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Original Article

Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: non-dividing cells express growth stimulating *Fgf-4* gene

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ABSTRACT The main morphological features of the mammalian tooth crown are cusps, but the developmental mechanisms that cause the formation of cusps are unknown. Tooth cusp formation commences at cap-stage with the appearance of the enamel knot, which is a cluster of non-dividing epithelial cells. In this study, enamel knot was first seen in embryonic mice molar teeth at the onset of cap-stage. Later in tooth development, secondary enamel knot structures were observed at the cusp tips and their appearance corresponded to the formation of individual cusp morphology. Comparisons of the pattern of cell proliferation in embryonic mouse molars and the expression of fibroblast growth factor-4 *(Fgf-4)* gene revealed that expression of *Fgf-4* mRNA is strictly localized to the non-dividing cells of the enamel knot. However, when FGF-4 protein was introduced onto isolated dental tissues *in vitro*, it stimulated the proliferation of both dental epithelial and mesenchymal cells. Based on these results, we suggest that the enamel knot may control tooth morphogenesis by concurrently stimulating cusp growth (via FGF-4 synthesis) and by directing folding of cusp slopes (by not proliferating itself).

KEY WORDS: *tooth development, enamel knot, cell proliferation, Fgf-4*

Introduction

Mammalian teeth are morphologically diverse and the evolutionary history of mammals is best documented by their teeth. While dental morphology is closely determined genetically (Butler, 1983), the epigenetic control mechanisms of dental morphology are unknown. During tooth development, the characteristic crown morphologies of mammalian teeth are produced by folding of the inner enamel epithelial-mesenchymal interface and growth of the dental papilla (Butler, 1956; Ruch, 1987). This commences with the appearance of the enamel knot, which is a morphologically distinct population of epithelial cells in the center of the tooth germ, facing the dental papilla mesenchyme (Butler, 1956). Cells from the inner enamel epithelium are included into the enamel knot and they are densely packed and non-dividing. The enamel knot has been suggested to function as a reservoir of cells for the fast growing enamel organ, and also to direct folding of the epithelial-mesenchymal interface (see Butler, 1956 for a review). However, it has only been described in association with the primary cusp and has not been suggested to have a role in the formation of other cusps later in tooth development.

In the present study we monitored cell proliferation by immunohistological detection of S-phase cells with incorporated 5-bromo-2'-deoxyuridine (BrdU) and analyzed the expression pattern of *Fgf-4* by *in situ* hybridization from serial sections of mouse molars and incisors from embryonic day 13 onwards until two days postnatally. First isolated from tumor tissues (Delli-Bovi *et al.*, 1987; Taira *et al.*, 1987) *Fgf-4* (also known as *hst* or *K-fgf*) encodes a protein (FGF-4) of the fibroblast growth factor family which, like other members of the FGF-family, is a potent mitogen for both epithelial and mesenchymal cells (Rifkin and Moscatelli, 1989; Niswander and Martin, 1993a). In the developing embryo, the *Fgf-4* gene is expressed during pre- and early postimplantation stages, and later in several tissues including branchial arches, limb buds and tooth epithelium (Niswander and Martin, 1992; Drucker and Goldfarb, 1993). To investigate the role of *Fgf-4* in tooth morphogenesis, we carried out a set of *in vitro* experiments in which the effects of recombinant FGF-4 protein on dental cell proliferation were analyzed. The result was an apparent paradox: why does a cluster of cells that does not proliferate (the enamel knot) express a gene which makes cells proliferate? We suggest

Abbreviations used in this paper: BrdU, 5-bromo-2'-deoxyuridine; *Fgf-4*, fibroblast growth factor-4 gene and transcript; FGF-4, fibroblast growth factor-4 protein; BSA, bovine serum albumin.

