

Localization of Putative Stem Cells in Dental Epithelium and Their Association with Notch and FGF Signaling

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Abstract. The continuously growing mouse incisor is an excellent model to analyze the mechanisms for stem cell lineage. We designed an organ culture method for the apical end of the incisor and analyzed the epithelial cell lineage by 5-bromo-2'-deoxyuridine and Dil labeling. Our results indicate that stem cells reside in the cervical loop epithelium consisting of a central core of stellate reticulum cells surrounded by a layer of basal epithelial cells, and that they give rise to transit-amplifying progeny differentiating into enamel forming ameloblasts. We identified slowly dividing cells among the *Notch1*-expressing stellate reticulum cells in specific locations near the basal epithelial cells expressing *lunatic fringe*, a secretory molecule modulating Notch signaling. It is known from tissue recombination studies that in the mouse incisor the mesenchyme regulates the

continuous growth of epithelium. Expression of *Fgf-3* and *Fgf-10* were restricted to the mesenchyme underlying the basal epithelial cells and the transit-amplifying cells expressing their receptors *Fgfr1b* and *Fgfr2b*. When FGF-10 protein was applied with beads on the cultured cervical loop epithelium it stimulated cell proliferation as well as expression of *lunatic fringe*. We present a model in which FGF signaling from the mesenchyme regulates the Notch pathway in dental epithelial stem cells via stimulation of *lunatic fringe* expression and, thereby, has a central role in coupling the mitogenesis and fate decision of stem cells.

Key words: ameloblast • Notch • fringe • Fgf-10 • epithelial–mesenchymal interactions

STEM cells are present in many vertebrate regenerative tissues including the hematopoietic system, nervous system, gut, gonads, skin, and olfactory epithelium. The stem cells are generally defined as cells that have the capacity to self-renew as well as to give rise to differentiated progeny. In invertebrates, stem cells have been shown to undergo asymmetric cell division resulting in one daughter cell remaining in the stem cell compartment and the other undergoing further cell divisions and giving rise to differentiated tissues (Morrison et al., 1997). The direct identification of stem cells in most mammalian tissues has been problematic because of the lack of specific stem cell markers.

Neural and hematopoietic stem cells have been the subject of specific interest recently, and multipotent neural

cells that could generate new neurons and glia were identified in the ependymal cells of the adult brain ventricles (Johansson et al., 1999). These cells were shown to divide rarely and asymmetrically, and the other daughter cell was shown to stay as an undifferentiated stem cell in the ependymal layer, whereas the other cell moved to the subventricular layer and gave rise to a rapidly dividing progenitor cell pool, the so-called transit-amplifying cells providing the source for neuronal and glial precursors. Hematopoietic stem cells have been isolated using antibodies to cell surface antigens (Spangrude et al., 1998), and their stem cell properties have been demonstrated by transplantation into irradiated host animals under conditions where the progeny of a single stem cell can be identified (Morrison et al., 1994). Recently, evidence was presented from similar transplantation experiments that neuronal stem cells may have the competence to give rise to hematopoietic cells (Bjornson et al., 1999).

The molecular mechanisms regulating the maintenance of the stem cells, their cell division, and differentiation remain largely unknown, but it has been suggested that there are common mechanisms shared by stem cells in different tissues that underlie their specific properties. The mainte-

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nance of the undifferentiated state of the stem cells has been thought to be regulated by cell–cell interactions involving the Notch signaling pathway (Fortini and Artavanis-Tsakonas, 1993; Bray, 1998; Carlesso et al., 1999). There is no experimental evidence for this assumption in vertebrate tissues, but it is supported by in situ expression data and the established role of Notch in the regulation of cell differentiation in invertebrates. *Notch* receptors have been shown to be expressed in undifferentiated neural cells as well as in keratinocytes, but expression is not confined to stem cells only (Zagouras et al., 1995; Johansson et al., 1999). The hematopoietic stem cells express *Notch* receptors, and the Notch ligand *Jagged1* (*Serrate1*) has been shown to be expressed by stromal cells in culture, suggesting roles in mediation of interactions regulating stem cell maintenance and/or differentiation (Jones et al., 1998; Varnum-Finney et al., 1998).

It is generally thought that stem cells respond to environmental cues that stimulate cell division both in stem cells and their multipotent progeny, the transit-amplifying cells. Some conserved diffusible signal molecules have been proposed to function in the regulation of self-renewal of the stem cells. Neural stem cells can be stimulated to divide in vitro by EGF and FGF (Reynolds and Weiss, 1996).

We have used the mouse incisor tooth as a model to analyze certain aspects of stem cell regulation and functions. The mouse incisor differs from mouse molars as well as from all human teeth in that it erupts continuously throughout the life of the animal. Cells in the apical end of the tooth proliferate and differentiate into the various tooth forming cells including the mesenchyme-derived odontoblasts producing dentin and the epithelium-derived ameloblasts producing enamel. The epithelial tissue at the labial aspect of the incisor apex forms the cervical loop, which consists of a core of stellate reticulum cells surrounded by basal epithelial cells contacting the dental mesenchyme (see Fig. 1, a–d). The cells in the apex of the tooth divide rapidly as compared with the more incisal region, and there is a gradient of cell differentiation from the apex towards the incisal direction (Smith and Warshawsky, 1975, 1977). Hence, it is generally thought that the apex is the reservoir for the cells producing the enamel and dentin of the erupting tooth. There is no evidence of stem cells among the differentiated ameloblasts (Nataatmadja et al., 1990). Therefore, the epithelial stem cells presumably reside within the cervical loop, but this has not been demonstrated directly and their identity has remained completely unknown.

A specific advantage of the tooth as a model is that the basic mechanisms regulating morphogenesis and differentiation have been analyzed in great detail both in classical experimental embryological studies as well as recently by molecular methods. Hence, although the previous studies have not focused on stem cells, there is a wealth of data on expression patterns of developmental regulatory molecules as well as their functions (Gene expression in tooth: www database, 1999). Several conserved signal molecules have been implicated in the mediation of the epithelial–mesenchymal interactions regulating morphogenesis and cell differentiation in teeth. These include FGFs, BMPs, Wnts, and Shh (Thesleff

and Sharpe, 1997; Peters and Balling, 1999). In addition, several Notch receptors and ligands have been localized in the dental tissues and they have been implicated in the regulation of cell determination and differentiation both in dental epithelium and mesenchyme (Mitsiadis et al., 1995, 1997, 1998). Furthermore, it is known from tissue recombination experiments that the mesenchymal tissue controls advancing morphogenesis after the initiation of tooth development (Kollar and Baird, 1969), and that mesenchyme also determines whether the tooth grows continuously or not (Jernvall, J., personal communication). Hence mesenchymal signals apparently regulate the continuous production of progenitor cells in the epithelium.

