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Pathway during Epithelial Morphogenesis of Teeth

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Teeth develop as epithelial appendages, and their morphogenesis is regulated by epithelial–mesenchymal interactions and conserved signaling pathways common to many developmental processes. A key event during tooth morphogenesis is the transition from bud to cap stage when the epithelial bud is divided into specific compartments distinguished by morphology as well as gene expression patterns. The enamel knot, a signaling center, forms and regulates the shape and size of the tooth. Mesenchymal signals are necessary for epithelial patterning and for the formation and maintenance of the epithelial compartments. We studied the expression of Notch pathway molecules during the bud-to-cap stage transition of the developing mouse tooth. *Lunatic fringe* expression was restricted to the epithelium, where it formed a boundary flanking the enamel knot. The *Lunatic fringe* expression domains overlapped only partly with the expression of *Notch1* and *Notch2,* which were coexpressed with *Hes1*. We examined the regulation of *Lunatic fringe* and *Hes1* in cultured explants of dental epithelium. The expression of *Lunatic fringe* and *Hes1* depended on mesenchymal signals and both were positively regulated by FGF-10. BMP-4 antagonized the stimulatory effect of FGF-10 on *Lunatic fringe* expression but had a synergistic effect with FGF-10 on *Hes1* expression. Recombinant Lunatic fringe protein induced *Hes1* expression in the dental epithelium, suggesting that Lunatic fringe can act also extracellularly. *Lunatic fringe* mutant mice did not reveal tooth abnormalities, and no changes were observed in the expression patterns of other *Fringe* genes. We conclude that Lunatic fringe may play a role in boundary formation of the enamel knot and that Notch-signaling in the dental epithelium is regulated by mesenchymal FGFs and BMP.

Key Words: **signaling center; enamel knot; tooth development; Lunatic fringe; Notch; Jagged; BMP; FGF; Hes1.**

INTRODUCTION

Like all epithelial–mesenchymal organs, the development of tooth depends on sequential and reciprocal interactions between the two tissues (Jernvall and Thesleff, 2000). In mice, the development of the molar tooth germs is initiated between embryonic days 10 and 11 by epithelial signals, and by E12, the potential for tooth formation has shifted to the underlying mesenchyme (Mina and Kollar, 1987; Lumsden, 1988). The mesenchyme regulates budding of the epithelium which is subsequently divided morphologically as well as by gene expression patterns into several compartments. At the transition of bud-to-cap stage, the cells at the tip of E13.5 tooth bud stop dividing and form an epithelial signaling center, the enamel knot, secreting growth factors, and other signaling molecules during the cap stage (E14) (Fig. 1) (Vaahtokari *et al.*, 1996). The central role of the enamel knot in the regulation of tooth shape has been verified in several studies (Laurikkala *et al.*, 2001; Jernvall *et al.*, 2000). The epithelial cells around the enamel knot proliferate and form the cervical loops, which will surround the mesenchymal dental papilla. In cap-stage tooth germs, the epithelium is divided to histologically different cell types including the inner and outer enamel epithelium, which surround a core of stellate reticulum and stratum intermedium cells (Ten Cate, 1994). During subsequent morphogenesis of molar teeth, secondary enamel knots appear in the dental epithelium and they initiate epithelial folding resulting in the characteristic cusp pat-

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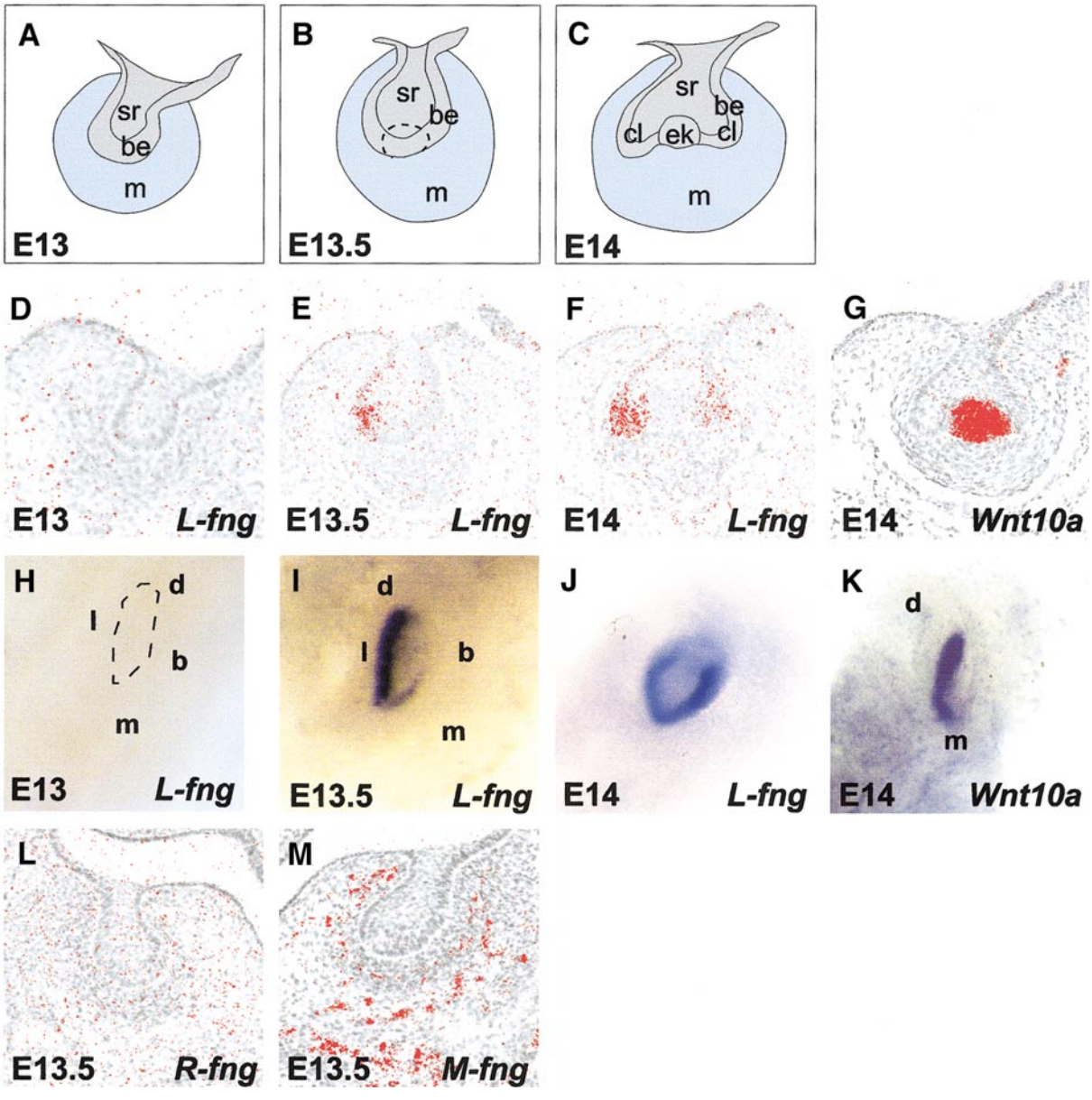