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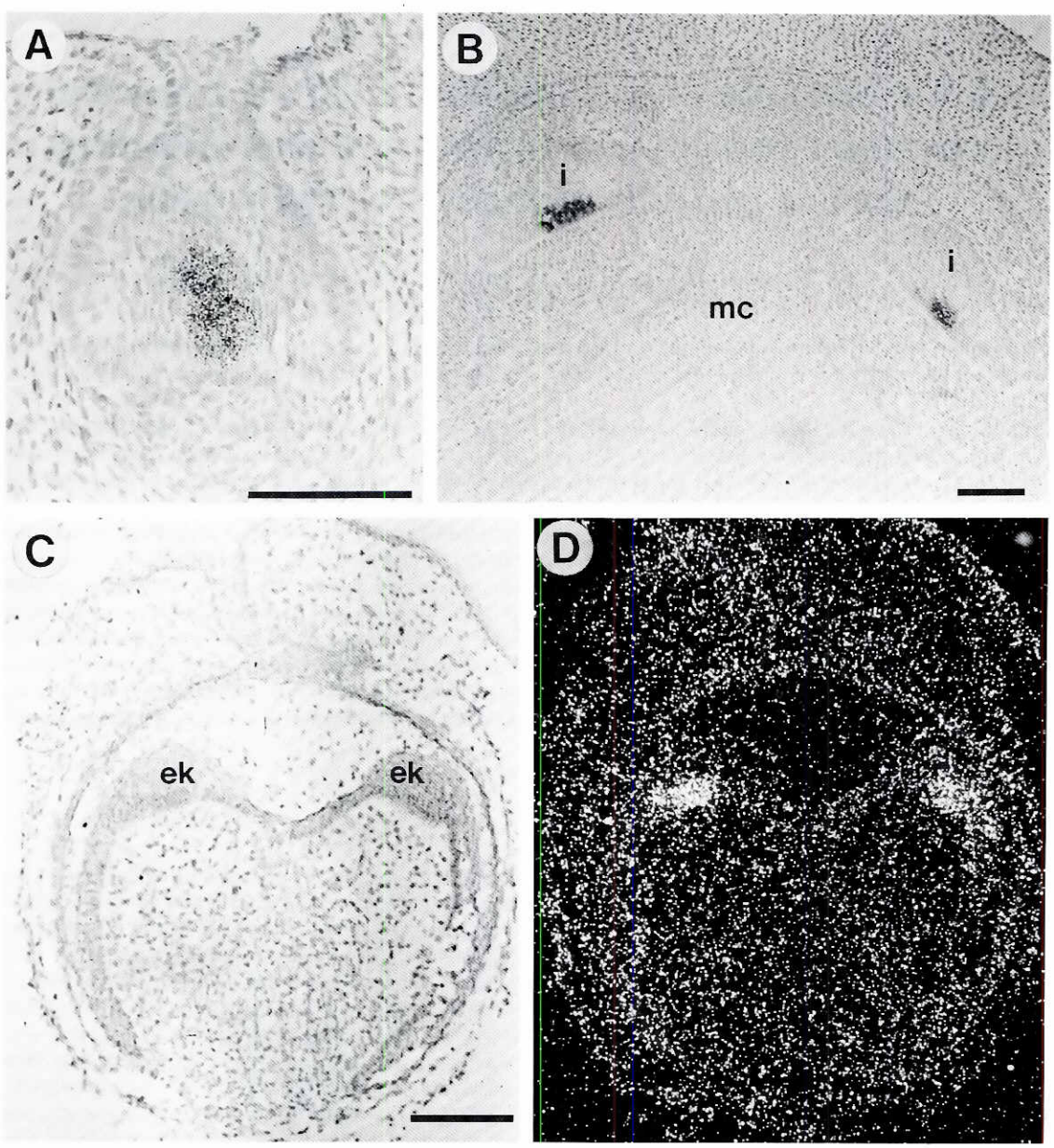

Fig. 1. *Fgf-4* expression in the cells of the enamel knot. **(A)** *Day-14 mouse embryonic lower molar (transverse section).* **(B)** *Day-14 mouse embryonic lower incisors (i), Meckel's cartilage (mc), (transverse section).* **(C)** *Day-18 mouse embryonic lower molar, distal cusps (hypoconid and entoconid) with secondary enamel knots (ek), (transverse section).* **(D)** *Dark field illustration of the section in* **(C)** *showing Fgf-4 expression in the secondary enamel knots. Scale bars, 100 μm.*

that the enamel knot may simultaneously stimulate and direct the growth of cusps in mammalian teeth and thus act as a control center during tooth morphogenesis.