In this study, we have designed an organ culture system for the apical end of the mouse incisor and used it for the analysis of cell division and cell fate in the epithelium. Although there is no universally accepted definition of a stem cell, we have identified a cell population in the dental epithelium that fulfills several criteria of the stem cells. We have shown by 5-bromo-2'-deoxyuridine (BrdU)¹ and [1,1'-dioctadecyl-6,6-di(4-sulfophenyl)-3,3,3',3'-tetramethylindocarbocyanine] (DiI) labeling that stem cells are present in the cervical loop epithelium. They give rise to transit-amplifying cells and their progeny differentiate into ameloblasts forming the enamel matrix. We have performed a detailed in situ hybridization analysis of the expression of certain genes in the Notch and FGF signaling pathway and studied experimentally the effects of FGF-10 on the epithelium. Slowly dividing putative stem cells expressing *Notch1* were located in close proximity with basal epithelial cells expressing *lunatic fringe*. Lunatic fringe is a secretory molecule involved in the modulation of Notch signaling in invertebrates (Cohen et al., 1997; Fleming et al., 1997; Panin et al., 1997; Klein and Arias, 1998) and in chicks (Sakamoto et al., 1998). Our studies also indicate that FGF-10 is a mesenchymal signal that in addition to stimulating division in stem cells and/or transit-amplifying cells, may regulate the fate of the stem cells by affecting the Notch pathway via stimulation of *lunatic fringe* expression. Our data are in line with FGF-10 being a signal that may couple the division of stem cells and their determination to the ameloblast cell fate.

Materials and Methods

Dissection and Culture of Tissues

The incisors were dissected carefully from the lower jaws (mandibles) of 2-d-old mice (CBA × NMRI) (see Fig. 1 b). This stage was selected for experiments because it was easy to dissect incisors from calcified mandibular bone. The apical ends of the incisors were dissected and cultured in Trowell-type organ cultures on 0.1- μ m pore-size. Nucleopore filters (Costar Corp.) supported by metal grids in a humidified atmosphere of 5% CO₂ in air at 37°C. The culture medium consisted of DME (GIBCO BRL) supplemented with 10% FCS (GIBCO BRL), penicillin/streptomycin, glutamate I (GIBCO BRL), and 100 μ g/ml ascorbic acid (Sigma Chemical Co.). Calcification of the enamel and dentin matrices was visualized by alizarin red staining.

For tissue recombination experiments, the apical ends of the incisors were incubated for 10 min in 2% collagenase (GIBCO BRL) in DME at 37°C, and the epithelia were mechanically separated from mesenchyme in PBS. The epithelia were placed in contact with mesenchyme and cultured

1. *Abbreviations used in this paper:* BrdU, 5-bromo-2'-deoxyuridine; DiI, 1,1'-dioctadecyl-6,6-di(4-sulfophenyl)-3,3,3',3'-tetramethylindocarbocyanine; FGF, fibroblast growth factor.

for 24 h on the filters as described above. Heparin acrylic beads (Sigma Chemical Co.) releasing FGF-10 (25 ng/μl; Amgen) were placed in cultures of whole apical ends of the incisors and on isolated epithelial tissue. Control beads were incubated in BSA (Sigma Chemical Co.). The preparation of the beads has been described in detail (Kettunen et al., 1998).

Processing of Tissues for In Situ Hybridization

Freshly dissected as well as cultured tissues were fixed in 4% paraformaldehyde in PBS, pH 7.2, overnight at 4°C, dehydrated, embedded in paraffin, and serially sectioned at 7 μm. Sections were placed on triethoxysilane- and acetone-treated slides, dried overnight at 37°C, and stored at 4°C until used. For histological analysis, the sections were stained with hematoxylin/eosin.

Immunohistochemistry

Cryostat sections were incubated with rabbit anti-CVADR, (1:50; Tomko et al., 1997) as a primary antibody for 1 h at room temperature, rinsed in PBS, and incubated with peroxidase-labeled anti-rabbit antibodies for 45 min at room temperature. For color reaction DAB (Vector Laboratories, Inc.) as chromogen was used and the sections were counterstained with hematoxylin.

DiI Labeling Analysis

The fluorescent DiI label was microinjected to the epithelium of dissected apical ends of the incisors that were subsequently cultured as described above. DiI [1,1'-diiododecyl-6,6-di(4-sulfophenyl)-3,3',3'-tetramethylindocarbocyanine; Molecular Probes, Inc.] was diluted in DMSO (0.2% wt/vol). The explants were fixed after 5 d and observed using a fluorescent microscope.

BrdU Incorporation Analysis

Cell division was analyzed by culturing the tissues in the presence of BrdU (Amersham Life Science, Inc.). BrdU (1:1,000) was present in the medium for 3, 24, or 72 h, and the tissues were fixed in 4% paraformaldehyde in PBS, pH 7.4, either directly after labeling or after a 7-d chase period. The tissues were embedded in OCT compound (Tissue Tek), and 14-μm frozen sections were cut. The incorporated BrdU was detected by indirect immunoperoxidase method with mouse mAb against BrdU (Amersham Life Science, Inc.) and peroxidase-labeled anti-mouse secondary antibody (ENVISION; DAKO). For color reaction, DAB (Vector Laboratories, Inc.) as chromogen was used and the sections were counterstained with hematoxylin.