FIG. 1. Expression patterns of mouse Fringe molecules at E13–E14 developing lower molar. (A–C) A schematic representation of the dental tissue sections. At early bud stage (E13), the dental epithelium (e) consisting of basal epithelial cells (b) surrounding the stellate reticulum (sr) grows into the dental mesenchyme (m); at late bud stage (E13.5), the enamel knot starts to form at the tip and the epithelium starts to fold; and at the cap stage E14, the epithelial compartments, including the cervical loops (cl) and the signaling center enamel knot (ek), have formed. *Lunatic fringe* expression cannot be seen at the early tooth bud (H). *Lunatic fringe* expression starts at E13.5 in the lingual aspect of the bud (E, I). At E14, the expression flanks the enamel knot on both sides (F, J). The enamel knot was visualized by *Wnt10a* probe (G, K). *Radical Fringe* is not expressed in the early developing tooth (L). *Manic Fringe* expression is seen in the blood vessels (M). (D–G) and (L, M) are radioactive *in situ* hybridizations of sections. (H–K) are whole-mount *in situ* hybridizations. The dotted lines indicate the level of sections in (D–G). l, lingual; b, buccal; d, distal; m, mesial.

terns of the teeth (Jernvall *et al.*, 1994). As development continues, the cells in the inner enamel epithelium differentiate terminally to ameloblasts secreting the components of the enamel and mesenchymal cells differentiate into odontoblasts secreting dentin.

Notch signaling controls cell fate and the formation of

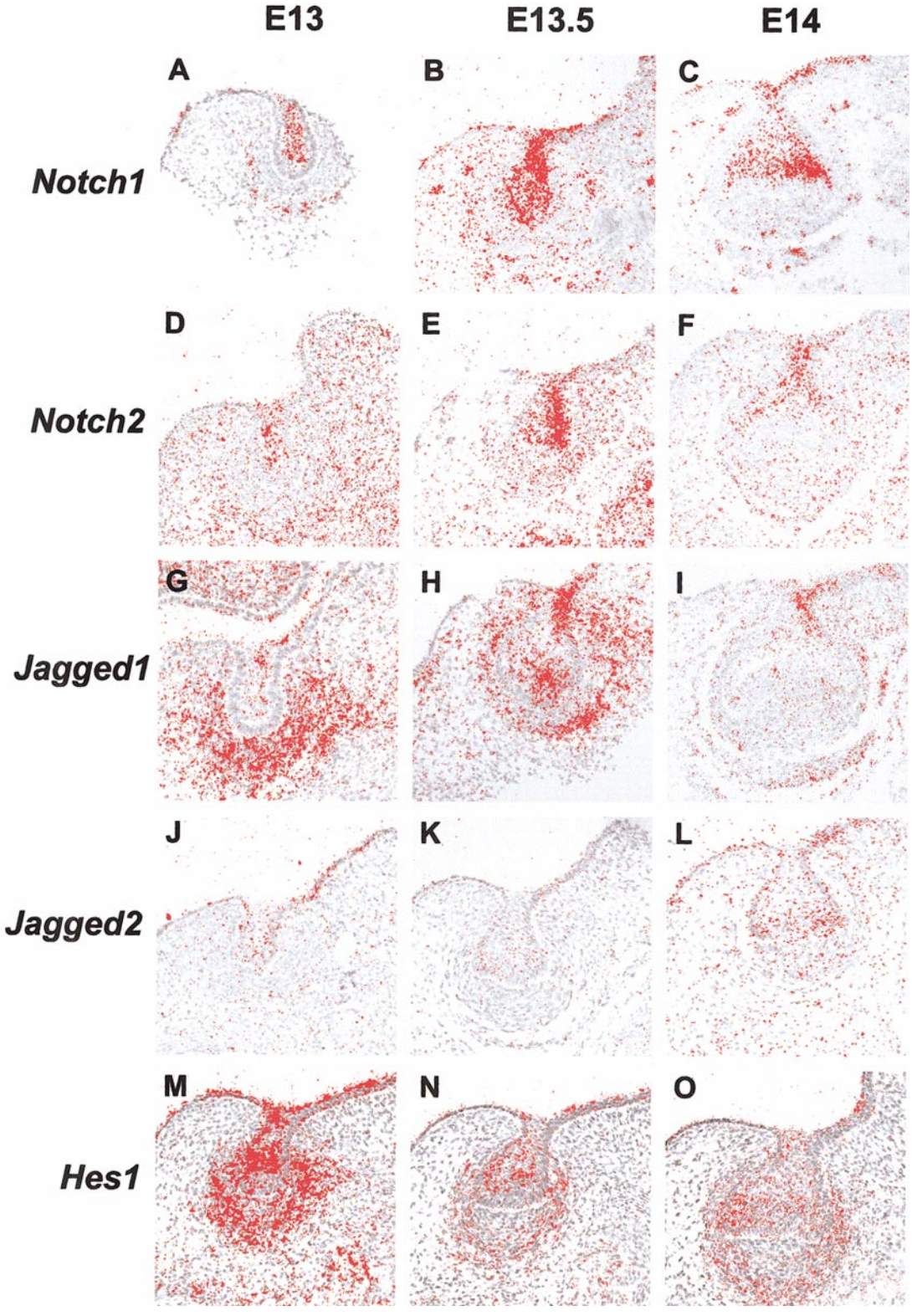


FIG. 2. Expression analysis of *Notch1* and *-2*, Notch ligands *Jagged1* and *-2*, and Notch target molecule *HES1* in the bud-to-cap stage transition of the developing tooth. Throughout these stages, the stellate reticulum cells express *Notch1*, *Notch2*, and *hes1* (A–C, D–F, M–O). In the mesenchyme, *Notch1* is expressed in the blood vessels (A) and *Notch2* and *HES1* in the outer dental mesenchyme (D, M). An interesting feature at the E13.5 tooth bud is the transient expression of *Jagged1* in the enamel knot (H); it is also expressed in the dental lamina and the outer parts of the dental mesenchyme (G–I). *Jagged2* is moderately expressed in the dental epithelium, including the basal cells during these stages (J–L).

tissue compartments in insects and vertebrates (Artavanis-Tsakonas *et al.*, 1999). Notch has also frequently been associated with stem cell development, e.g., in the developing brain where Notch1 may serve as a marker for immature neural cell precursors (Johansson *et al.*, 1999). Notch is a cell membrane receptor for membrane bound ligands Delta, Delta-like, Serrate, and its mammalian homologue Jagged. Ligand activation causes the release of the Notch intracellular domain and its transport into the nucleus (Schroeter *et al.*, 1998; Struhl and Adachi, 1998). The *Drosophila* Notch target gene *Enhancer of Split* encodes a transcription factor that has several mammalian homologues named *HES* (Hairy/Enhancer of Split) (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Mammalian *HES* genes have also been shown to be activated by Notch (Jarriault *et al.*, 1998). HES molecules are basic helix–loop–helix transcription factors inhibiting cellular commitment and differentiation, e.g., in inner ear and pancreas development (Zheng *et al.*, 2000; Jensen *et al.*, 2000).