Results

The first signs of ceasing proliferation were observed in dental epithelium during late bud-stage (day 13) at the tip of the bud as the epithelium started to broaden bucco-lingually (not shown). This marks the beginning of cap-stage where the enamel knot starts to form. No transcripts of *Fgf-4* were detected at this stage. By day 14 the first molar had reached cap stage. The epithelium had started to grow to encompass the mesenchymal dental papilla and the enamel knot had formed (Figs. 1A, 2A). The enamel knot is marked both buccally and lingually by enamel grooves (Butler, 1956) which, when observed from above, form two ridges parallel to the tooth germ's mesio-distal axis (Fig. 3A). Essentially, no incorporation of BrdU was detected in cells of the enamel knot (Figs. 2A, 3B), whereas the surrounding buccal and lingual processes showed intense incorporation and apparently grew to surround the

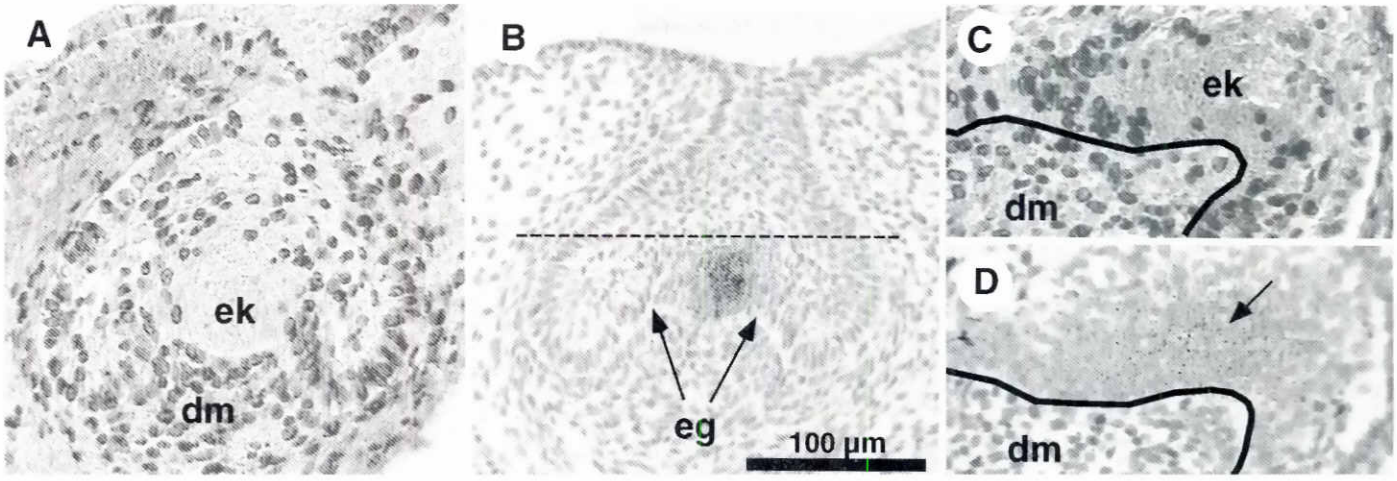

Fig. 2. Cell proliferation and *Fgf-4* expression in bucco-lingual (transverse) sections of day-14 cap-stage (A,B) and day-16 bell-stage (C,D) first lower molars. (A) *Cell proliferation has ceased in the enamel knot (ek) whereas it is intense in the surrounding tooth epithelium. Dental mesenchyme (dm) is proliferating strongly.* **(B)** *Fgf-4 expression is observed only inside the enamel knot. Enamel grooves (eg) mark the extent of the enamel knot. The dashed line represents the plane of exposure for the digitized tooth germ in Fig. 3.* **(C)** *Secondary enamel knot surrounded by intensely proliferating cells. The histology of the secondary enamel knots is identical to the primary enamel knot.* **(D)** *Fgf-4 expression is co-localized with the secondary enamel knot (arrow). The amount of transcripts is decreased but still detectable in a bright-field image. Black lines in* **C** *and* **D** *represent the basement membrane.*

mesenchymal dental papilla. The dental mesenchyme surrounded by epithelial outgrowths was also proliferating intensely (Figs. 2A, 3D). In contrast, expression of *Fgf-4* was detected only in the enamel knot where the signal of expression was very strong and sharply delineated (Figs. 1A, 2B). Also day-14 mouse incisors had similar *Fgf-4* expression in their enamel knots (Fig. 1B).