Construction of Probes and In Situ Hybridization

For in situ hybridization analysis, single stranded [³⁵S]UTP- and digoxigenin-labeled antisense riboprobes for mouse *Notch1*, *Notch2*, *Notch3* (Mitsiadis et al., 1995), *Fgf-3*, *Fgfr1b*, and *Fgfr2b* (Kettunen et al., 1998) were synthesized as described. *Lunatic fringe* antisense probe of 570 bp was obtained by linearizing the plasmid (pBS SKII(-)) with 860 bp 5' coding region of mouse *lunatic fringe* with BamHI and transcribing with T7 RNA polymerase. For the sense probe, XhoI and T3 RNA polymerase were used, respectively. *Serrate 1* probe was a gift from Domingos Henrique (University of Lisbon), *Fgf-3* probe from David Wilkinson (NIMR, London), and *Fgf-10* probe from Nobuyuki Itoh (Kyoto University). The procedures for in situ hybridization of paraffin sections and whole mounts of cultured explants have been described previously (Kettunen and Thesleff, 1998; Kettunen et al., 1998).

Results

The Progenitors of Ameloblasts Reside in the Cervical Loop Epithelium

The appearance of the dissected incisor tooth of a 2-d-old mouse in a stereo microscope is shown in Fig. 1 b. The cervical loop epithelium appears translucent as compared with the mesenchymal tissue. The histology of the apical end of the incisor and its schematic representation show

the different cell types (Fig. 1, c and d). The cervical loop is composed of basal epithelium and a central core of stellate reticulum. A gradient of an increasing level of cell differentiation is evident in the basal epithelium contacting the pulp mesenchyme from apical to incisal direction. The inner enamel epithelial cells differentiate into tall columnar ameloblasts secreting the enamel matrix.

We devised an organ culture method for the dissected apical ends of the incisors to analyze cell kinetics and fate. The appearance of an explant as photographed through a stereomicroscope during a 7-d culture period is shown in Fig. 1, e–i. The cervical loop produced new dental epithelium, which was seen as an upward movement of the apical end of the epithelium to encompass the apical mesenchyme. This differs from the in vivo situation where the growing epithelium moves towards the incisal direction, probably because of mechanical factors as in vitro the tissues are attached to the filter. After 3 d of culture, production of new enamel and dentin by differentiated ameloblasts and odontoblasts, respectively, was evident (Fig. 1 g). The thickness of the enamel and dentin matrices increased with time when the explants were cultured for 5 and 7 d, respectively (Fig. 1, g–i). Histological sections revealed normal morphology of the ameloblasts that had differentiated in vitro into tall columnar cells with nuclei polarized to their distal ends facing the normal looking stratum intermedium cells. Tomes processes were evident in the secretory ends of the ameloblasts and they appeared as a picket fence in the enamel matrix (Fig. 1 m). Whole mount staining of the explants after 7 d of culture with alizarin red showed that the extracellular enamel and dentin matrices, formed in vitro, had calcified (Fig. 1, k and l).

To visualize the fate of the cervical loop cells, they were labeled with the fluorescent DiI stain at the onset of culture, and the labeled cells were examined after 5 d of culture. Care was taken to microinject DiI to the central cells in the cervical loop, and the restriction of the dye was checked at the onset of culture (not shown). After 5 d of culture, fluorescent cells were seen to extend from the cervical loop to the differentiated ameloblasts (Fig. 2, a and b). The original site of DiI injection could still be seen (Fig. 2, a and b, arrows), but, interestingly, the highest intensity of fluorescence was observed at some distance incisally from the original site. These presumably represent daughter cells, which proliferated rapidly. Extending incisally from this site were labeled cells that had entered the stage of ameloblast differentiation. In conclusion, the DiI injection experiments showed that the cells in the cervical loop give rise to the differentiated dental epithelial cells, in particular the ameloblasts.

Only Cervical Loop Epithelium Has Regenerative Capacity

The DiI labeling experiments suggested that the stem cells for differentiated dental epithelium reside in the cervical loop, but they did not rule out the possibility that there would be stem cells also among the more differentiated cells. To answer this question, we experimentally removed either the cervical loop epithelium or the more differentiated epithelial cells before culture of the apical ends of the incisors. Excision of the epithelium was done at the junc-

