hematoxylin and Eosin. Apoptotic cell death was determined, following treatment with 5 μg/ml of proteinase K for 25 minutes at room temperature, by the TUNEL procedure (Gavrieli et al., 1992) using a Boehringer Mannheim kit. DNA replication was scored by injection of BrdU into pregnant females 1 hour before recovery of 14.5 d.p.c. pups. BrdU analysis was performed as described (Nowakowski et al., 1989).

In situ hybridization

In situ hybridizations and β-galactosidase staining procedures have previously been described (Whiting et al., 1991; Wilkinson et al., 1987). ^{35}S-UTP-labeled RNA probes were prepared according to Wilkinson et al. (1987). *Bmp2*, *Bmp4*, *Gli1*, *Msx1*, *Msx2*, *Ptch*, *Shh* and *Wnt10b* probes were made as in Dassule and McMahon (1998). Full-length amelogenin cDNA, full-length 2.9 kb dentin matrix protein 1 cDNA, a 550 bp cDNA fragment of dentin sialoprotein, a 479 bp *Gli2* cDNA fragment corresponding to residues 1815-2293 of

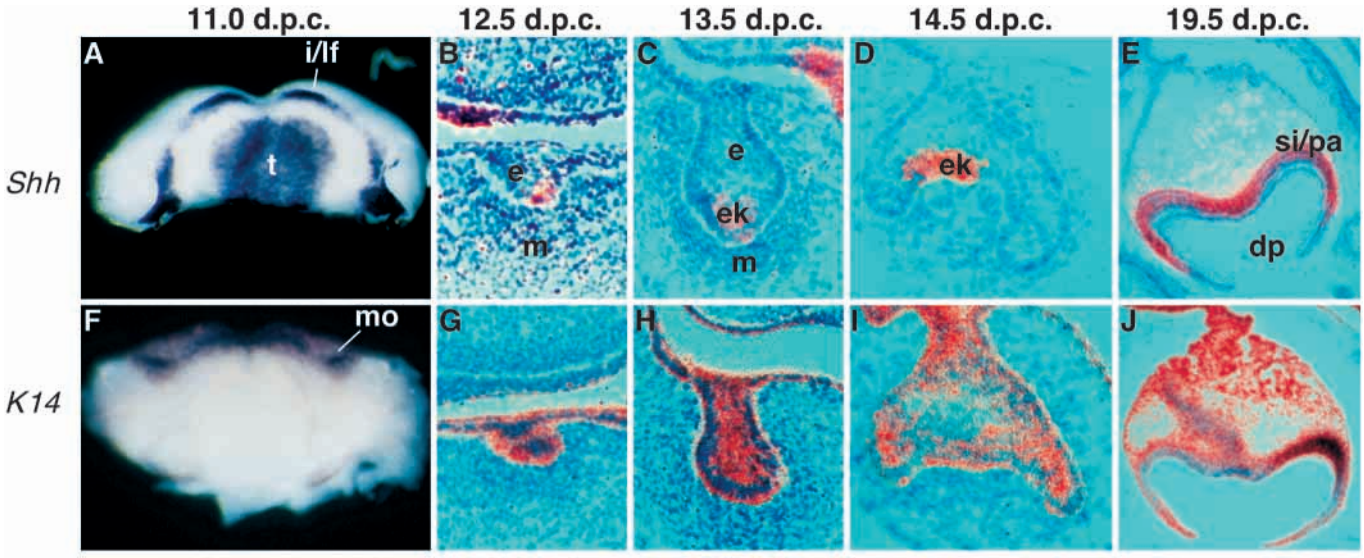

Fig. 1. *K14* and *Shh* are expressed in overlapping domains. (A,F) Oral views of the whole mandibles, (B,G) parasagittal sections, (C-E,H-I) frontal sections. (A) *Shh* is expressed in the incisor/lip furrow (i/lf) domain and in the presumptive tongue (t) at 11.0 d.p.c., (B) at the tip of the invaginating epithelium (e) but not in the mesenchyme (m) of the molar at 12.5 d.p.c., (C) at the tip of the epithelial bud just prior to enamel knot (ek) formation at 13.5 d.p.c., (D) in the enamel knot of the epithelial cap at 14.5 d.p.c., (E) in the pre-ameloblasts (pa) and in the stratum intermedium (si) at 19.5 d.p.c. *K14* is expressed throughout the mandibular molar epithelium and the oral ectoderm, including the molar (mo) at 11.0 d.p.c. (F), 12.5 d.p.c. (G), 13.5 d.p.c. (H), 14.5 d.p.c. (I) and 19.5 d.p.c. (J).

GenBank Accession number X99104, a 690 bp *Gli3* cDNA fragment, full-length 620 bp *Fgf4* cDNA, full length 1251 bp *K14* cDNA and a 700 bp *Pitx1* cDNA fragment were used to generate antisense RNA probes. The *Ptch2* probe is described in St-Jacques et al. (1998). *Shh* exon2 probe corresponds to 252 bp of exon2 of *Shh.*

Morphometrical analysis

Sections of teeth were photographed, imaged and traced. Areas of the traced teeth were measured graphically using NIH image. Tooth size was calculated by plotting the areas of each section and numerically estimating the area under the curve.

Kidney capsule grafts

Wild-type and $Shh^{c/n}$ molar tooth rudiments from 13.5 d.p.c. to 15.5 d.p.c. embryos were collected in phosphate buffered saline, then transplanted under the kidney capsule of adult male nude mice (M. Bei and R. Maas, unpublished). Transplants were allowed to develop for 9 to 20 days. The stage and the extent of tooth development were assessed by histological analysis.

RESULTS

Generation of a tissue specific knockout of *Shh* in the developing tooth

We used the Cre/lox system to remove Shh activity in the teeth, while leaving first branchial arch outgrowth intact. Mice were generated in which the second exon of *Shh*, which is essential for Shh function (Chiang et al., 1996), was flanked by the DNA recognition sites (loxP sites) for Cre recombinase (*Cre*), a P1 bacteriophage enzyme (Metzger et al., 1995). Mice homozygous for this conditional allele of *Shh* ($Shh^{c/c}$) or heterozygous for this allele and the null allele ($Shh^{c/n}$) were viable and fertile with no discernible phenotype.

To initiate a recombination event in developing teeth, we generated mice expressing *Cre* under the regulation of the keratin-14 *(K14)* promoter (Turksen et al., 1992). *K14* expression has been extensively described in the hair and in the skin, and its promoter element widely used for ectodermal expression (Byrne et al., 1994). In the first branchial arch, *K14* is expressed in the oral ectoderm and in the tooth ectoderm (Fig. 1F-J). This domain overlaps with that of *Shh* (Fig. 1A-E) in the developing teeth from 11.0 d.p.c. (Tabata et al., 1996; Zhou et al., 1995).