The close association of *Fgf-4* expression and lack of cell proliferation in the enamel knot was even more evident in a three dimensional reconstruction (Fig. 3B,C), although the match was not entirely complete. Based on morphology, the extent of enamel knot matched exactly with the area of the non-proliferative cells, but the area where *Fgf-4* transcripts were detected was slightly smaller (up to 5 cells in every direction). This finding, together with the observation that during the bud-stage (day 13) *Fgf-4* transcripts were not detected in the first cells, which ceased to incorporate BrdU, suggests that the cessation of cell proliferation in the enamel knot precedes the start of *Fgf-4* expression.

From cap-stage onwards, the non-proliferative areas (called here secondary enamel knots), and also *Fgf-4* transcripts, were transiently present in the epithelial cells at the future cusp tip regions. The amount of transcripts appeared to decrease, but their co-localization with the secondary enamel knots remained consistent (Figs. 1C,D, 2C,D, 3E,F). In Fig. 3F expression of *Fgf-4* is evident in the developing mesial buccal and lingual cusps in a 16-day embryonic first molar. Mesenchyme continued to proliferate intensely (not shown). *Fgf-4* expression was quite transient in the inner enamel epithelial cells, disappearing (with the secondary enamel knots) prior to their differentiation into enamel-forming ameloblasts. As *Fgf-4* expression was absent from other areas of inner enamel epithelium that differentiate into ameloblasts, *Fgf-4* appears not to be directly associated with ameloblast cell differentiation.

The effects of FGF-4 protein on dental cell proliferation were analyzed by introducing recombinant FGF-4 locally to tissues by heparin-acrylic beads. The beads were placed in contact with day-13.5 isolated tooth epithelium or mesenchyme and cultured for 24 h. In mesenchyme the FGF-4 beads caused the formation of a translucent area around the beads and the proliferation of cells was stimulated in this area (Fig. 4A, B is the control). A similar translucent area is induced in early dental mesenchyme by the epithelium (Vainio *et al.*, 1989) and also by beads releasing bone morphogenetic protein (Vainio *et al.*, 1993). In epithelium cultured in isolation, FGF-4 beads had a similar but weaker effect (Fig. 4C,D is the control), which could be due to the generally slow rate of proliferation when epithelium is cultured in isolation.

In a second set of experiments epithelial and mesenchymal tissue components were cultured in isolation, and 100 ng/ml FGF-4 was added to the culture medium. ^{3}H-thymidine incorporation into both mesenchymal and epithelial cells increased by about three-fold (Fig. 4E). These results suggest that, *in vivo*, FGF-4 may have a growth stimulating effect on both dental mesenchyme and epithelium.

Discussion

Our observation that *Fgf-4*, a potent mitogen, is expressed by cells that do not themselves proliferate is striking. Since exogenous FGF-4 increased proliferation in dental epithelium (Fig. 4), at least some epithelial cells apparently have cell surface receptors that transmit the signal. The FGF-receptor (FGFR) family consists at present of four members (Partanen *et al.*, 1992) of which FGFR-1 and FGFR-2 are known to be expressed in the developing tooth at the stages examined here, but no apparent regional differences have been detected that would explain the lack of responsiveness of the enamel knot cells to FGF-4 (Orr-Urtreger *et al.*, 1991; our unpublished observations). It is possible that enamel knot cells lack some necessary non-signaling receptors such as proteoglycans that may be needed for interaction of FGF-4 with the signaling receptor (Guimond *et al.*, 1993). It is also possible that the withdrawal of the enamel knot cells from the cell cycle involves nuclear or other unknown mechanism which prevents the response to FGF-4.