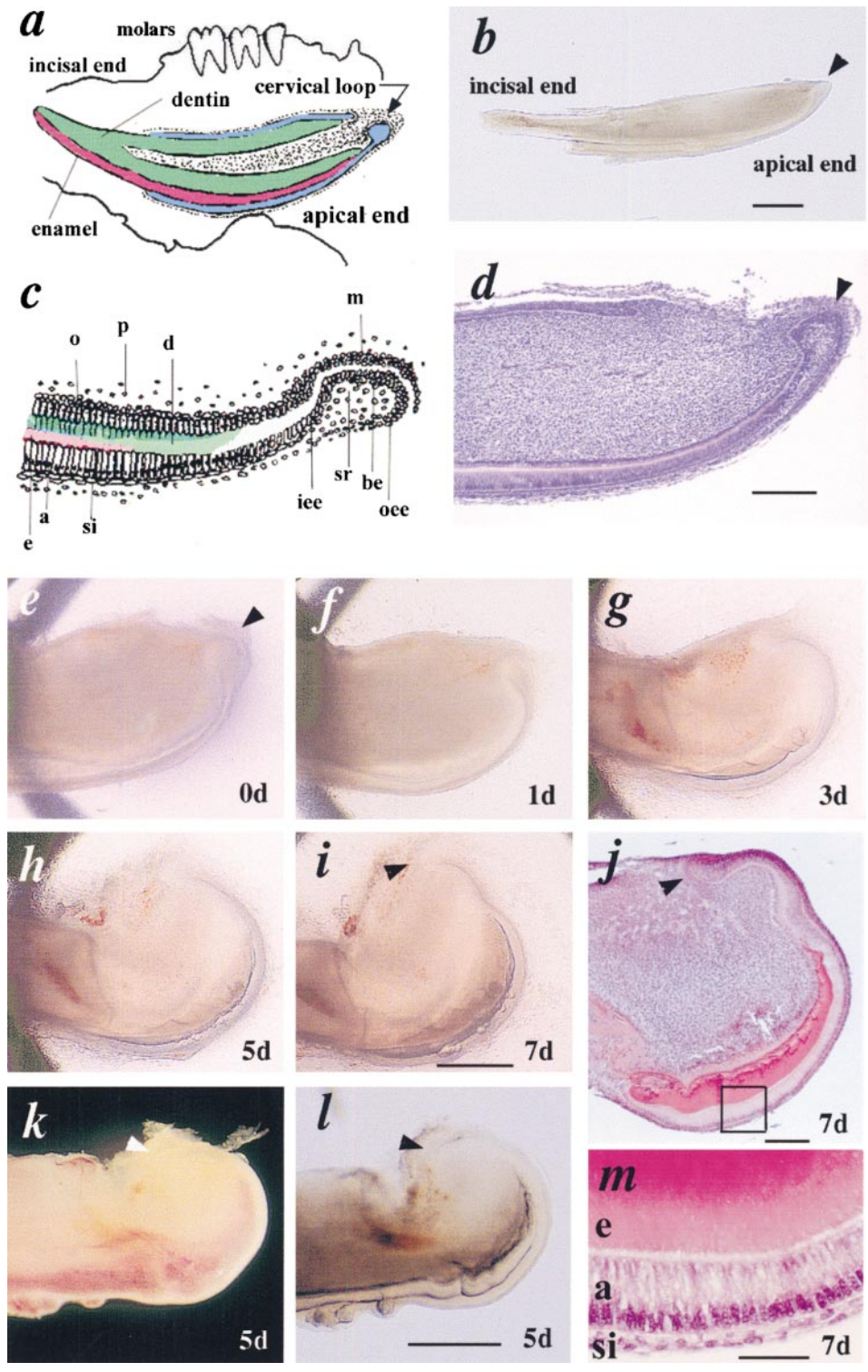


Figure 1. Development of the apical end of mouse incisor in organ culture. (a) Schematic appearance of the incisor tooth in a sagittal section through the mouse mandible. Enamel (red) is covered by odontogenic epithelium (blue) that forms the cervical loop in the apical end of the tooth. Dentin is shown in green. (b) The appearance of the dissected incisor tooth of a 2-d-old mouse in stereomicroscope. (c and d) Histology of the apical end of the incisor showing the different epithelial layers in the cervical loop and the gradual differentiation from the apical to incisal direction of epithelial ameloblasts and mesenchymal odontoblasts depositing enamel (red) and dentin (green), respectively. (e–i) Development of the dissected apical end of a 2-d-old mouse incisor in organ culture for 7 d as seen in stereomicroscope. (j) H-E-stained section of the explant in i, and (m) higher magnification of ameloblasts and enamel. (k) Whole mount alizarin red staining of an explant (l) cultured for 5 d shows mineralization of the extracellular matrices formed in vitro. Abbreviations: a, ameloblast; be, basal epithelium; d, dentin; e, enamel; iee, inner enamel epithelium; o, odontoblast; p, pulp cell; si, stratum intermedium; sr, stellate reticulum. Bars: (b) 1 mm; (d and i) 500 μm ; (j) 200 μm ; (l) 700 μm ; and (m) 100 μm .

tion of the cervical loop and the inner enamel epithelium. When the cervical loop was removed (five explants) and the explants were cultured for 9 d, it was evident that the wounded end of the epithelium did not grow like in the controls (Fig. 3, a–e, compare with Fig. 1, e–i). Instead, the epithelial cells underwent differentiation into ameloblasts. The cells produced enamel matrix, and after 9 d of culture, a sharp end of matrix production was seen and its calcification was visualized by alizarin red staining (Fig. 3

f). These observations indicate that the inner enamel epithelial cells did not exhibit regenerative capacity, and that they were already committed to differentiation into enamel-producing ameloblasts. In the second set of experiments, the inner enamel epithelium and ameloblast zones were removed from the apical ends of the incisors and only the cervical loop epithelium was left intact (five explants). During the 7-d culture, the cervical loop was shown to grow remarkably and to

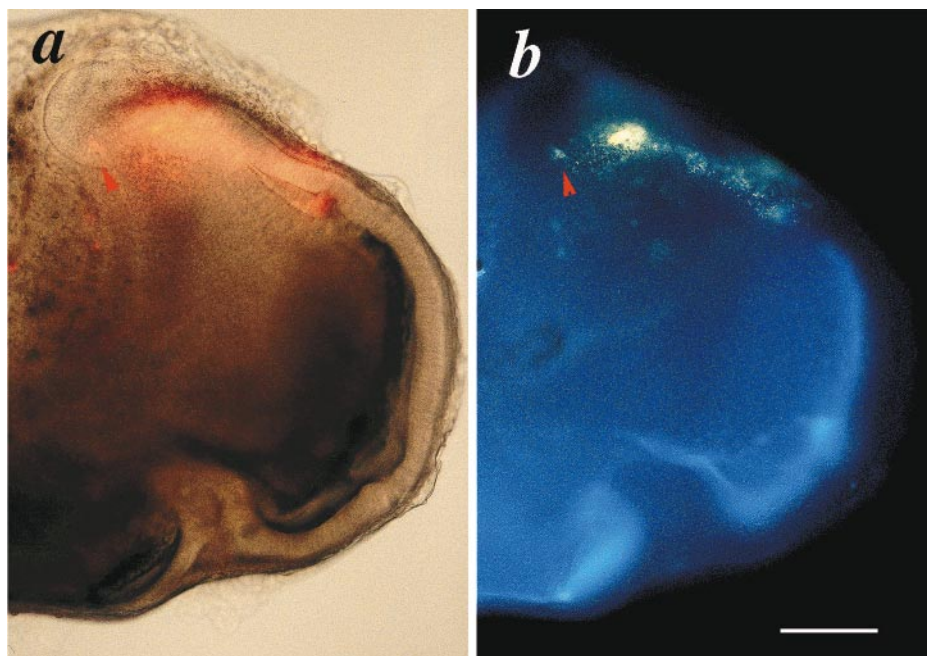


Figure 2. Analysis of the cell fate of cervical loop epithelial cells. DiI was injected in the center of the cervical loop and the explants were cultured for 5 d. (a) Appearance of the explant in phase-contrast and (b) fluorescent microscope. DiI-labeled cells have extended from the point of injection incisally, and the highest intensity of fluorescence is in the inner enamel epithelium corresponding to the location of rapidly dividing cells. Arrowheads show the point of injection of DiI at the onset of culture. Bar, 250 μ m.

give rise to inner enamel epithelium (Fig. 3, g–k). After 7 d of culture, matrix production by new epithelium was seen and its calcification was visualized by alizarin red staining (Fig. 3 l, small arrowheads). Therefore, these results gave evidence that cells with significant growth potential reside exclusively in the cervical loop epithelium.