The Notch signaling activity is modified by Fringe (Irvine and Wieschaus, 1994; Kim *et al.*, 1995; Johnston *et al.*, 1997). Fringe plays an essential role in the formation of tissue boundaries that have a developmental organizer function in the *Drosophila* wing imaginal disc (Kim *et al.*, 1995) and in the developing vertebrate limb bud (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997; Hicks *et al.*, 2000). Fringe is also involved in the development of the somites (McGrew *et al.*, 1998). The three vertebrate *fringe* genes have been named *manic*, *radical*, and *lunatic fringe* (Cohen *et al.*, 1997; Johnston *et al.*, 1997; Laufer *et al.*, 1997). Fringe is suggested to either potentiate or suppress Notch activation by its ligands in a context-dependent manner (Wu and Rao, 1999). The amino acid sequence of Fringe is weakly but significantly homologous to bacterial Lex-1 family glycosyl transferases and it has been shown that Fringe affects glycosylation of Notch. Fringe has been reported to affect Notch–ligand interactions by specific glycosylation of Notch extracellular EGF repeats, which occurs in the Golgi (Moloney *et al.*, 2000; Bruckner *et al.*, 2000; Munro and Freeman, 2000). However, it is not clear whether Fringe is also secreted out of the cell and whether it mainly acts intra- or extracellularly (Wu and Rao, 1999).

The expression and regulation of some genes in the Notch pathway have been previously analyzed in developing teeth. The downregulation of *Notch1* in basal epithelial cells during the early stages of dental development, possibly associated with fate determination of the epithelial ameloblasts, was shown to be dependent on the mesenchymal signals. (Mitsiadis *et al.*, 1995). More recently, *Notch* expression was shown to be present in the stellate reticulum compartment of the cervical loop in the continuously erupting incisor of the mouse, and it was suggested that *Notch* is associated with stem cell properties of these epithelial cells, which were regulated through mesenchymal FGF10 (Harada *et al.*, 1999).

To understand the molecular basis of the transition from the bud into cap stage, we studied the expression and regulation of Notch signaling pathway genes. The analysis included receptors *Notch1*, *Notch2*, *Notch3*; ligands *Jagged1*, *Jagged2*, *Delta1*; target genes *HES1* and *HES5*; and modulators *Manic fringe*, *Radical fringe*, and *Lunatic fringe*. *Lunatic fringe* was associated with the formation of tissue boundaries around the enamel knot, the signaling center in the developing tooth. Tissue recombination experiments showed that epithelial *Lunatic fringe* and *HES1* were induced by the underlying mesenchyme. This induction was mimicked by FGF4 and FGF10 beads in dental epithelial explants. We also found that BMP4 inhibits the FGF10 induction of *Lunatic fringe* but stimulates *HES1* expression. Lunatic fringe protein stimulated *HES1* expression, suggesting that Lunatic fringe can act also extracellularly and regulates Notch signaling as a paracrine factor. *Lunatic fringe* mutant mice showed no defects during tooth development and no upregulation of other fringe genes to compensate for the loss of *Lunatic fringe*.

MATERIALS AND METHODS

Preparation of Tissues, Explant Cultures, and Bead Implantation Experiments

Dental tissues from NMRI mouse embryos were prepared as earlier described (Åberg *et al.*, 1997). Generation of *Lunatic fringe* mutant mice has been described previously (Zhang and Gridley, 1998). For explant cultures, E11–E14 lower molar tooth germs were dissected and the epithelium was separated from the mesenchyme by pancreatin-trypsin treatment as described previously (Kettunen *et al.*, 2000). In recombination experiments, the epithelium was cultured with the mesenchyme of the same developmental stage. In bead experiments, E12 dental epithelia were cultured on a drop of Matrigel with reduced amounts of growth factors (Collaborative Biochemical Products, Bedford, MA) on a piece of filter (polycarbonate Nucleopore filters, pore size 0.1 μm; Costar). E13 epithelia were placed directly on the filter. For the localized protein delivery, approximately 100 Affigel-Blue agarose (BioRad) or heparin acrylic beads (Sigma H-5263) were soaked in 5 μl of protein in PBS, 0.1% BSA for 30–60 min in 37°C. Agarose beads were soaked in 100 ng/μl BMP4 (kind gift from J. Wozney, Genetics Institute, MA), and heparin beads were soaked in 75 ng/μl FGF4 or 24–100 ng/μl of FGF10 (R&D Systems, UK). Both agarose and heparin acrylic beads were soaked in Lunatic fringe protein (50 ng/μl). Number of samples indicated in Table 1.

Preparation of Recombinant Rat Lunatic fringe

Rat *Lunatic fringe* cDNA was cloned, and sequence analysis showed that it encodes a 378-amino-acid protein with 97.1% identity to mouse *Lunatic fringe*. Rat *Lunatic fringe* has a typical signal sequence for secretion and a conserved internal proteolytic processing site RARR at amino acids 82–85 (T. Mikami and N. Itoh, unpublished observation). *Lunatic fringe* cDNA encoding a mature form of rat *Lunatic fringe* (amino acids 86–378) with a DNA fragment encoding an E tag (GAPVPYPDPLEPR) and a hexameric His tag (HHHHHH) at the 3′ terminus was constructed in a transfer vector DNA, pAcGP67A (PharMingen). Recombinant baculovirus containing the cDNA was obtained by using the

BaculoGold system (PharMingen). High Five cells (approximately 2×10^6 cells/ml) were infected with the resultant recombinant baculovirus and incubated at 27°C for 60 h in serum-free medium EX-CELL 400 (JRH Biosciences). The culture medium was dialyzed against phosphate-buffered saline (PBS), pH 7.4, and applied to a column of Ni^{2+}-nitrilotriacetic acid-agarose (QIAGEN) in PBS, pH 7.4, containing 20 mM imidazole and 0.5 M NaCl. After washing the column with PBS, pH 7.4, containing 20 mM imidazole and 0.5 M NaCl, the recombinant Lunatic fringe was eluted from the column with PBS, pH 7.4, containing 250 mM imidazole and 0.5 M NaCl, and desalted by gel filtration using Bio-Gel P-6 DG (Bio-Rad). Protein product was analyzed in a 12.5% polyacrylamide gel electrophoresis and a single band of circa 37 kDa consistent with the calculated size 36.7 kDa was detected (T. Mikami and N. Itoh, unpublished observation).

In Situ Hybridization

The *in situ* hybridization with [^{35}S]UTP (Amersham)-labeled riboprobes was performed as described previously (Wilkinson and Green, 1990). Whole-mount *in situ* hybridization protocol using digoxigenin-labeled probes has been described earlier (Kettunen *et al.*, 1998). The proteinase K concentration was 10 μg/ml, and the length of the proteinase K treatment was modified according to the size of the tissue. Cultures of isolated dental tissues were treated for 8 min at 37°C, and whole jaw cultures for 25 min.

The slides from the radioactive *in situ* hybridization were photographed with Olympus Provis microscope equipped with CCD camera (Photometric Ltd.). Figures were processed by using ImagePro and NIH Image 1.61 software and further manipulated with Adobe Photoshop (Adobe Systems, CA; the dark field images were inverted and artificially stained red and combined with the bright field image) and Micrographx Designer software. Whole-mount *in situ* hybridization results were photographed with a digital camera (Kodak), and the data were downloaded from the digital film with Adobe Photoshop and combined with the Micrographx Designer software.