Seven founder mice were generated and Cre recombinase activity was analyzed in two lines in which we detected high levels of *Cre* expression in the skin at 14.5 d.p.c. (data not shown). Cre activity was examined by crossing each founder mouse to a reporter line (R26R) in which the ubiquitously expressed *Rosa26* locus is targeted with a *lacZ* transgene that requires Cre-mediated recombination for expression (Fig. 2A, Soriano, 1999). Cre activity leads to the excision of the PGKneo cassette and to transcription and translation of *lacZ* message. As expected, we found β-galactosidase activity in the skin (data not shown), the oral ectoderm including the dental lamina at 11.75 d.p.c. and throughout the dental epithelium by 14.5 d.p.c. (Fig. 2B,C).

To effectively generate a dental epithelium homozygous for the null allele of *Shh*, we crossed the *K14-Cre* mice onto a $Shh^{+/n}$ background. These mice ($K14\text{-}Cre;Shh^{+/n}$) were then crossed to $Shh^{c/c}$ mice. In this cross, a single recombination event is needed within a $Shh^{c/n}$ cell to remove *Shh* activity completely. At birth, we recovered $K14\text{-}Cre;Shh^{c/n}$ embryos at the expected Mendelian frequency of 25%. We will refer to $K14\text{-}Cre;Shh^{c/n}$ embryos and pups as mutants, and they will in all cases be compared with *Shh* heterozygote embryos ($Shh^{c/n}$), which were phenotypically indistinguishable from wild-type embryos.

At birth (19.5 d.p.c.), mutant pups from both independent

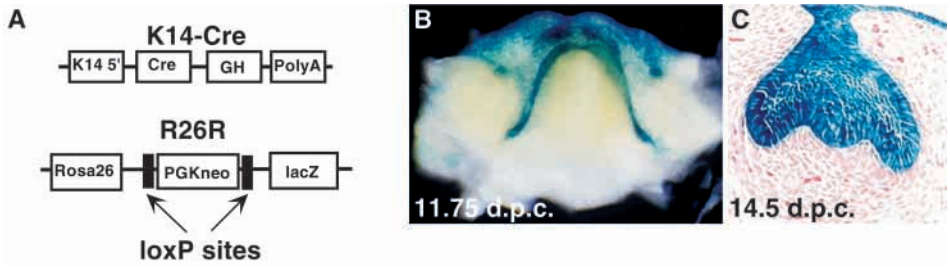

Fig. 2. Cre is active in the K14 domain. (A) Schematic showing the K14-Cre transgene and the R26R reporter construct. (B,C) β-galactosidase activity in the K14 domain in response to Cre activity in *Cre;R26R*$^{+/-}$ mice in the mandible at 11.75 d.p.c. (B) and in the molar at 14.5 d.p.c. (C).

lines displayed a similar phenotype: flattened skulls, open eyes, no whiskers and a small frontal nasal process. Pups were observed gulping air. The pups from both lines died within one day of birth. Skeletal preparations of newborn mutant pups revealed that, despite the small size of the frontal-nasal process, all of the skeletal elements were present and both the upper and lower jaws were of normal length. However, no teeth were visible and the nasal passageways were severely narrowed (data not shown).

Small abnormally shaped teeth develop in the absence of Shh

In order to investigate the absence of obvious teeth, we analyzed histological sections of mandibles at 19.5 d.p.c. In the mutants, first molars and incisors were present in both the mandible and the maxilla. However, in comparison with teeth from heterozygous littermates, those from mutant pups were small and abnormally shaped (Fig. 3). The phenotype of the maxillary teeth was consistent with, but somewhat less severe than, that of the mandibular teeth (data not shown). Our analysis focuses on the development of the mandibular molars and incisors.

Morphometrical analysis revealed that the mutant molars were 25% the size of those of their heterozygous littermates (n=8) and the incisors only 5% (n=3). Both parasagittal and frontal views of the mandibular first molar showed that morphogenesis of the mandibular molars was severely disrupted. In heterozygous pups, the molars were separated from the oral epithelium by the dental cord and surrounded by alveolar bone. A crown had formed that contained well-developed cusps and cervical loops, enclosing the mesenchymally derived dental papilla (Fig. 3A,B). In the mutant, the dental cord was absent, the tooth was fused with the oral ectoderm, and the alveolar bone was absent from the oral part of the tooth. A single, irregularly shaped cusp was visible, but additional cusp formation appeared to be arrested (arrowheads in Fig. 3F,G). In the incisor, a single cusp with two symmetrical cervical loops was present (compare Fig. 3D with 3I).

Closer histological examination revealed that the organization of both dental epithelium and mesenchyme was also abnormal. In heterozygous pups, which have a wild-type phenotype, polarized pre-odontoblasts were present in an epithelial monolayer at the tip of the cusp where they produced and secreted pre-dentin matrix (Fig. 3C). Adjacent to them, polarized pre-ameloblasts were differentiating (Fig. 3C). In the mutant, substantial matrix was observed in the intercellular space between the dental epithelium and mesenchyme papilla, suggesting that functional pre-odontoblasts were present (Fig. 3H). However, unlike the heterozygote (Fig. 3C),