Slow Cycling Putative Stem Cells Reside in the Cervical Loop Epithelium

The kinetics of epithelial cell division was examined by labeling the apical ends of the incisors with BrdU for various times and examining the BrdU incorporating cells in tissue sections after culture. When the explants were labeled for 3 h and immediately fixed, labeled cells were seen throughout the cervical loop epithelium extending to the inner enamel epithelium zone (Fig. 4, a–c). Presumably both stem cells and transit-amplifying cells were labeled with BrdU during this period, as recently shown for skin keratinocytes *in vitro* (Jensen et al., 1999). When the explants were cultured for 24 h in the presence of BrdU, labeled cells were observed in the same locations as in the 3-h cultures and in the ameloblast zone, indicating that the inner enamel epithelium, which had undergone their last divisions during the culture, had differentiated into ameloblasts (Fig. 4, d and f). When the labeling period was extended to 72 h, BrdU incorporation was seen to extend further to the zone of differentiated, postmitotic ameloblasts (Fig. 4 i). These results are in line with the observations on DiI-labeled explants, indicating that proliferating cells in the apical region of the incisor give rise to ameloblasts.

To analyze kinetics of cell division, we cultured the explants for 7 d after they had been incubated for 3 h with BrdU. The rapidly proliferating cells were, thereby, given time to dilute the label. After the 7-d chase period, very

few labeled cells were seen in the inner enamel epithelium area, indicating that the vast majority of cells had diluted the label by repeated divisions or differentiated into ameloblasts after their last divisions. BrdU-labeled ameloblasts were present near the inner enamel epithelium zone, and they were grouped into clusters. Hence, they appeared as clones of cells that presumably were descendants of individual inner enamel epithelial cells, which had undergone their last cell divisions and incorporated BrdU during the 3-h labeling period (Fig. 5, a and c). Similarly, clusters of labeled cells were seen in the epithelial cells overlying the labeled ameloblasts, suggesting that they had differentiated from precursor cells that had been labeled during the first 3 h of culture.

Interestingly, intensely labeled epithelial cells were observed within the cervical loop. They were located among the peripheral stellate reticulum cells in close vicinity to the basal epithelial cells that contact the basement membrane and dental mesenchyme at their basal end (Fig. 5, a and b). We suggest that the labeled cells represent a population of slowly dividing stem cells in the cervical loop. They did not have neighboring labeled cells, suggesting that the stem cells had undergone asymmetric cell division. It is conceivable that one daughter cell had remained as a stem cell and the other daughter cell had generated a transit-amplifying population of cells, which are the progenitors of ameloblasts.

As a potential stem cell marker, we used the antibody against the adenovirus receptor CVADR (previously known as CAR), which has been associated with stem cells in the brain (Tomko et al., 1997; Johansson et al., 1999). Strong expression was seen in the cervical loop epithelium and, at higher magnification, the expression appeared more intense in the stellate reticulum cells than in the basal epithelium. The transit-amplifying cells of the inner enamel epithelium and the differentiated ameloblasts did not ex-