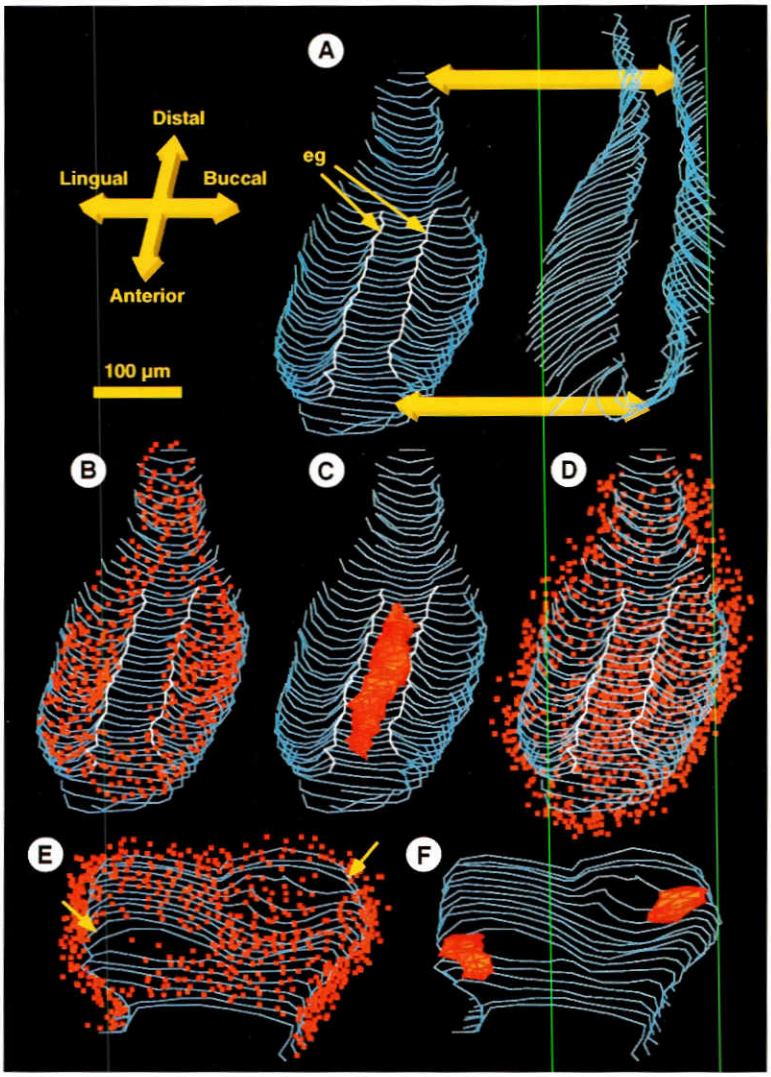

Fig. 3. Three-dimensional reconstructions of day-14, (A-D) and day-16, (E,F) tooth germs showing distributions of BrdU incorporating cells and *Fgf-4* mRNA. *An oblique view plane is used better to show the morphology of epithelial-mesenchymal interface (blue); teeth are rotated 57° (counterclockwise) about the x-axis and 22° (clockwise) about the y-axis. The anterior end is towards the observer.* **(A)** *Lower portion (on the left) is separated from upper portion (on the right) exposing the enamel knot area (on the left). Enamel grooves (eg) are about 230 µm long and they mark the extent of the enamel knot.* **(B)** *The proliferation of epithelial cells is strongest around enamel grooves, but the area of enamel knot is negative.* **(C)** *Fgf-4 expression is restricted to a torpedo-shaped area between enamel grooves inside the enamel knot.* **(D)** *Dental mesenchyme is proliferating strongly underneath the enamel knot.* **(E)** *Anterior part of day-16 tooth germ's inner enamel epithelium (cell proliferation is mapped ≤40 µm from the basement membrane, hidden lines removed) is shown where the proliferation of epithelial cells is prevalent except for two areas that correspond to the future two anterior cusps (arrows).* **(F)** *Fgf-4 is expressed in these non-proliferative areas.*