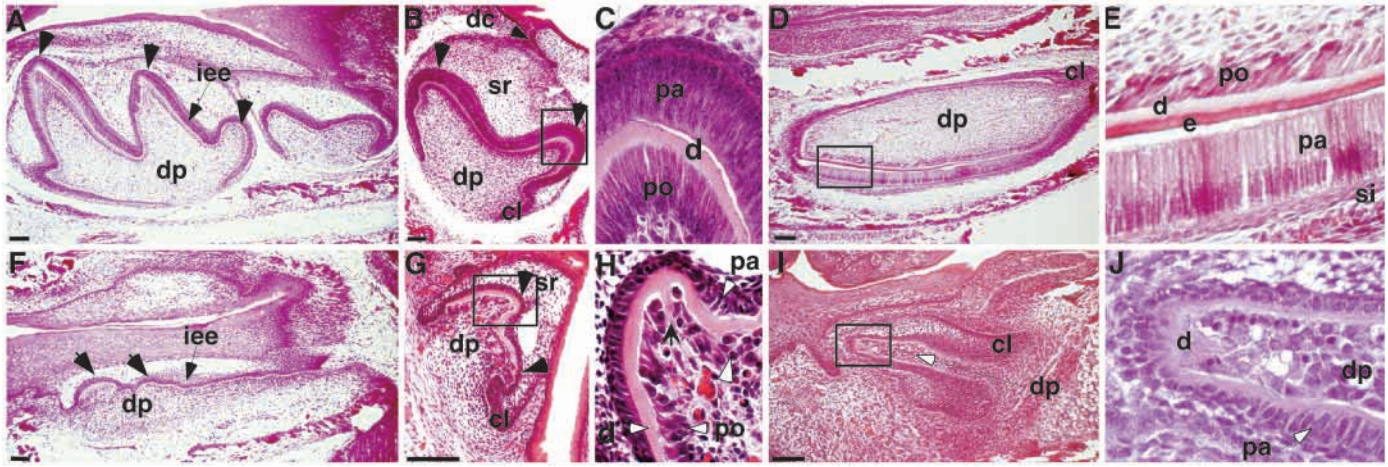

Fig. 3. Histological analysis of mutant mandibular teeth at 19.5 d.p.c. (A,F) Parasagittal sections through the first and second molars in the heterozygote (A) and through the first molar in the mutant (F). (B,G) Frontal sections through the first molar in the heterozygote (B) and in the mutant (G). Mutant teeth are small and abnormally shaped. However, cervical loops (cl), dental papilla (dp), inner enamel epithelium (iee), pre-dentin (d), and stellate reticulum (sr) are present in the mutant. The dental cord (dc) is absent in the mutant (G). Tips of the cusps are marked by black arrowheads. (C,H) High-power view of boxed areas. pre-dentin (d) is present but cytodifferentiation is disrupted in the mutants (H) in comparison with the heterozygotes (C); pre-ameloblasts (pa), pre-odontoblasts (po), stratum intermedium (si). Black arrowhead marks abnormal location of matrix, white arrowheads show unpolarized, stacked pre-odontoblasts. Incisor development in the heterozygote (D) and in the mutant (I). (E,J) High-power magnification of boxed areas in D and I, respectively. pre-enamel (e). Scale bars: 0.1 mm in A,B,D,F,G,I.

mesenchymal cells at the epithelial-mesenchymal interface had not formed a continuous monolayer. Some of these cells were partially polarized and irregularly shaped; others were stacked having neither polarized nor elongated. In both the mutant and the heterozygotes, cellular processes extended into the matrix (Fig. 3H). In addition, in contrast to the elongated and polarized pre-ameloblasts of the heterozygote pup (Fig. 3C), the cells of the inner enamel epithelium of the mutant were small and cuboidal (Fig. 3H). A similar result was observed in the incisor which is developmentally more advanced than the molar (compare Fig. 3E with 3J).

Functional Shh is not detectable at 19.5 d.p.c.

In order to establish that the phenotype we observed was due to an absence of functional Shh, we examined transcripts by in situ hybridization. *Shh* is normally expressed in the inner enamel epithelium (pre-ameloblasts and the stratum intermedium) (Fig. 4A). In the mutant, a full-length *Shh* RNA probe detected *Shh* expression discontinuously in the inner enamel epithelium (Fig. 4I). However, transcripts entirely lacked exon 2 in the mutant tooth (compare Fig. 4B with 4J) indicating that Cre-mediated recombination of the Shh^{c} allele had occurred. Consequently these transcripts did not encode functional Shh protein. Consistent with the absence of functional Shh, we could not detect *Ptch* expression in the mutant tooth (Fig. 4K), whereas in the heterozygote, *Ptch* was expressed in the outer enamel epithelium, the pre-odontoblasts and the dental papilla (Fig. 4C).

Shh is not required for tooth differentiation

To address the state of differentiation in the mutant teeth, we examined the expression pattern of a number of markers of epithelial and mesenchymal cell types. We confirmed that the mesenchymal cells at the epithelial-mesenchymal interface were pre-odontoblasts by in situ hybridization. Both dentin matrix protein 1 (*Dmp1*), which is expressed by pre-odontoblasts (Fig. 4D) and dentin sialo protein (*Dsp*), which is expressed by pre-odontoblasts and functional odontoblasts (Fig. 4E) (Begue-Kirn et al., 1998), were expressed along the mutant cusp. Together with the observed matrix deposition, these results suggest that functional pre-odontoblasts formed, even though their cellular architecture was abnormal.

In the inner enamel epithelium, we also observed evidence of pre-ameloblast differentiation. As in the teeth of the heterozygous pups (Fig. 4E,F), cells facing the pre-odontoblasts expressed *Dsp* and amelogenin (Fig. 4M,N), both markers of functional pre-ameloblasts. *Msx2* was also expressed in the inner enamel epithelium and the pre-odontoblasts (Fig. 4H,P) of the mutants, and *Pax9* was expressed in the deep papilla cells (Fig. 4G,O). We also observed *Bmp2* expression in the pre-odontoblasts and in the dental papilla (data not shown). *Bmp4* expression in the pre-ameloblasts and the pre-odontoblasts was also normal (data not shown). In summary, there does not appear to be an absolute requirement for Shh signaling to form distinct cell identities in the late tooth.

To address the extent of tooth development at later stages, we performed grafts of early tooth rudiments (13.5 to 15.5 d.p.c.) to kidney capsules. This technique allows normal tooth rudiments to complete organogenesis and terminal differentiation. At all stages tested, wild-type tooth germs (15/16) yielded teeth that had differentiated to advanced stages of enamel and dentin matrix formation (Fig. 5A,B).

In contrast, the developmental capacity of the mutant tooth rudiments depended on the stage at which the teeth were isolated. Tooth rudiments isolated at 13.5 d.p.c. failed to develop fully differentiated teeth. In 2/15 cases, bell stage teeth formed, however in most cases (13/15) keratinized cysts formed (data not shown). In contrast, mutant tooth rudiments isolated at 14.5 d.p.c. yielded teeth in 70% of cases (7/10). Teeth were significantly smaller than their wild-type counterparts, and in all cases the molar morphology was abnormal (Fig. 5C,D). Either a single cusp or a partial crown had formed. However, enamel and dentin matrices were deposited despite the lack of ameloblast elongation and the disorganization of the odontoblasts (Fig. 5D). The data suggest