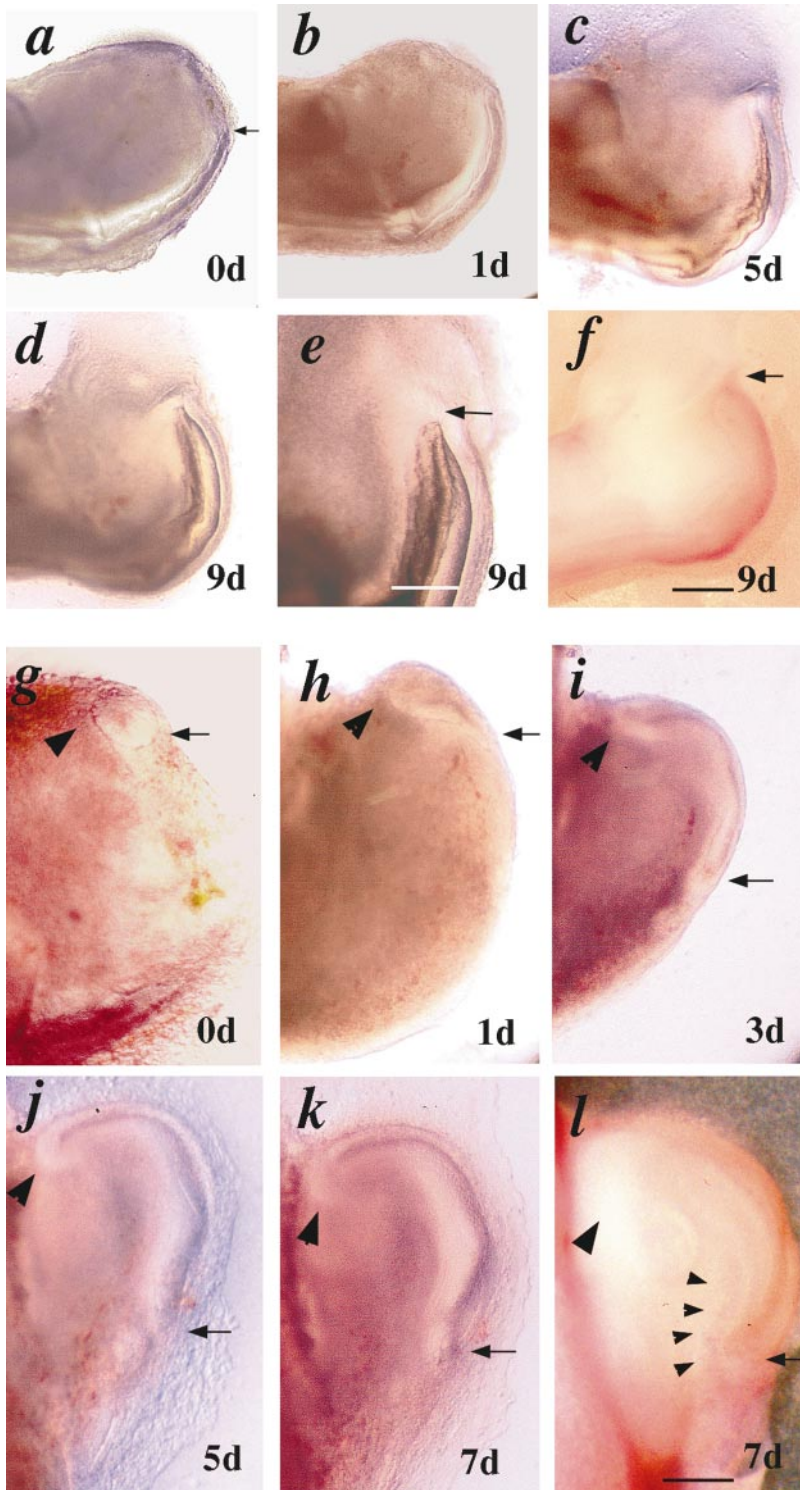


Figure 3. Observations of cultured apical explants after removal of the cervical loop epithelium or inner enamel epithelium and ameloblasts. (a–f) After the removal of the cervical loop epithelium, no apical extension of the epithelium was seen during 9 d of culture. Epithelial cells differentiated and produced enamel matrix, which calcified (f). The arrows point to the cut end of the epithelium. (g–l) After removal of all dental epithelium except the cervical loop, new epithelium was produced by the cervical loop, which grew rapidly to incisal direction during 7 d of culture. Large arrowheads indicate the cervical loop. Small arrowheads point to the edge of the regenerating epithelium. Bars: (a–d and f–l) 300 μm ; (e) 100 μm .

press CVADR (Fig. 5, d and e). Hence the expression of CVADR correlated well with the suggested stem cell distribution within the cervical loop epithelium.

Expression of Genes in the Notch Signaling Pathway Is Associated with the Localization of Stem Cells

Because the Notch pathway has an important role in cell fate determination in invertebrates and it has been specifi-

cally implicated in the determination of stem cell fate in vertebrates, we performed a careful in situ hybridization analysis of the expression patterns of several genes in this pathway. Notch receptors and ligands previously have been shown to be associated with early tooth morphogenesis and cell differentiation (Mitsiadis et al., 1995, 1997, 1998), and we have recently shown that *lunatic fringe*, a homologue of a gene that is linked with Notch signaling in *Drosophila*, has an expression pattern during early tooth

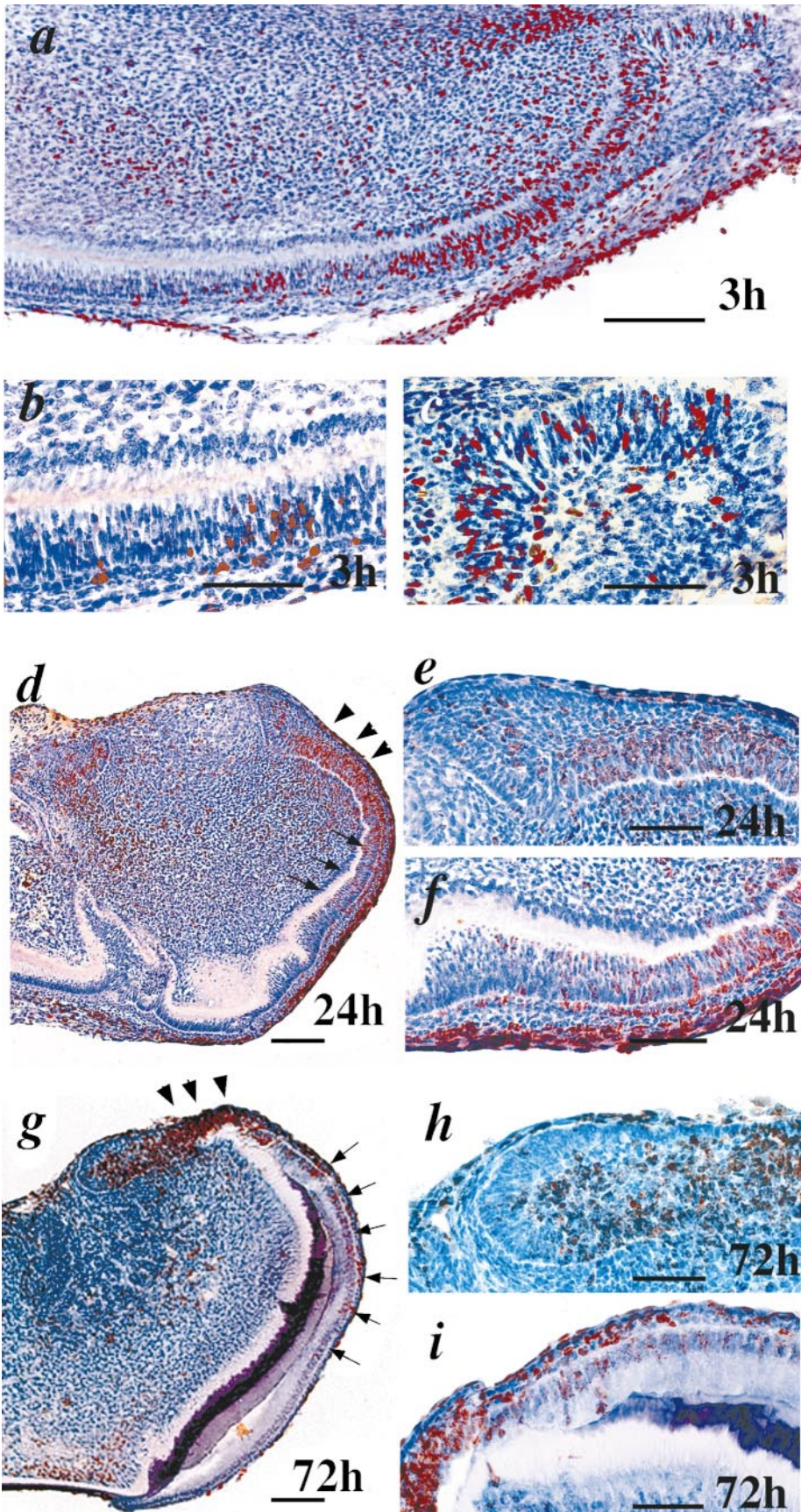


Figure 4. Analysis of cell division in the dental epithelium by BrdU labeling. (a–c) After 3 h labeling, BrdU incorporating cells are located in the cervical loop (c) and inner enamel epithelium extending to the zone of terminal ameloblast differentiation (b). (d–i) After 24 (d–f) and 72 h of labeling (g–i), BrdU incorporation is seen also among the newly differentiated ameloblasts (arrows). Particularly high incorporation was evident in the zone of inner enamel epithelium (arrowheads). Bars: (a, d, and g) 200 μm; (b, c, e, f, h, and i) 100 μm.