Probes

The following plasmids were used for both [^{35}S]UTP and digoxigenin–UTP labeling. *Jagged2* plasmid was found by sequence comparisons, and ordered from the NCBI EST clone library. The clone number 717B16 (image ID 317487) contains pT7T3D vector and a 1.0-kb *Jagged2* insert, which was verified by sequencing. For the *Jagged2* antisense probe, we used *Eco*RI for linearization and T7 polymerase for transcription, *Not*I and T3 were used for the sense probe. *Lunatic fringe*, *Manic fringe*, and *Radical fringe* probes were a kind gift from A. Wang and have been described earlier (Harada *et al.*, 1999). *Jagged1*- and *Delta1*-containing plasmids were a kind gift from Domingos Henrique, and probes have been described earlier (Mitsiadis *et al.*, 1997; Bettenhausen *et al.*, 1995). *Notch1*, *-2*, and *-3* probes have been described earlier (Lardelli *et al.*, 1994; Larsson *et al.*, 1994). *Engrailed1* and *Wnt10a* containing plasmids were a kind gift from A. McMahon (Danielian and McMahon, 1996; Wang and Shackleford, 1996), *Engrailed2* plasmid from A. Joyner (Liu *et al.*, 1999), and *HES1* and *HES5* from Royuchiro Kageyama (Sasai *et al.*, 1992).

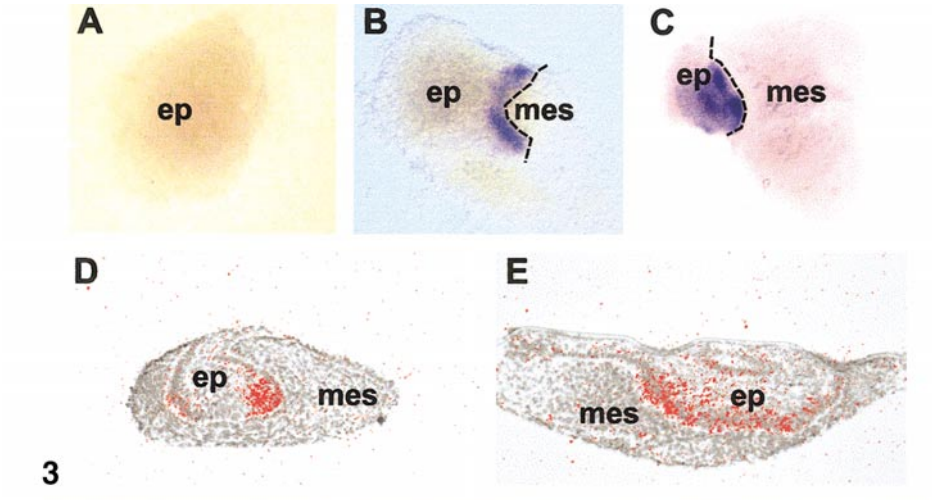
RESULTS

Localization of Notch Pathway Molecules during the Bud-to-Cap-Stage Transition of Developing Tooth

Notch1, *Notch2*, *Notch3*, *Lunatic fringe*, *Jagged1*, and *Delta1* expression in developing teeth have been partly described earlier (Mitsiadis *et al.*, 1995, 1996, 1997; Pouyet and Mitsiadis, 2000). We analyzed the expression in more detail by radioactive *in situ* hybridization on serial tissue sections in parallel with *Radical fringe*, *Manic fringe*, *Jagged2*, *HES1*, *HES5*, *Engrailed1*, and *Engrailed2*. Gene expression patterns were analyzed at early bud stage (E13), late bud stage (E13.5), and early cap stage (E14) (Figs. 1A–1C). At early bud stage, the dental mesenchyme has condensed around the epithelial bud, which consists of an outer layer of basal epithelial cells, continuous with the basal cell layer of the oral epithelium, and of central loosely arranged epithelial cells, the stellate reticulum cells. At advanced bud stage, the transition from bud-to-cap stage becomes evident through the formation of the enamel knot at the tip of the epithelial bud. These cells start to express several signal molecules and exit the cell cycle. This results in the growth of the epithelium flanking the enamel knot and the formation of the cervical loops. The cervical loops grow downwards and subsequently surround the mesenchymal dental papilla at the cap stage. The enamel knot is fully developed at the cap stage and consists of densely packed epithelial cells that are characterized by a unique gene expression profile, including more than 10 signal molecules (Jernvall and Thesleff, 2000).

Fringe Genes

Of the three vertebrate *Fringe* genes analyzed, only *Lunatic fringe* showed developmentally regulated expression in tooth germs. *Lunatic fringe* expression was restricted to the dental epithelium. *Lunatic fringe* was not detected in oral epithelium, and also all mesenchymal tissue was negative. In our radioactive *in situ* analysis, the dental epithelium did not express *Lunatic fringe* during the initiation of tooth development (data not shown) and transcripts were still absent in E13 tooth bud (Fig. 1D), although it has been reported earlier that *Lunatic fringe* transcripts could be detected as early as E11 (Pouyet and Mitsiadis, 2000). *Lunatic fringe* expression was first seen during late bud stage (E13.5) in epithelial cells at the lingual aspect of the dental bud (Fig. 1E). Interestingly, the *Lunatic fringe*-expressing cells flanked the forming enamel knot. This was more evident at cap stage when the expression had appeared also on the buccal side of the enamel knot and continued at high intensity in the lingual cervical loop (Fig. 1F). Some *Lunatic fringe* expression was seen in the outer enamel epithelium, but most of the stellate reticulum cells and the whole enamel knot were negative (Fig. 1F). The enamel knot was visualized with a W*nt10a* probe (Dassule and McMahon, 1998) (Fig. 1G).



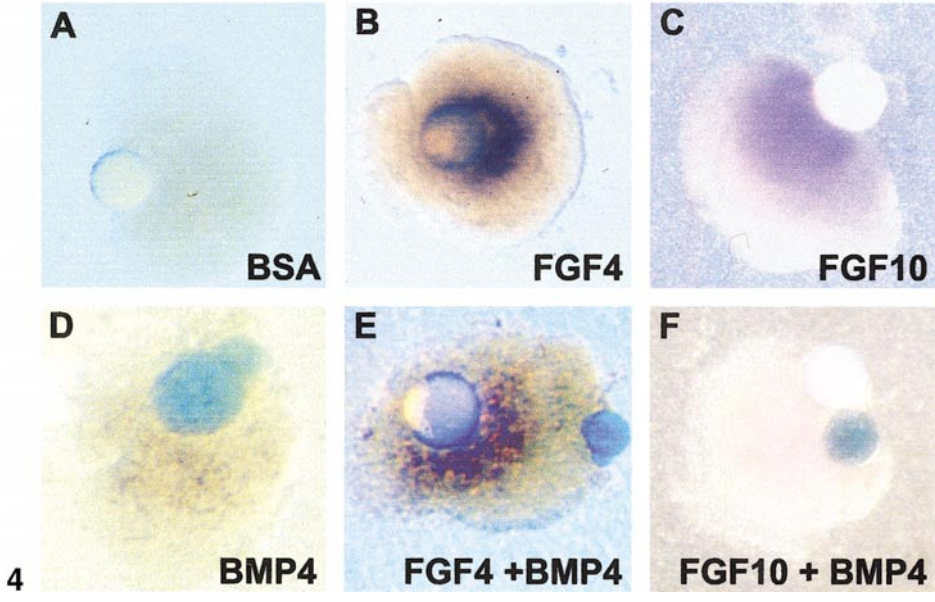


FIG. 3. Expression of *Lunatic fringe* in cultured dental tissues. E13 dental epithelium cultured alone does not express *Lunatic fringe* (A), but when it is recombined with dental mesenchyme of the same stage, *Lunatic fringe* is induced in the epithelial cells close to the mesenchyme (B–E). (A–C) Whole-mount *in situ* hybridizations. (D, E) Sections of cultured explants hybridised with a radioactive riboprobe.