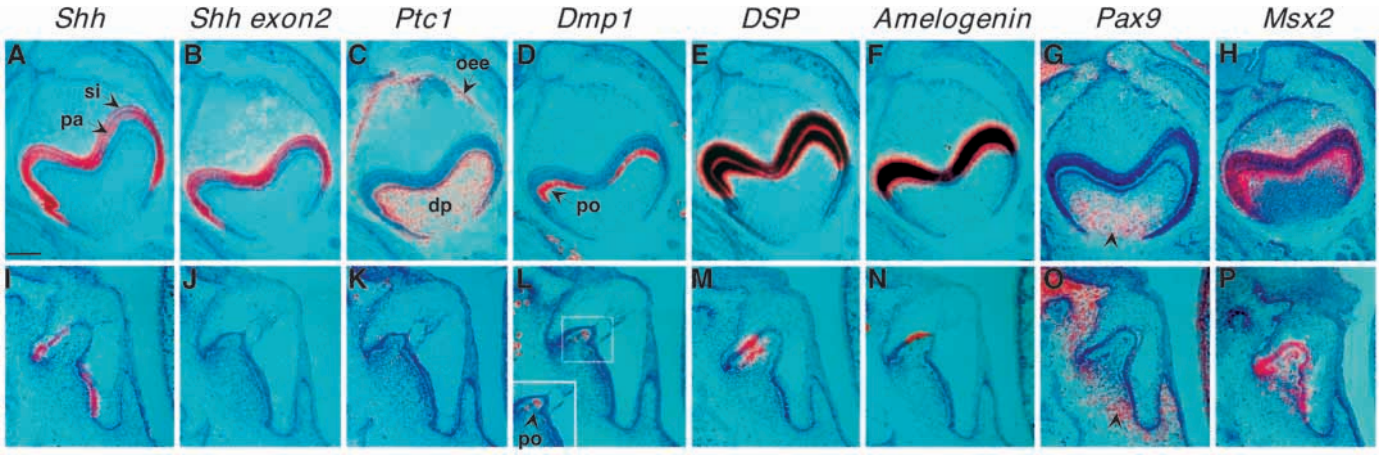

Fig. 4. RNA in situ hybridizations in the first molar at 19.5 d.p.c. Frontal sections through heterozygote (A-H) and mutant (I-P) molars. Full-length *Shh* is expressed in the stratum intermedium (si) and the pre-ameloblasts (pa) in the heterozygote (A) and in the mutant (I). (B,J) Exon 2 of *Shh* is not expressed in the mutant. (C,K) *Ptch* is expressed in the dental papilla (dp) and the outer enamel epithelium (oee) of the heterozygote (C), but its expression is completely absent in the mutant (K). (D,L) *Dmp1* expression, pre-odontoblasts (po). Inset in (L) is a higher magnification of the boxed area. (E,M) *Dsp*, (F,N) *Amelogenin*, (G,O) *Pax9* and (H,P) *Msx2* are expressed in the mutant as in the heterozygote. Scale bar: 0.1 mm.

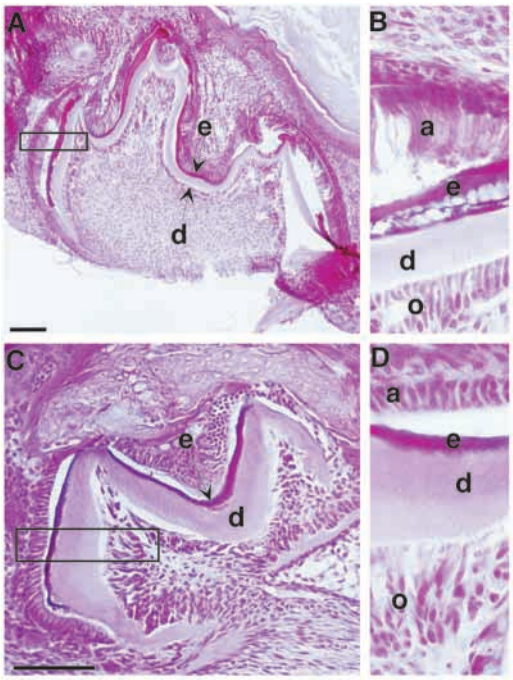

Fig. 5. In grafts, enamel and dentin are secreted. (A,B) Well-developed cusps form with enamel (e) and dentin (d) deposits in the heterozygotes after 9-20 days in culture under kidney capsules. (C,D) Enamel and dentin deposits are deposited in the mutant. However, the crown is incomplete and the tooth is small (C). Higher magnification of boxed area shows no ameloblast (a) elongation and disorganized odontoblasts (o) in the mutant (D). Scale bars: 0.1 mm.

that Shh is not essential for the differentiated properties of either ameloblasts or odontoblasts in the mammalian tooth. The reason why only the 14.5 d.p.c. grafts developed is unclear.

Shh is required for growth and morphogenesis of the tooth

In order to establish when tooth development was disrupted and how the phenotype correlated with the loss of Shh activity following removal of exon 2, we analyzed molar tooth germs at different stages of development.

At 11.5 d.p.c., molars of heterozygous and mutant pups were histologically indistinguishable, and functional Shh was present in the mutants (data not shown). Consequently, we cannot address whether there is a requirement for Shh signaling for tooth initiation. At 12.5 d.p.c., in the mutant, the molar epithelial bud was wider and it had not invaginated as deeply as in the heterozygote (Fig. 6A,B). Expression of *Shh* exon 2 was barely detectable at this stage in the mutant (Fig. 6C,D). Together with the observed phenotype, these data indicate that Shh activity is largely absent as early as 12.5 d.p.c. At 13.5 d.p.c., the mutant epithelial molar bud was still wider and less invaginated than that of the heterozygote (Fig. 6E,H). However, a condensed dental papilla was observed in the mutants as in the heterozygotes. At this stage, *Shh* is transiently silent in the molar dental epithelium (Fig. 6F,G). In contrast, we detected high levels of full-length *Shh* but not of exon 2 in the mutant buds (Fig. 6I,J), suggesting that the normal mechanism that downregulates *Shh* in the tooth germ at this time requires Shh activity.

By 14.5 d.p.c., the molars of the *Shh* mutants were 25% (*n*=7) smaller than those of heterozygotes, and cap formation had been significantly disrupted. In heterozygous embryos, the molars had developed to an advanced cap stage. The tooth displayed a polarized morphology, with a lingual invagination that had ingrown deeper into the jaw mesenchyme than the buccal invagination (Fig. 7A). The tooth cap narrowed at its apex and was connected to the oral ectoderm in this region by the dental cord. A primary enamel knot, flanked by two enamel grooves, was visible. In the mutant, morphology had been disrupted in several ways (Fig. 7D). First, there was no clear dental cord; rather the tooth lay closer to the oral ectoderm and was not well separated from it. Second, there appeared to be a dramatic failure of ingrowth of the epithelium on the lingual side resulting in the loss of the lingual enamel groove. This phenotype was somewhat less severe posteriorly where a very reduced lingual protrusion could be observed (arrowhead in Fig. 8K). These results indicate that Shh has differential growth effects in the tooth, and that it may play a significant role in determining tooth pattern.