In limb buds, *Fgf-4* is expressed by the apical ectodermal ridge. It has recently been suggested that FGF-4 plays an important role in limb outgrowth by stimulating mesenchymal cell proliferation (Niswander and Martin, 1993a; Niswander *et al.*, 1993). Our results indicate that, in addition to stimulating mesenchymal cell division, FGF-4, diffusing from the enamel knot, may also enhance proliferation of adjacent epithelial cells. This could provide a mechanism whereby the enamel knot controls the formation of tooth cusps: it may simultaneously stimulate cusp growth (via FGF-4 synthesis) and direct folding of the inner enamel epithelial-mesenchymal interface (by not proliferating itself). In this model the unequal distribution of cell proliferation in the inner enamel epithelium causes changes in tooth germ form (Fig. 5). In the case of cap-stage tooth germ, the only direction the growing tooth epithelium can expand is lateral to the enamel knot (Fig. 5). Simultaneously FGF-4 would stimulate growth of both epithelium and adjacent dental mesenchyme. Dental epithelium has been shown to stimulate cell proliferation in dental mesenchyme *in vitro* (Vainio *et al.*, 1989; Vainio and Thesleff, 1992), which is also indirectly supported by the intense proliferation of dental mesenchyme *in vivo* (this study; Figs. 2A, 3D). Furthermore, the proliferating and condensing dental mesenchyme may be partially responsible for keeping the enamel knot area stationary. Thus the non-proliferative area between enamel grooves corresponds to the former tip of the tooth bud.

Interestingly, Fisher (1971) found that developmentally early tooth germs, which are halved and grown *in vitro*, are capable of developing a full cusp configuration. This ability disappears when tooth germs reach the developmental stage corresponding to the appearance of the enamel knot, supporting the suggestion that the «cusp-making program» is switched on with the appearance of the primary enamel knot. Also developmental morphology of very different molars of mouse and vole *(Clethrionomys glareolus)* indicate that the first differences appear in the shape of the primary enamel knot (Jernvall, in preparation)

The transient appearance of secondary enamel knot structures later in development (bell-stage), corresponds to the foldings (and number of cusps) of epithelium that create individual cusps and subsequently make cusp tips angular. This observation is consistent with earlier studies where unequal growth of epithelium is