FIG. 4. Stimulation of *Lunatic fringe* expression by FGFs and inhibition of the stimulation by BMP4. BSA bead had no effect on *Lunatic fringe* expression (A), but FGF4- and FGF10-releasing beads stimulated *Lunatic fringe* expression in the epithelium around the bead (B, C). BMP-4 did not stimulate *Lunatic fringe* expression (D) but had a counteracting effect on induction by FGFs (E). When FGF and BMP beads were placed at opposite end of the tissue explant, FGF4 could induce Lunatic fringe (F), but when placed next to each other, the BMP4-releasing bead inhibited the stimulatory effect of the FGF10 (E).

Whole-mount *in situ* hybridization analysis of dissected tooth germs allowed the visualization of *Lunatic fringe* expression in three dimensions. The appearance of *Lunatic fringe* expression first at the lingual side of the enamel knot was evident, as well as the subsequent extension of expression along the buccal side from mesial-to-distal direction eventually surrounding the enamel knot at cap stage (Figs. 1I and 1J). This pattern corresponds exactly to the formation of the enamel knot between late bud and early cap stages as earlier visualized by three-dimensional reconstructions of enamel knot markers (Vaahtokari *et al.*, 1996; Jernvall *et al.*, 1998). Here, the enamel knot was visualized by *in situ* hybridization with *Wnt10a* probe (Fig. 1K). Based on the expression patterns, we propose that Lunatic fringe may

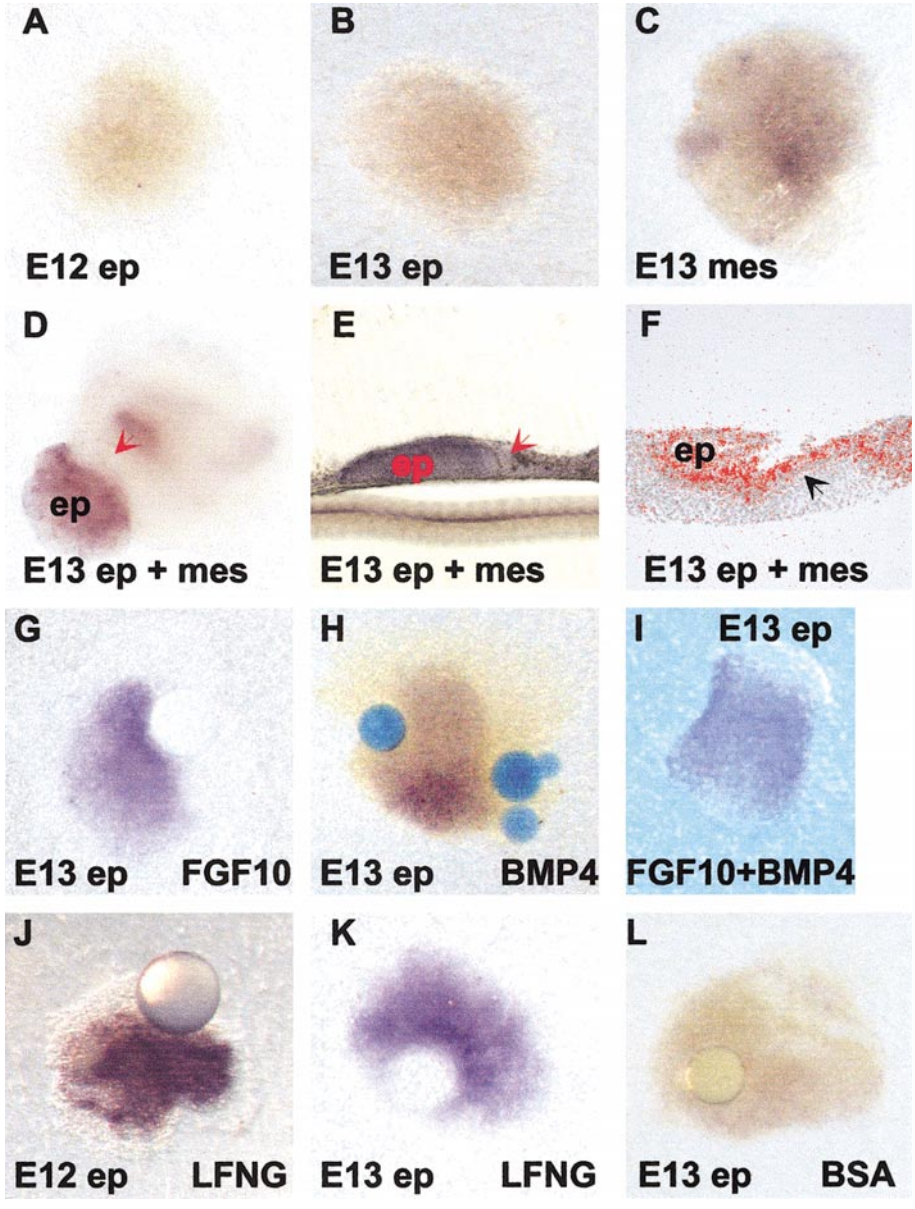


FIG. 5. Expression of *HES1* in the dental tissue explants. Epithelium at E12 or E13 is negative when cultured in isolation (A, B) and only faint *HES1* expression is seen in dental mesenchyme cultured in isolation (C). Mesenchymal signals stimulate *HES1* expression in the dental epithelium (D–F). The epithelial cells next to the basal lamina (arrow in D–F) appear negative in whole-mount *in situ* stained tissue (D), in vibratome section of the whole-mount *in situ* stained tissue (E), and in radioactive *in situ* hybridization of a paraffin section of the recombinant tissue (F). Both FGF10- and BMP4-soaked beads stimulate *HES1* expression in isolated epithelium (G–I). Also Lunatic fringe protein stimulates *HES1* expression in isolated dental epithelium (J, K). BSA-soaked bead has no effect on the expression of *HES1* in cultured epithelium (L).

play a role in the creation of tissue boundaries here in an analogous manner to its functions in *Drosophila* signaling centers (Kim *et al.*, 1995). *Radical fringe* was not detected in the dental tissues (Fig. 1L). *Manic fringe* was intensely expressed in patches within the mesenchymal tissue, which were identified as blood vessels (Fig. 1M).