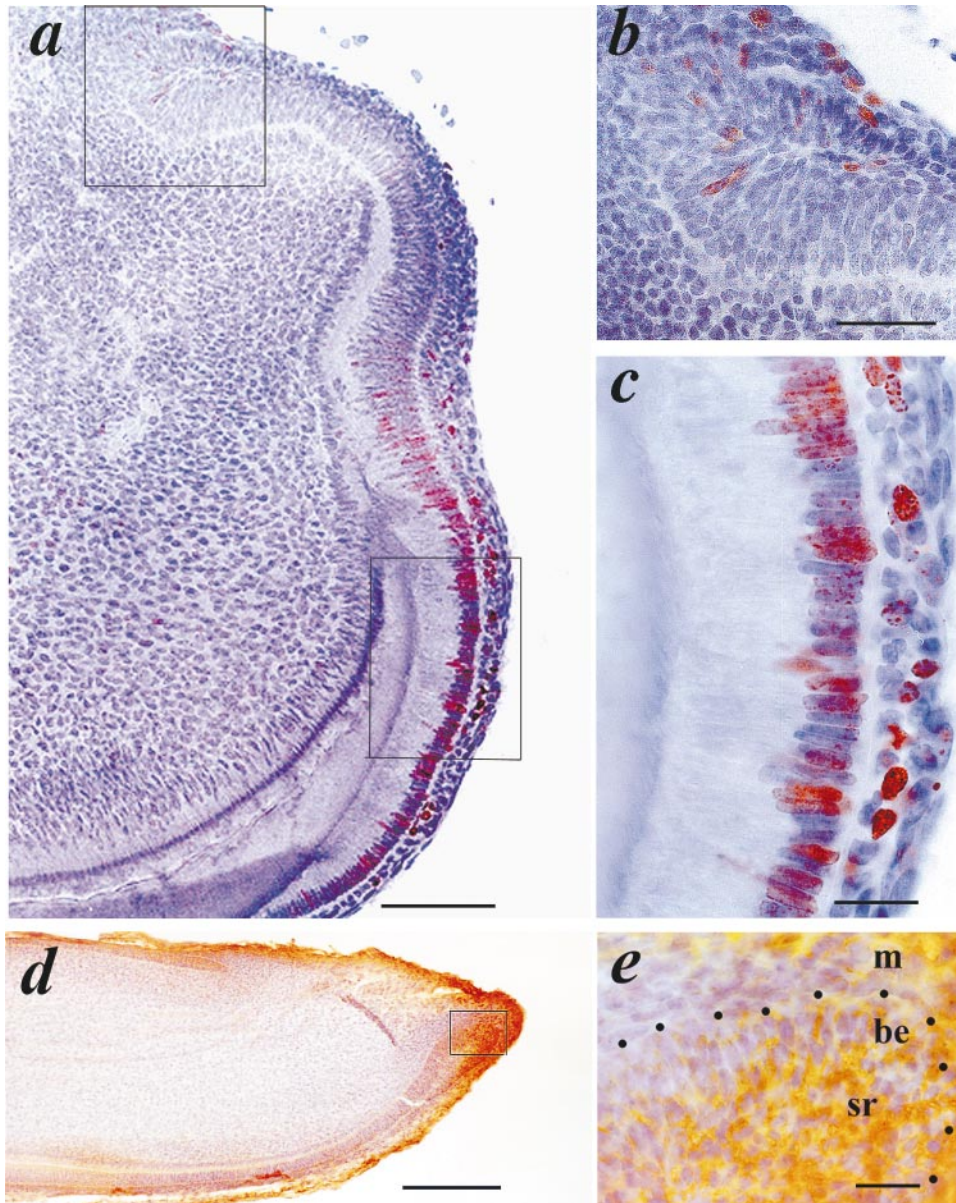


Figure 5. Localization of stem cells by BrdU pulse-chase analysis and by anti-adenovirus receptor antibody (anti-CVADR). 3 h BrdU labeling was followed by 7 d chase period. (a and b) Labeled cells were detected in the cervical loop at the border between basal epithelium and stellate reticulum. (a and c) Labeled cells represent ameloblasts derived from the last cell division of inner enamel epithelial cells. Stellate reticulum cells in cervical loop expressed CVADR strongly (d and e). The expression in basal epithelium was at lower level. The staining at the border of the frozen section is due to nonspecific binding of antibodies. m, mesenchyme; be, basal epithelium; sr, stellate reticulum; black dots, basement membrane. Bars: (a and d) 200 μm ; (b) 100 μm ; (c and e) 50 μm .

development suggesting developmental roles (Mustonen et al., in preparation).

Expression of the receptors *Notch1*, *2*, and *3* mRNA, the Notch ligand *Serrate1* mRNA as well as *lunatic fringe* mRNA was localized in longitudinal sections through the 2-d mouse incisors. In the cervical loop, *Notch1* was restricted to stellate reticulum cells and was most intense in the cells facing inner enamel epithelium. On the other hand, *Notch2* was expressed in the outer enamel epithelium and the underlying stellate reticulum cells, and *Notch3* was not detected in the cervical loop (Fig. 6, a–c and a'–c'). Expression of *Serrate1* was not detected in the cervical loop (Fig. 6, d and d'). Interestingly, *lunatic fringe* was expressed in the inner enamel epithelium starting from the cervical loop (Fig. 6, e and e'). This correlates closely with the distribution of BrdU incorporating cells in the enamel epithelium (Fig. 4 a). Furthermore, the localization of the slowly dividing putative stem cells in our

BrdU incorporation study in the peripheral stellate reticulum cells correlates with the boundary between *lunatic fringe* and *Notch1*, suggesting that the maintenance and fate of the stem cells may be influenced by Notch signaling.