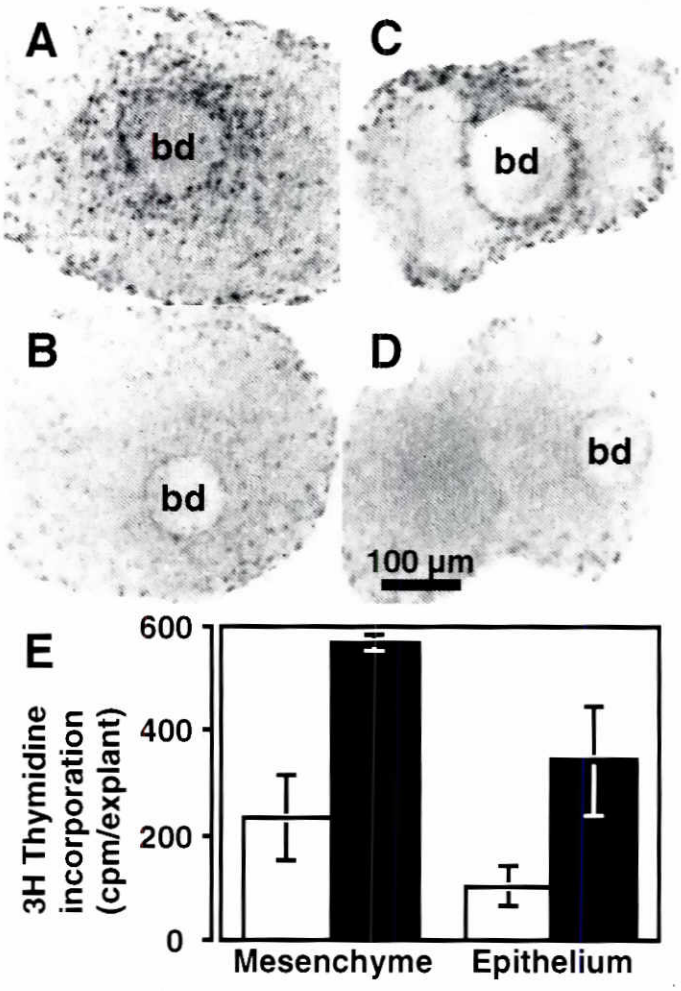

Fig. 4. Stimulation of cell proliferation by FGF-4 in isolated dental mesenchymes and epithelia. (A-D) *The effect of FGF-4 releasing beads on tooth epithelium and mesenchyme in vitro as detected using BrdU incorporation. Whole-mount immunoperoxidase staining.* **(A)** *mesenchyme and* **(C)** *epithelium show increased incorporation of BrdU around beads (bd) whereas no increase is detected around beads incubated in BSA* **(B,D)**. **(E)** *The increase in ^{3}H-thymidine incorporation (as cpm) into the mesenchymes and epithelia cultured in the presence of 100 ng/ml FGF-4 in the culture medium (black bars, white bars medium only). Compared to mesenchyme, the lower level of ^{3}H-thymidine incorporation into epithelium reflects the slower rate of epithelium proliferation when cultured in isolation. The mean cpm/ng of DNA were 0.8 and 2.4 for mesenchyme, and 0.5 and 1.9 for epithelium.*

postulated to create the cusp patterns (Butler, 1956, 1967; Butler and Ramadan, 1962), stressing close similarities with other structures of skin such as avian feathers (Desbiens *et al.*, 1992). It is plausible that the non-proliferative zones of tooth epithelium (and *Fgf-4* expression) are associated with allometric changes in developing tooth shape (Fig. 5). Rather than acting as a general growth stimulus only, the *Fgf-4* expression may be more associated with the control of individual cusp shape. In genetic terms, the control of tooth form would be exercised only transiently in localized areas.

Tooth development is controlled by epithelial-mesenchymal interactions (see Ruch, 1987; Thesleff *et al.*, 1990; Weiss, 1993; Thesleff *et al.*, 1994), and early oral epithelium has been shown to have the potential to induce tooth formation (Mina and Kollar, 1987; Lumsden, 1988). After day 11 in the mouse, this potential transfers to dental mesenchyme, which is capable of instructing the morphogenesis of a complete tooth (Kollar and Baird, 1969; Richman and Kollar, 1986; Mina and Kollar, 1987; Lumsden, 1988). It is important to note that the inductive role of dental mesenchyme does not have to be in any conflict with the role of enamel knot. The dental mesenchyme may induce the activation of enamel knot and the specific tooth-making program (e.g. incisor, molar). The primary enamel knot and *Fgf-4* expression was also present in cap-stage mouse incisor. Available evidence shows that once the tooth epithelium has reached enamel knot stage (day 14 in mouse), the tooth type can not be changed (Kollar and Baird, 1969), which suggests the intriguing possibility that the induction of the enamel knot (and the cusp-making program) is needed only once.

The expression of a number of transcription factors, growth factors and structural molecules have been analyzed in developing teeth (see Thesleff *et al.*, 1992; Weiss, 1993; Thesleff *et al.*, 1994), but so far the extremely restricted pattern of *Fgf-4* expression is unique. The closest correlation with *Fgf-4* expression in teeth has been reported for the homeobox-containing gene *Msx-2*, which is also expressed in the enamel knot and has been proposed to be involved in tooth patterning (MacKenzie *et al.*, 1992; Jowett *et al.*, 1993). It is possible that *Msx-2* is involved in regulating transcription of *Fgf-4* and/or the gene(s) responsible for cessation of proliferation in the enamel knot. *Fgf-4* could also be a factor in signaling positional information in teeth through activating homeobox-containing genes as recently shown for limbs (Niswander and Martin, 1993b).

In conclusion, this work shows that the enamel knot is a dynamic structure the first appearance of which corresponds to the beginning of tooth cusp formation. By remaining non-proliferative while expressing the growth stimulating *Fgf-4* gene, the enamel knot may both direct and stimulate tooth cusp formation.