Notch Receptors

Notch1 and *Notch2* showed similar although not identical expression patterns (Figs. 2A–2F), whereas *Notch3* transcripts were not detected at these early stages of tooth development (data not shown). *Notch1* and *Notch2* were

both expressed in the stellate reticulum cells of the dental epithelium, but the surrounding basal epithelial cell layer was devoid of expression (Figs. 2A–2F). At E14, *Notch1* was expressed throughout the stellate reticulum and overlapped the enamel knot region (Fig. 2C), whereas *Notch2* expression continued only in the oral half of the stellate reticulum and was weak or absent in the enamel knot and cervical loop compartments (Fig. 2F). *Notch1* and *Notch2* appeared to be absent from the dental mesenchymal cells, but *Notch1* was expressed in a patchy pattern around the tooth germ, presumably in blood vessels.

Notch Ligands

Of the Notch ligands, *Jagged1* was intensely expressed and showed developmentally regulated patterns (Figs. 2G–2I), whereas *Jagged2* expression was quite weak and appeared to be restricted to epithelial tissue (Figs. 2J–2L). *Jagged2* transcripts were seen in the oral epithelium already at E11 (data not shown). *Delta1* expression was not detected at these developmental stages of the tooth (data not shown). *Jagged1* was expressed in the stellate reticulum cells of the epithelium, and its expression domain overlapped with *Notch* expression. Expression was specifically intense in the region of the dental lamina connecting the tooth germ to the oral epithelium, and at late bud stage (E13.5), it was transiently expressed in the forming enamel knot (Fig. 2H). Like *Notch1* and *Notch2*, *Jagged1* was absent from the basal epithelial cells. Unlike *Notch* genes, *Jagged1* was intensely expressed in the dental mesenchyme. At early bud stage (E13; Fig. 2G), it was seen throughout the condensed dental mesenchyme, and at late bud stage (Fig. 2H), it was downregulated in the mesenchyme closest to the epithelial bud forming the dental papilla. However, cells in the periphery of the dental mesenchyme forming the dental follicle that may present dental mesenchymal stem cells maintained *Jagged1* expression. The dental papilla cells continued to be *Jagged1*-negative at cap stage.

HES1 and HES5

Of the Notch target genes, the expression of *HES1* and *HES5* was analyzed. *HES5* expression was not detected in epithelium and mesenchyme during the bud and cap stages (data not shown). *HES1*, in contrast, showed intense and developmentally regulated expression. It was largely coexpressed with *Notch1* and *Notch2* in the epithelium. Expression was intense in oral epithelium and in the stellate reticulum, whereas the basal epithelial cells of the tooth bud were mostly negative (Figs. 2M–2O). The enamel knot appeared mostly negative for *HES1* expression at bud stage and cap stage. Interestingly, in the mesenchyme, which was negative for *Notch*, *HES1* was coexpressed with *Jagged1*. It was intensely expressed in the condensed mesenchyme at early bud stage, and at late bud and cap stages, it was restricted to the peripheral mesenchyme forming the dental follicle (Figs. 2N and 2O).

Mesenchyme Is Required for the Expression of Lunatic fringe in Dental Epithelium

It has been shown that mesenchymal signals are not needed for the maintenance of *Notch* expression in stellate reticulum cells, but that the downregulation of *Notch* in the basal epithelial cells depends on mesenchymal signals (Mitsiadis *et al.*, 1995). We wanted to analyze whether Lunatic fringe was regulated by epithelial–mesenchymal interactions and whether it could be involved in enamel knot formation. Isolated dental epithelia from E12 and E13 (early and late bud stage) tooth germs were cultured with and without dental mesenchyme for 24 h, and *Lunatic fringe* expression was monitored with *in situ* hybridization analysis of whole mounts and tissue sections. *Lunatic fringe* was never detected in epithelia cultured in isolation (Fig. 3A), suggesting that the expression is induced and maintained by mesenchymal signals at these stages. In E13 recombinants, the mesenchyme induced *Lunatic fringe* in restricted regions of epithelium next to the mesenchyme (Figs. 3B and 3C, whole-mount *in situ*; and Figs. 3D and 3E, radioactive *in situ* on sections from cultured dental explants). *Lunatic fringe* could never be induced in the epithelium of E12 or younger tissue recombinants (data not shown).

Lunatic fringe Expression Is Induced by FGF4 and FGF10 in Cultured Dental Epithelium

The tissue recombination studies indicated that the mesenchyme is required for *Lunatic fringe* expression in epithelium, and the *in vivo* expression analysis showed that *Lunatic fringe* expression appears in cells flanking the enamel knot. We decided to analyze the function of several candidate signal molecules in the regulation of *Lunatic fringe* expression. Our recent data indicate that *FGF10* is expressed in the dental mesenchyme and that it stimulates epithelial proliferation (Kettunen *et al.*, 2000). We have also shown that FGF-10 protein stimulates *Lunatic fringe* expression in the cervical loop of epithelium of postnatal incisors (Harada *et al.*, 1999). On the other hand, the forming enamel knot expresses several signal molecules, including FGF4, that stimulate proliferation of cells in both dental epithelium and mesenchyme (Jernvall *et al.*, 1994). When E13 dental epithelium was cultured with beads soaked in FGF4 or FGF10, the expression of *Lunatic fringe* was induced around the bead (Figs. 4B and 4C). However, FGF-soaked beads did not induce *Lunatic fringe* in E12 dental epithelium (data not shown). Together with the epithelium–mesenchyme recombinant experiments, this suggests that the dental epithelium is not yet competent to express *Lunatic fringe* at E12.

BMP4 Inhibits the FGF10-Induced Expression of Lunatic fringe

Evidence from tissue cultures and mutant mice indicates that mesenchymal BMPs are required for the formation of

TABLE 1

Regulation of *Lunatic fringe* and HES1 Expression by FGF4, FGF10, BMP4, and *HES1* Expression by Lunatic fringe in the Cultured E12 and E13 Dental Epithelia

	Lunatic fringe induction		Hes-1 induction	
Protein bead	Positive (*n*)	Negative (*n*)	Positive (*n*)	Negative (*n*)
FGF4 (E12)	0	13	na	na
FGF4 (E13)	21	0	na	na
FGF10	24	2	7	1
BMP4	0	17	2	0
Lunatic fringe	na	na	19	5
BSA control	0	20	0	7

Note. n, number of samples; na, not analyzed.

the enamel knot (Bei *et al.*, 2000; Jernvall and Thesleff, 2000). In explant cultures of isolated dental epithelium, BMP4 induces enamel knot markers, including *p21* and *Msx2* (Jernvall *et al.*, 1998). We wondered whether BMPs would regulate *Lunatic fringe* expression as well. BMP2 and BMP4 beads did not induce *Lunatic fringe* expression in E12 and E13 dental epithelia (Fig. 4D). Instead, BMP4 beads inhibited the induction of *Lunatic fringe* by FGF4 and FGF10 beads in E13 epithelia (Fig. 4E). This inhibitory effect depended on the positioning of the beads. When the beads were placed near the FGF bead, the inhibition was complete, whereas when the beads were placed at opposite sides of the explant, no inhibition was seen (Fig. 4F). *Lunatic fringe* expression was never observed near the BMP4 bead. In conclusion, BMP4 appeared to prevent FGF10- and FGF4-induced *Lunatic fringe* expression in dental epithelium in *in vitro* cultures.