To examine whether the lack of lingual invagination was due to an absence of the enamel knot, to an absence of enamel knot signaling or to an absence of lingual cells, we looked at cell proliferation, cell death and marker gene expression. As

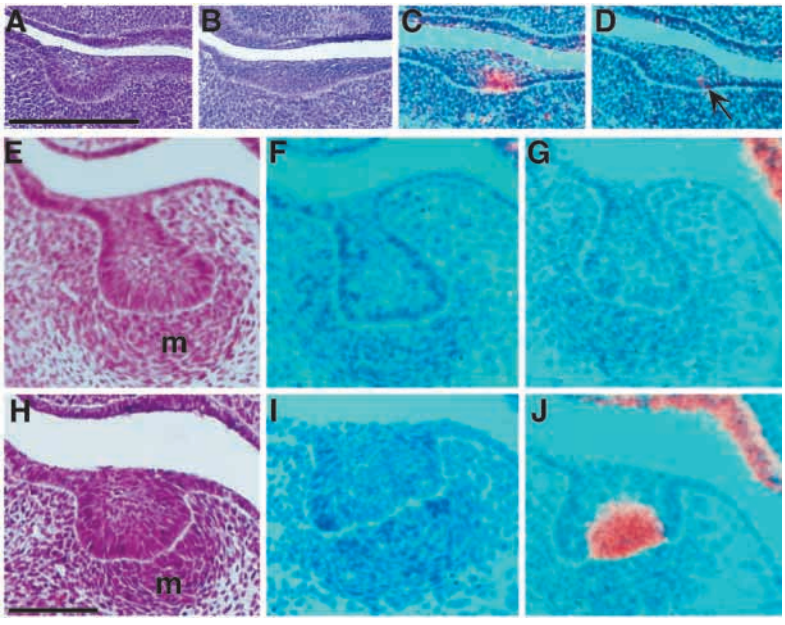

Fig. 6. Analysis of mandibular molars at 12.5 d.p.c. and at 13.5 d.p.c. (A,E-G) Heterozygote and (B-D,H-J) mutant. (A-D) Parasagittal sections at 12.5 d.p.c. show that the mutant tooth invagination (B) is less deep and broader than the heterozygote tooth (A), and that very few *Shh* transcripts containing exon 2 can be detected (arrow) (D) in comparison with full-length *Shh* expression in the mutant (C). Histological analysis at 13.5 d.p.c. reveals that the mesenchyme (m) has condensed in both the heterozygote (E) and the mutant (H). (F,I) *Shh* exon 2 expression; (G,J) *Shh* expression. Scale bars: in A, 0.1 mm for A-D; in H, 0.1mm for E-J.

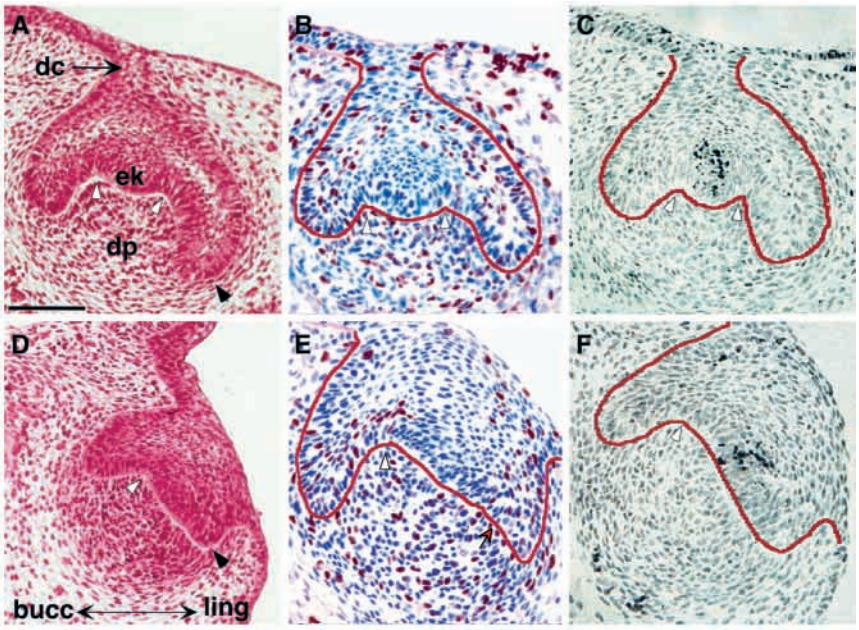

Fig. 7. Morphogenesis is disrupted at 14.5 d.p.c. Frontal sections through heterozygote (A-C) and mutant (D-F). (A,D) Histological analysis. (A) A well-formed cap with a dental cord (dc), a dental papilla (dp) and an enamel knot (ek) is formed. In the mutant (D), the dental cord and the lingual epithelial invagination (black arrowhead) are missing. Enamel grooves are marked with white arrowheads. (B,E) BrdU analysis, dividing nuclei are reddish-brown, non-dividing are blue. The red arrowhead in E demarcates the lingual extent of the non-proliferating cells in the mutant. White arrow marks buccal enamel groove. (C,F) TUNEL analysis. Apoptosis in the heterozygote and mutant enamel knot. (F) Apoptosis in the dental follicle and in the buccal epithelium is normal. Buccal (bucc) is towards the left and lingual (ling) towards the right in all panels. Epithelium is traced in red. Scale bar: 0.1 mm.

reported previously (Jernvall et al., 1994), cells of the enamel knot show low rates of proliferation and high incidence of programmed cell death (Fig. 7B,C). A similar population was observed in the mutants, suggesting that an enamel knot was present (Fig. 7E,F). Further, given the placement of the enamel knot it would appear that growth of the lingual epithelium was dramatically reduced. To determine whether loss of Shh activity in the enamel knot altered the expression of enamel knot-derived signals, we examined the expression of a number of signaling factors known to be expressed in this putative organizer. The population of non-proliferating cells expressed all the enamel knot markers tested: *Fgf4* (Fig. 8H,I), *Bmp2* (Fig. 8H,I), full-length *Shh* (Fig. 8B,J) and *Wnt10b* (Fig. 8D,L). Thus, we conclude that formation of the enamel knot was independent of Shh signaling after 12.5 d.p.c. and that the observed growth retardation in tooth development did not appear to result indirectly from the loss of the Wnt, Bmp or Fgf signals analyzed.