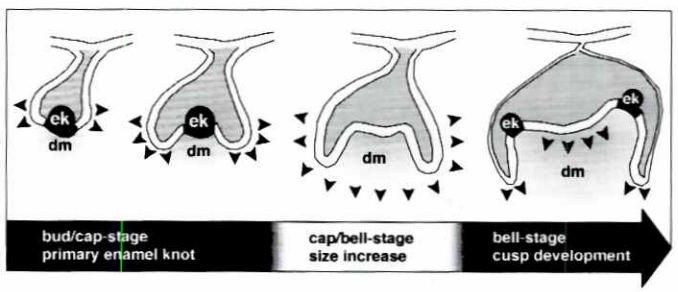

Fig. 5. Generalized illustration (transverse sections) of the possible role of enamel knot in the development of tooth crown form. *Non-proliferative enamel knot (ek) with its adjacent inner enamel epithelium shown black. Arrowheads show the direction of growth. At cap-stage, tooth epithelium grows lateral to the primary enamel knot and around dental mesenchyme (dm). When tooth germ increases in size only (isometric growth) the tooth epithelium proliferates uniformly and no enamel knot is present. The final development of individual cusps in multi-cusped teeth corresponds to the secondary enamel knots at the cusp tips and unequal (allometric) growth of the inner enamel epithelium (epithelium facing the dm). After the development of primary enamel knot, there is an increasing overlap of adjacent developmental stages between different parts of tooth germ.*

Materials and Methods

The age of the embryos of (CBAxC57BL) mice was estimated by morphological criteria (day of appearance of the vaginal plug was day 0). BrdU-labeling and detection: BrdU (Boehringen-Mannheim, 1.5 ml/100 g) was administered to etherized mice (2-3 mice/each day age group), and, after 2 h, they were sacrificed and teeth were dissected, fixed in 70% ethanol at 4°C and embedded in paraffin, serially sectioned at 10 μm. The immunoperoxidase detection of incorporated BrdU was done as described (Vainio *et al.*, 1991). The experiments were in accordance with the guidelines of the Animal Welfare Committee of University of Helsinki. For *in situ* hybridization (Wilkinson and Green, 1990), the teeth were fixed with 4% paraformaldehyde at 4°C and embedded in paraffin, serially sectioned at 7 μm (Vainio *et al.*, 1991). The Bluescript vector (Stratagene, La Jolla, CA, USA) containing 620 bp *Fgf-4* cDNA (Hébert *et al.*, 1990) was from Dr. C. Basilico (NYU Medical center, NY, USA).

Individual sections were digitized using Hitachi KP-M1U CCD camera and QuickImage framegrabber-board (Data Translation, MA, USA) with NIH-Image 1.5 software. Alignment of adjacent sections was done manually using oral epithelium as landmark. The difference between the shrinkage of *in situ* hybridization and BrdU sections was adjusted using number of epithelial cells/100 μm for calibration. Superimposition of *in situ* and BrdU teeth was based on enamel grooves (day 14) or cusps (day 16). The illustration was prepared with MacSpin 3 (Abacus Concepts, CA, USA) and Presto3D (humanOs technologies, CA, USA). No perspective projection was used.

In vitro experiments

First mandibular molar tooth germs were dissected from day 13.5 mouse embryos (CBAxNMRI) and epithelium and mesenchyme were separated and cultured on Nuclepore filters in Trowell-type organ cultures (Vainio *et al.*, 1993). Heparin-acrylic beads (SIGMA H-5263) were incubated in 5 μg/μl recombinant FGF-4 (human sequence, British Bio technology Products, UK) or in BSA for 30 min in 37°C, washed rapidly and placed in contact with the tissues and cultured for 24 h. Tissues were labeled by BrdU (Boehringen-Mannheim) for 1 h, fixed 5 min in MeOH and overnight in 4% paraformaldehyde 4°C, and BrdU incorporation was detected by whole-mount immunoperoxidase staining (Amersham primary antibody, Vector ABC kit). As positive controls, beads were placed in contact with day-12 limb buds after removing the epithelium. The effect was a substantial outgrowth of limbs in contact with FGF-4 beads (not shown). For ^{3}H-thymidine-assay, separated tooth tissues (5 epithelia or mesenchymes per filter) were cultured in the presence of FGF-4 (100 ng/ml in the medium). After 20 h, tissues were labeled with ^{3}H-thymidine for 4 h (Amersham) and processed for determination of radioactivity (Partanen *et al.*, 1985). Each bar represents the mean of 3 (mesenchyme) or 8 (epithelium) independent assays. Mesenchyme control versus FGF-4, $p<0.05$; epithelium control versus FGF-4, $p<0.001$ (Mann-Whitney *U*-test).

Acknowledgments

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