HES1 Expression in Cultured Dental Epithelium Requires Mesenchyme and Is Stimulated by Both FGF10 and BMP4

HES1 was downregulated in dental epithelium when cultured in isolation, similar to *Lunatic fringe* (Figs. 5A and 5B). In the presence of mesenchyme, *HES1* expression was stimulated in the epithelium of both E12 and E13 recombinants, unlike *Lunatic fringe*, which was only upregulated at E13. A closer look at the epithelial–mesenchymal interface revealed that expression was not induced in the epithelial cells directly facing the mesenchyme, i.e., the basal epithelial cells, which *in vivo* do not express *HES1* (Figs. 5D–5F). This is consistent with the earlier report that *Notch1* and *Jagged1* expression in dental recombinant explants is downregulated by the mesenchyme in the basal epithelial cells (Mitsiadis *et al.*, 1995, 1997). *HES1* expression was also detected in parts of the mesenchyme when cultured in isolation and with epithelium (Figs. 5C and 5D). These results indicated that, like *Lunatic fringe*, *HES1* expression in epithelium requires mesenchymal signals and that the dental epithelium is capable of expressing *HES1* earlier than *Lunatic fringe*. This is in line with the *in vivo* situation where *HES1* expression precedes that of *Lunatic fringe*.

HES1 expression was induced by FGF10-soaked beads in both E12 and E13 dental epithelia (data not shown; and Fig. 5G). FGF10 thus mimicked the effect of the mesenchyme on epithelial *HES1* expression. The induction of *HES1* expression by FGF10 was not disturbed by the application of a BMP4-containing bead (Fig. 5I). In contrast, BMP4 beads were capable of inducing *HES1* expression (Figs. 5H and 5I).

Lunatic Fringe Protein Induces HES1 Expression in Cultured Dental Epithelium

Fringe molecules modulate Notch signaling, but it is still unclear whether they are secreted from cells and can affect cells extracellularly. Furthermore, the effect on Notch pathway may be stimulatory or inhibitory, depending on the cellular context (Wu and Rao, 1999). In order to shed more light on these questions, we analyzed the effects of Fringe protein on dental epithelium. Lunatic fringe is suggested to be produced as an inactive protein precursor and processed to a mature form (Johnston *et al.*, 1997). We produced an affinity-purified recombinant mature form of rat Lunatic fringe protein, which was applied with either agarose or heparin acrylic beads on isolated E12 and E13 dental epithelia. The *in situ* hybridization analysis after 24 h showed that *HES1* expression was induced around the beads at both stages (Figs. 5H and 5I). This showed that the recombinant Lunatic fringe protein had extracellular effects and that it stimulated the Notch signaling pathway.

Tooth Morphogenesis Is Not Altered in the Lunatic fringe Mutant Mice

Lunatic fringe null mutant mice have defects in somitogenesis (Zhang and Gridley, 1998; Evrard *et al.*, 1998). We studied the tooth phenotype of the *Lunatic fringe* heterozygous and homozygous mutant mice at E14 and E17. We prepared serial paraffin sections and studied the histological appearance of the lower molar tooth germs. The sizes of the mutant tooth germs did not differ from those of the heterozygotes and no morphological differences were observed in the shapes of the tooth germs or in tissue histology. Also, the enamel knots appeared normal both in morphology and gene expression as shown by *Wnt10a* expression in mutants (data not shown). No alterations were observed in the expression patterns of genes of the Notch pathway, including *Jagged1*, *Notch1*, *Notch2*, *Notch3*, *Radical fringe*, and *Manic fringe*, analyzed by *in situ* hybridization analysis between mutant and wild type embryos. Particular attention was paid to the expression of *Manic fringe* (Fig. 6A) and *Radical fringe* (Fig. 6B), but neither of these was upregulated or misexpressed.

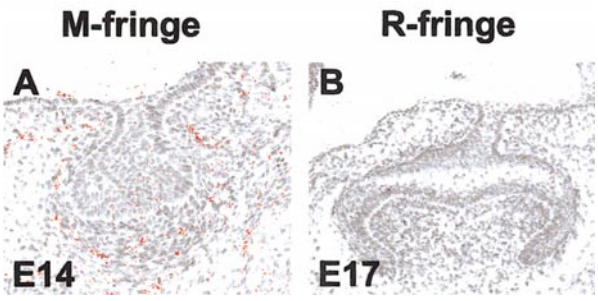


FIG. 6. Tissue sections of the Lunatic fringe knockout embryonic teeth indicate normal tooth development and do not show increased levels of *Manic fringe* (A) or *Radical fringe* (B) expression.

DISCUSSION

Signaling or organizing centers often form at tissue boundaries and *Fringe* genes are known to play an important role in establishing these boundaries both during *Drosophila* and vertebrate development (Wu and Rao, 1999). During tooth development, the enamel knot represents the signaling center. This epithelial structure expresses more than 10 signaling molecules and has been implicated in the regulation of tooth shape and in patterning of the tooth cusps (Jernvall and Thesleff, 2000; Pispa *et al.*, 1999; Dassule *et al.*, 2000). The enamel knots form at the tips of tooth buds as epithelial ridges between the buccal and lingual aspects of teeth, and they resemble morphologically the AER in limbs.

We propose that *Lunatic fringe* plays a role in the initiation and maintenance of the compartment boundary between enamel knot and the remainder of the dental epithelium, similar to the situation in other signaling centers. Both initiation and maintenance could be regulated by similar mechanisms involving Notch signaling. We reported here a striking association between the edge of *Lunatic fringe* expression and the enamel knot boundary. The *Lunatic fringe* expression domain flanked the forming enamel knot first from the lingual aspect at E13.5 and subsequently spread to the buccal side continuing in distal direction closely accompanying the growth of the enamel knot. This expression formed a sharp boundary with the enamel knot, which was completely negative. At the cap stage, when the enamel knot reaches its final extent, *Lunatic fringe*-expressing cells enclosed the enamel knot completely. We propose that the initiation and prolongation of this *Fringe* boundary is established by a set of complex interactions between mesenchyme and epithelium.

Fringes act through the modulation of the Notch receptor, resulting in a change of Notch activity. Areas with high Notch activity have been shown to regulate boundary formation (de Celis *et al.*, 1996). As we have shown, *Notch* itself is expressed in rather broad domains and lacks a sharp boundary with the enamel knot. *Notch* is differently regulated than *Fringe* and *HES1* since its expression does not require mesenchyme. We tried to ascertain whether the edge of *Lunatic fringe* expression corresponded with high Notch activity. *HES1* has been reported as a marker for Notch activity (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). *HES1* expression did not correspond to the boundaries set by *Lunatic fringe* expression. It was clear, however, that at E13–E14 it was expressed at higher mRNA levels at the lingual side compared to the buccal corresponding to the higher *Lunatic fringe* levels at the lingual side.