To determine the identity of the non-proliferating cells lingual to the mutant enamel knot, we looked at *Msx2* and *Pitx1* expression (St Amand et al., 2000). In the heterozygotes, cells in the lingual invagination did not express *Msx2* (Fig. 8F), but strongly expressed *Pitx1* (Fig. 8G). In the mutant, *Msx2* was appropriately restricted to the buccal epithelium (Fig. 8N). However, *Pitx1* was barely detectable in the lingual epithelial population (Fig. 8G,O). Furthermore, *Wnt10b* and *Lef1*, which are normally expressed in the enamel knot (Fig. 8D,E), extended beyond the non-proliferating cells of the enamel knot into presumptive lingual epithelium (Fig. 8L,M). These results suggest that the lingual epithelium may have undergone a partial fate change and have assumed some characteristics of the less proliferative enamel knot population.

High levels of *Ptch* expression indicate where the Hh pathway is transduced and as *Ptch* is expressed in the mesenchymal dental papilla (Fig. 9B, Hardcastle et al., 1998), we asked if dental papilla development was normal. Wild-type condensed dental

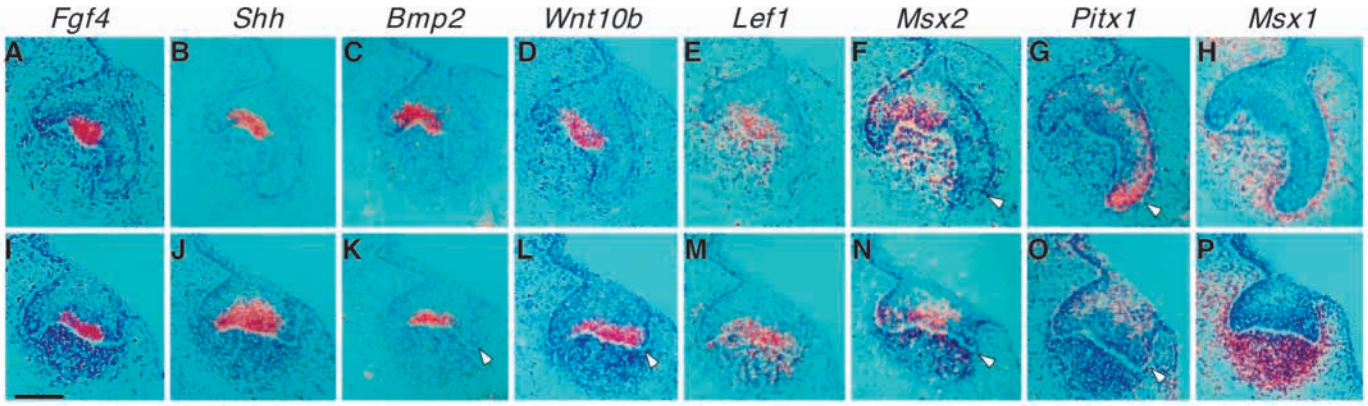

Fig. 8. The enamel knot forms. Frontal sections through heterozygote (A-H) and mutant (I-P) molars. (A,I) *Fgf4*, (B,J) *Shh*, (C,K) *Bmp2*, (D,L) *Wnt10b*, (E,M) *Lef1*, (F,N) *Msx2*, (G,O) *Pitx1* and (H,P) *Msx1*. White arrowheads point to the lingual epithelial invagination in F,G,K,L, to the non-*Msx2*-expressing cells in N and to the reduced *Pitx1* expression in O. Buccal is towards the left and lingual towards the right in all panels. Scale bar: 0.1 mm.

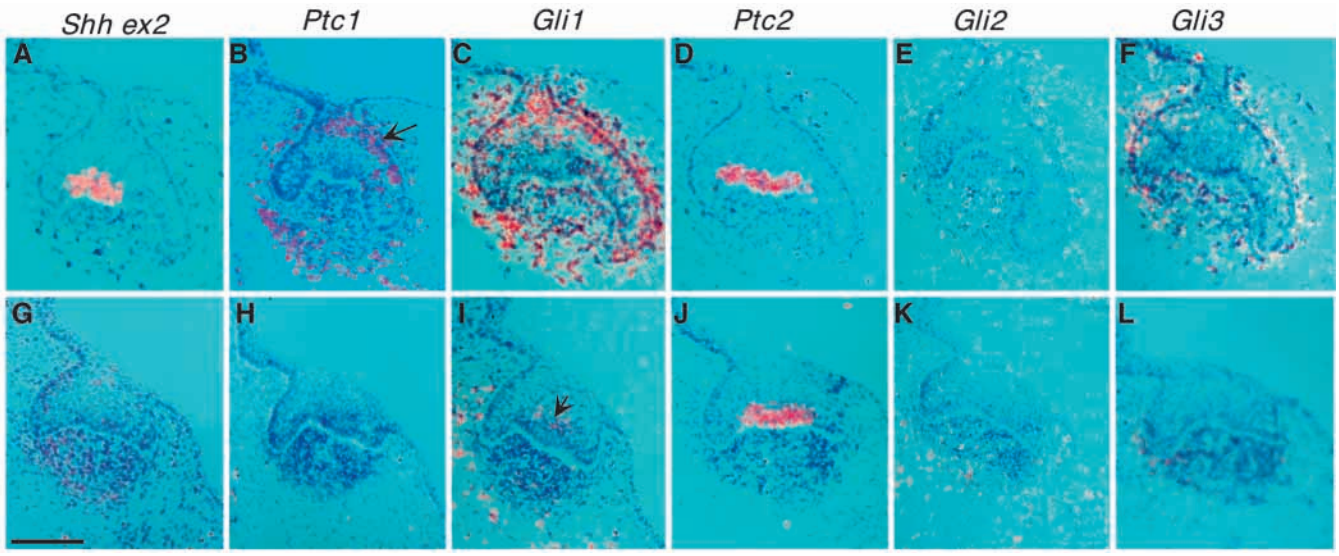

Fig. 9. Expression of members of the *Shh* pathway at 14.5 d.p.c. Frontal sections through heterozygote (A-F) and mutant (G-L) molars. (A,G) *Shh* exon 2, (B,H) *Ptc1*, (C,I) *Gli1*, (D,J) *Ptc2*, (E,K) *Gli2* and (F,L) *Gli3*. In the mutant, expression of *Shh* exon 2, *Ptc1* and *Gli1* are downregulated. Expression of *Ptc2*, *Gli2* and *Gli3* are unaffected. In B, the arrow points to expression in the lingual outer enamel epithelium and stellate reticulum and in I, the arrow points to residual expression of *Gli1*. Buccal is towards the left and lingual towards the right in all panels. Scale bar: 0.1 mm.

papilla expresses *Lef1* (Fig. 8E), *Msx1* (Fig. 8H) and *Pax9* (data not shown), all of which are essential for dental papilla condensation (Chen et al., 1996; Kratochwil et al., 1996; Peters et al., 1998). We observed normal condensation of the dental papilla in the mutant and expression of *Msx1*, *Lef1* and *Pax9* (Fig. 8M,P and data not shown), suggesting that several aspects of dental papilla development were unaltered.

Our results indicate that Shh is required for asymmetrical development of the dental cap, an essential aspect of molar morphogenesis. To determine how this might occur we examined the expression of a number of key genes in the *Shh* signaling pathway at advanced cap stage, 14.5 d.p.c. As expected, *Shh* transcripts containing exon 2 (Fig. 9A,G) and *Ptch* expression (Fig. 9B,H), were undetectable in the mutant. Interestingly, in the wild-type tooth, *Ptch* was asymmetrically expressed in the lingual epithelium including the dental cord, suggesting that Shh signaling is indeed asymmetric in the molar at this time (Fig. 9B, Hardcastle et al., 1998). In contrast to *Ptch*, *Gli1*, a putative transcriptional effector of hedgehog signaling, which is upregulated in response to Shh signaling, was expressed at high levels both buccally and lingually (Fig. 9C). Its expression was largely absent in the *Shh* mutants, except in the region above the enamel knot (Fig. 9I). *Gli3*, a putative negative regulator of Shh signaling, showed reduced levels of expression close to the oral ectoderm, but similar expression levels in the dental papilla of the heterozygote and *Shh* mutant (Fig. 9F,L). *Ptch2* expression in the enamel knot (Fig. 9D,J) and low levels of *Gli2* expression in the epithelium and mesenchyme (Fig. 9E,K) were also maintained in the absence of Shh activity.

DISCUSSION

In this study, we removed Shh activity from the developing tooth germ at early bud stage to address the role of Shh signaling in growth, morphogenesis and differentiation of the mammalian tooth. Our results indicate that Shh activity is crucial for normal development of both incisors and molars. The most striking feature of the mutant phenotype is a severe retardation in tooth growth, which leads to abnormal placement of the tooth in the jaw and disrupted tooth morphogenesis. Despite the abnormal cellular organization of ameloblast and odontoblast cell layers, enamel and dentin matrices were deposited. Thus, Shh is essential for growth and morphogenesis but not for differentiation of the mammalian tooth.

Shh is essential for growth of the tooth

The reduced size of the teeth indicates that Shh is important for growth. In wild-type mice, *Ptch* is expressed in cells to which Shh signals. As *Ptch* is expressed in the epithelium and the mesenchyme and as *Ptch* expression is downregulated in both these tissues in the mutant, it is likely that Shh signals directly to both tissues. Interestingly, *Ptch* is absent from the enamel knot, the only tissue that does not proliferate. Since *Shh* is transcribed in the mutants, we cannot absolutely exclude the existence of rare and isolated cells in which the Cre-mediated recombination event has not taken place. However, the absence of *Ptch* expression and the strong downregulation of *Gli1* are indications that the pathway is not active.

Our data contribute to the accumulating body of evidence that hedgehogs act as growth factors during embryonic and postnatal life. Previous studies have demonstrated the role of Shh in growth and cell proliferation in the skin, lung and cerebellum (Goodrich et al., 1997; Matise and Joyner, 1999; Pepicelli et al., 1998; Ruiz i Altaba, 1999a). Null mutations in *Ptch*, which is a negative regulator of Shh signaling, also lead to over-proliferation of the skin, to basal cell carcinomas and to widespread growth anomalies throughout the embryos

(Goodrich et al., 1997; Milenkovic et al., 1999; Oro et al., 1997). Studies in hair, an organ that shares a set of early, developmentally conserved steps with the tooth, show that complete absence of Shh leads to a reduction in growth, but not to a complete arrest in growth (Chiang et al., 1999; St-Jacques et al., 1998).

Shh patterns the developing cusps

Interestingly, the growth defects in *Shh* null teeth are not proportionate. Thus, by 14.5 d.p.c., in *Shh* mutants, the development of the lingual side of the tooth is more severely affected than the buccal side. Very little outer enamel epithelium on the lingual side is present, and the lingual inner enamel epithelium has not invaginated. This disruption in morphogenesis suggests that Shh signaling patterns the development of the tooth crown.

In wild-type and heterozygote mice at 14.5 d.p.c., highest levels of *Ptch* expression are found in the outer enamel epithelium and the adjacent stellate reticulum on the lingual side of the cap, indicating that the Shh pathway is normally transduced at high levels in these cells. Considering the role of Shh in proliferation, it is possible that lateral invagination is driven by the proliferation of the outer enamel epithelium and of the stellate reticulum, although no increase in mitotic index has been reported for this population of cells (Osman and Ruch, 1976). In addition, Shh may direct the growth of stellate reticulum downwards. The absence of Shh-directed growth in the mutants may result in the absence of the dental cord. Last, *Ptch* expression observed in the lingual epithelial invagination during normal development suggests that Shh also signals directly to cells lingual to the enamel knot, resulting in ingrowth of the lingual epithelial invagination.

An alternative view, which we cannot rule out on the basis of available data, is that *Shh* controls the balance between proliferative and non-proliferative cell fates. This hypothesis receives some support from the fact that, in the inner enamel epithelium of the mutant, non-proliferating cells extend several cell diameters on either side of the zone of apoptotic cells in the enamel knot and express several enamel knot markers including *Wnt10b*. Thus, a role of *Shh* may be to restrict the enamel knot to a specific population of cells that expands at the expense of proliferating cells in *Shh* mutants. The observation that Shh is essential for the development of the lingual side of the cusp is of particular interest because it provides the first evidence that a signaling protein expressed in the enamel knot is involved in tooth morphogenesis. Although the cellular mechanisms remain to be clarified, these results raise the possibility of directional signaling within the tooth, particularly with respect to the buccal-lingual axis.

Shh signaling and genetic control of tooth development

Our data provide some potentially interesting insights into the Shh signaling pathway. Several lines of evidence support the model that Gli proteins, and their *Drosophila* counterpart cubitus interruptus (ci), are transcriptional effectors of hedgehog signaling (for review, Ruiz i Altaba, 1999a). ci acts as a constitutive repressor which is converted into an activator form in response to hedgehog. Although the precise details of Gli action in vertebrates is not fully resolved, there is evidence that, in the absence of Shh, Gli2 and Gli3 repress the pathway (Ruiz i Altaba, 1999b; Sasaki et al., 1999; Wang et al., 2000).