We have shown with tissue explants and bead experiments that *Lunatic fringe* and *HES1* were downregulated in the dental epithelium in the absence of mesenchyme, indicating the requirement of a mesenchymal signal. The effects of the mesenchyme on dental epithelium could be mimicked with beads containing FGF and BMP. *Lunatic fringe* expression was induced by FGF10 and FGF4 beads. *FGF10* is normally expressed in the mesenchyme and *FGF4* in the epithelial enamel knot. The induction of *Fringe* was

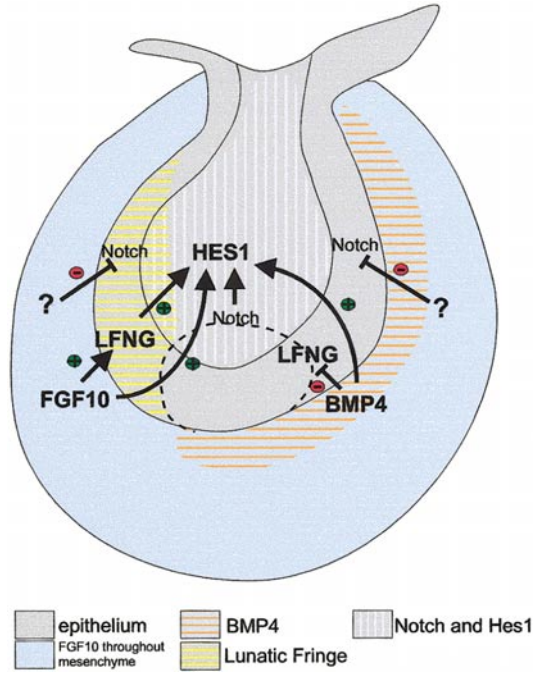


FIG. 7. Schematic view of the regulation of *Lunatic fringe*, *Notch1*, and *HES1* in the dental epithelium during advanced bud stage of tooth development. At the buccal side, mesenchymal BMP4 prevents the induction of *Lunatic fringe* expression by FGF10, restricting *Lunatic fringe* expression to the lingual side. *Lunatic fringe* expression forms a sharp boundary with the enamel knot and is expressed in the basal epithelium and partly in the stellate reticulum. It upregulates *HES1* where it overlaps with the expression domain of *Notch*. *HES1* is also upregulated by FGF-10 and BMP-4 signals from the mesenchyme.

counteracted by adding a BMP4 bead. Similar interactions between BMPs and FGFs have been commonly noted in developmental processes, and in teeth, the genes in which upregulation by FGF are counteracted by BMPs include *Pax9*, *Pitx1*, and *Pitx2* (Neubuser *et al.*, 1997; St. Amand *et al.*, 2000). The *Lunatic fringe* expression domain partly overlaps that of *Notch* and *HES1*, which are similar in dental epithelium.

We also addressed the question of whether Lunatic fringe could act extracellularly. Fringe has a sequence similarity with glycosyl transferases and is shown to act intracellularly by glycosylating the extracellular EGF domains of Notch (Moloney *et al.*, 2000; Bruckner *et al.*, 2000; Munro and Freeman, 2000). However, in our *in situ* hybridization analysis, the expression domain of *Lunatic fringe* includes the outer enamel epithelium cells facing the basal membrane without *Notch* expression (Figs. 1 and 2). Here, we showed that a source of extracellular Lunatic fringe protein in the form of beads affected the Notch pathway in explants of dental epithelium *in vitro*. Whole-mount *in situ* hybridization analysis showed induction of *HES-1* in the epithelium surrounding these beads. Another set of results shows that extracellular Radical fringe protein downregulates *HES1* in neuronal cells (Mikami *et al.*, 2001). It is known that Fringe molecules contain a signal sequence for secretion and therefore can stay in the Golgi or can be secreted out from the cell or both (Irvine and Wieschaus, 1994). It is also known that Lunatic fringe is processed from a precursor to an active form (Wu *et al.*, 1996; Johnston *et al.*, 1997). This active form seems to be able to function outside the cell in our culture experiments. A non-cell-autonomous function for Fringe is also supported by the observation that *Lunatic fringe* and *Notch* expression domains were mainly complementary in epithelium, overlapping only in a few cells at the border of basal epithelium and stellate reticulum. We propose that *in vivo* Fringe enhances the Notch signaling pathway on the lingual side adjacent to the basal epithelium. We have also shown that FGF4, FGF10, and interestingly also BMP4 could upregulate *HES1*. The upregulation of *HES1* could therefore be dependent on at least two different pathways (Fig. 7). In one pathway, Lunatic Fringe is induced by FGFs and upregulates *HES1*. However, BMP4 inhibits this induction by FGF. In the other pathway, *HES1* is upregulated by FGFs and BMP4 independent of Lunatic fringe.

We therefore propose a model in which, during the initial phase of boundary formation of the enamel knot at E13.5, FGFs upregulate the expression of *Lunatic fringe* throughout the epithelium of the tooth bud (Fig. 7). FGF receptors 1b, 1c, and 2b are present in most of the epithelium, excluding the enamel knot (Kettunen *et al.*, 1998), and will therefore be able to transduce both signals. However, expression of mesenchymal BMP4 at the buccal side prevents the induction of *Lunatic fringe* expression in the buccal epithelium, limiting the *Lunatic fringe* expression domain to the lingual side. Interestingly, at E13.5, *Jagged1* showed a transient expression in the forming enamel knot at E13.5. This suggests a possible change in cell identity or differentiation, representing a change in the maturation of the enamel knot. At E14, the *Lunatic fringe* expression has completely enveloped the enamel knot, forming a sharp boundary with this structure. The *Fringe* expression domain is probably extended around the enamel knot by employing mesenchymal–epithelial interactions similar to the initiation phase involving BMP4, FGF4, and FGF10. The contact area of *Lunatic fringe* and *Notch* expression could also represent an important site for the regulation of epithelial stem cells as earlier suggested in the cervical loop of the continuously growing incisor (Harada *et al.*, 1999). This area could be responsible for the recruitment of stem cells from the stellate reticulum and their differentiation into transit-amplifying cells, resulting in the formation and extension of the cervical loop by means of cell proliferation. Interestingly, the lingual cervical loop develops faster than the buccal one, which corresponds with the earlier expression of *Lunatic fringe* at the lingual side.

One unexpected finding was that the *Lunatic fringe* mutant mice did not show any obvious abnormalities in tooth morphogenesis, in the formation of the enamel knots or in cusp patterning. This was not due to compensation by other *Fringe* genes since *Radical fringe* and *Manic fringe* were not differently expressed in wild type or mutant teeth. Hence, it is obvious that there are additional molecular mechanisms regulating the formation of tissue boundaries during the formation of enamel knot. Recently, it was reported that in developing inner ear *Lunatic fringe* has a developmental role, which was only revealed in a mouse double mutant strain with *Lunatic fringe* and *Jagged2* null mutation. Nonsensory supporting cells of the cochlea normally expressing *Lunatic fringe* at E16–E18 (Morsli *et al.*, 1998) were normal as well as the hair cell number and patterning in the *Lunatic fringe* null mutant, but interestingly, in double mutant animals, the null mutation of *Lunatic fringe* gene could partially suppress the phenotypic effect of *Jagged2* null mutation in cochlea (Zhang *et al.*, 2000).

In summary, we have shown that the expression patterns of the Notch pathway genes are dynamic during the early events of tooth morphogenesis and that they are regulated by epithelial–mesenchymal interactions. The mesenchymal signals FGF and BMP have antagonistic effects on the expression of epithelial *Lunatic fringe*, which is seen at boundaries of epithelial compartments, suggesting roles in regulation of stem cell development and in formation of the signaling center at the enamel knot.

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