In our study, we observe more advanced development of the tooth than was observed in the *Gli2;Gli3* null teeth, which arrest before the initiation of morphogenesis (Hardcastle et al., 1998). This difference may lie in the temporal differences between the null mutations. In the *Gli2;Gli3* null, Gli2 and Gli3 function is absent prior to tooth development, whereas the conditional removal of Shh activity occurs shortly after the tooth bud forms. Alternatively, the difference in phenotypes may reflect additional roles for Gli2 and Gli3 in transcriptional repression. Removal of Shh signaling is expected to leave Gli-mediated repression of Shh targets intact. In contrast loss of Gli2 and Gli3 activity may remove the ability to respond to Shh signal, as well as to Gli-mediated repression of targets. Whether this accounts for the different phenotypes remains to be determined.

Several signaling pathways have been described in the tooth and it is thought that they mediate the reciprocal interactions between the epithelium and the mesenchyme to control growth, patterning and differentiation of the tooth. A central issue is how does Shh signaling interact with these pathways? Both *Bmp2* and *Bmp4* are expressed in the tooth, and *Bmp4* has been shown to be a target of Shh signaling in the mouse limb and gut (Drossopoulou et al., 2000; Roberts et al., 1998). We were not able to detect downregulation of *Bmp2, Bmp4, Pax9, Msx1* or *Msx2* in the teeth of our mice, suggesting that *Shh* is not upstream of these genes. These results are consistent with our studies in the early mandible (Dassule and McMahon, 1998; Hardcastle et al., 1998). Studies of *Msx1* null mutants have shown that between 12.5 d.p.c. and 13.5 d.p.c. there is a downregulation of *Shh* and *Bmp2* in the epithelium and of *Ptch* in the mesenchyme, whereas *Gli1* expression appears to be unaltered (Zhang et al., 1999, 2000). Here, we show that both *Ptch* and *Gli1* are downregulated in the absence of Shh. Together these results suggest that *Msx1* may repress a gene that can activate *Gli1* transcription independently of Shh signaling.

Zhang et al. (2000) also show that overexpression of *Bmp4* in the mesenchyme leads to downregulation of *Shh* and *Bmp2* in the overlying epithelium, although no phenotype is visible. In this paper, we clearly demonstrate that when Shh signaling is absent, *Bmp2* expression is unaltered and tooth development is severely affected. These two observations may be reconciled in a number of ways. For example, sufficient Shh signaling may remain in the *Bmp4* overexpression model to lead to normal growth of the tooth. Alternatively Shh and Bmp2 may play opposing roles: Shh in promoting growth and Bmp2 in inhibiting growth. In such a case, overexpression of *Bmp4*, which downregulates both genes, would maintain a balance between the two functions, whereas loss of Shh leaves Bmp2 signaling intact.

Shh is not required for tooth cytodifferentiation

Our graft data show that, although odontoblasts are disorganized and ameloblasts do not elongate, enamel and dentin secretion proceeds normally. It seems unlikely that the kidney supplies Shh, as we do not observe a rescue of the cellular phenotype. Further, the only Shh activity that has been documented in this organ is associated with deep medullary cells during development. Thus, the simplest interpretation is

that this specific aspect of odontoblast and ameloblast differentiation is independent of Shh.

Classical embryology has shown that pre-odontoblast differentiation requires a signal from the inner enamel epithelium (Thesleff and Hurmerinta, 1981). As *Shh* is expressed in the inner enamel epithelium and *Ptch* in the dental papilla prior to pre-odontoblast differentiation, Shh has been suggested to be the signal that initiates pre-odontoblast differentiation (Koyama et al., 1996). Organ culture experiments have shown that elongation and differentiation of odontoblasts can be induced separately by different secreted factors but the properties of Shh have not been tested (Martin et al., 1998). Shh signaling may only be required for the elongation of the odontoblasts. In the mutant, the unusual stacking of the cells suggests that during the final division, the spindle does not always reorient itself perpendicularly to the epithelial-mesenchymal interface. Alternatively, the lack of organization of the odontoblasts may be a secondary effect due to the reduced size of the dental papilla.

The phenotype of pre-ameloblasts is more severe as there is little observable elongation or polarization. Classical embryological experiments indicate that the pre-odontoblasts must signal to the inner enamel epithelium to initiate cytodifferentiation of ameloblasts (for a review, see Thesleff and Hurmerinta, 1981). As *Ptch* does not appear to be expressed in the inner enamel epithelium coincident with pre-ameloblast differentiation, it seems unlikely that the abnormal morphology of the pre-ameloblasts in the *Shh* mutants reflects a direct role for Shh in this population. Rather, the hedgehog-dependent development of normal pre-odontoblasts may lead to secondary abnormalities in pre-ameloblasts. For example, alteration in the normal polarity of the pre-odontoblasts may lead to abnormal signal trafficking that may influence cell interactions post-transcriptionally. Alternatively, the mesenchymal signal that induces pre-ameloblast elongation may be a downstream target of Shh signaling in the mesenchyme. Several factors have been shown to have this property in vitro (Coin et al., 1999).

Similarities between Shh action in tooth and hair

Finally, our results on Shh function in the tooth show striking parallels with earlier reports on Shh action in the hair (Chiang et al., 1999; St-Jacques et al., 1998). Both teeth and hair are structures derived from epidermal placodes, and the early stages of their development are morphologically similar. Also, many of the same genetic pathways are in place. As in the tooth, *Shh* is expressed at the tip of the invaginating hair bud and expression continues in the epithelial component throughout hair development. *Ptch* is expressed in both the epithelium and the mesenchyme of the early bud; *Ptch2* is expressed in the epithelium only. Interestingly, in the *Shh* null mutants, the hair phenotype is very similar to the tooth phenotype we have observed by conditionally inactivating Shh. The hair bud is smaller and remains closer to the surface of the skin as morphogenesis occurs. An organized hair follicle is never formed. However, an abnormal follicle which fails to separate from the skin does develop. Despite the disruption in growth and morphogenesis, differentiated hair keratins are produced. Thus, as in the tooth, the primary role of Shh is in growth and morphogenesis and not in induction of differentiation. These results suggest that Shh plays a conserved role in epidermal placode morphogenesis. Further, they raise the interesting possibility that modulation of Shh activity in these structures may have played an important role in the evolution of the size and shape of these particular organs.

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