The expression patterns of the Notch pathway genes were also correlated with terminal cell differentiation. In the zone of ameloblast differentiation, *Notch1* was expressed in the stratum intermedium cells, whereas *Notch2* was intensely expressed in the stellate reticulum cells (Fig. 6, a'' and b''). *Notch3* was expressed in stellate reticulum cells and, in addition, intense expression was seen in blood vessels and in the subodontoblastic mesenchyme (Fig. 6, c and c''). In contrast to Notch genes that were absent from ameloblasts, the ligand *Serrate1* was intensely expressed in terminally differentiated ameloblasts (Fig. 6, d and d''). These findings are in line with the recent data by Mitsiadis et al. (1998) and, thus, support a role for Notch signaling in

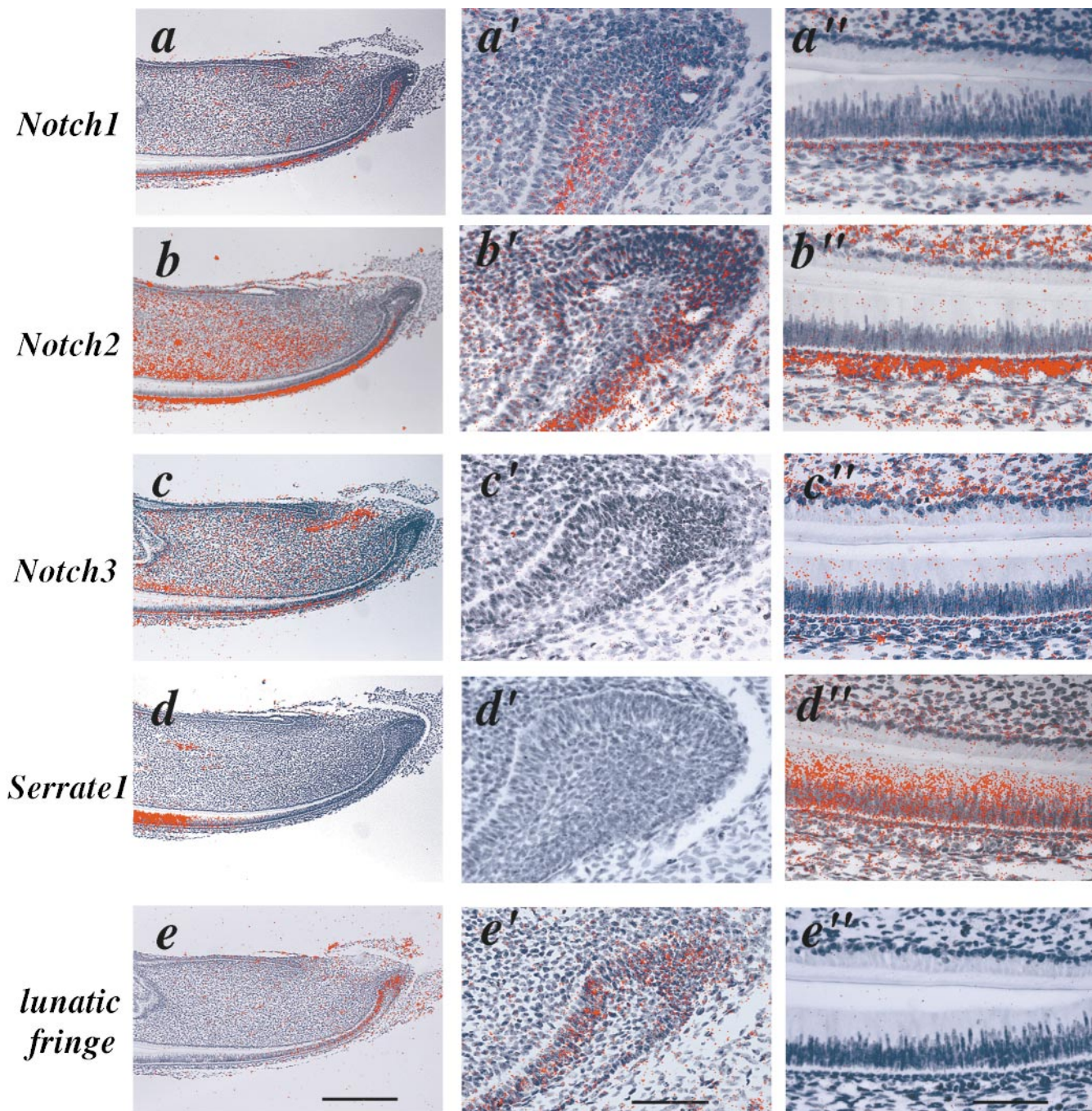


Figure 6. In situ hybridization analysis of *Notch1*, *2*, *3*, *Serrate1*, and *lunatic fringe* expression in the mandibular incisor of 2-d-old mice. (a–a'') *Notch1* is expressed in stellate reticulum in cervical loop (a) and in stratum intermedium and stellate reticulum in the zone of cell differentiation (a''). (b–b'') *Notch2* is expressed in outer enamel epithelium and stellate reticulum in cervical loop (b) and in stratum intermedium and stellate reticulum in the zone of cell differentiation (b''). (c–c'') *Notch3* is absent from the cervical loop (c), but is expressed in stellate reticulum and in the zone of cell differentiation (c''). (d–d'') *Serrate1* is absent from the cervical loop (d), but is expressed in ameloblasts (d''). (e–e'') *Lunatic fringe* is expressed in basal epithelium in cervical loop continuing in the inner enamel epithelium (e'), and is downregulated in differentiated ameloblasts (e''). Bar, 1 mm (χ). Bar, 200 μ m (χ' and χ'').

the maintenance of the differentiated state of ameloblasts. *Lunatic fringe* was not expressed in the zone of differentiated ameloblasts (Fig. 6 e''), and its functions, therefore, appear to be confined to the regulation of their determination and differentiation.

***Fgf-3* and *Fgf-10* Are Expressed in Dental Mesenchyme and Their Receptors in Overlying Cervical Loop Epithelium**

Earlier tissue recombination experiments have shown that dental mesenchyme controls the morphogenesis of dental

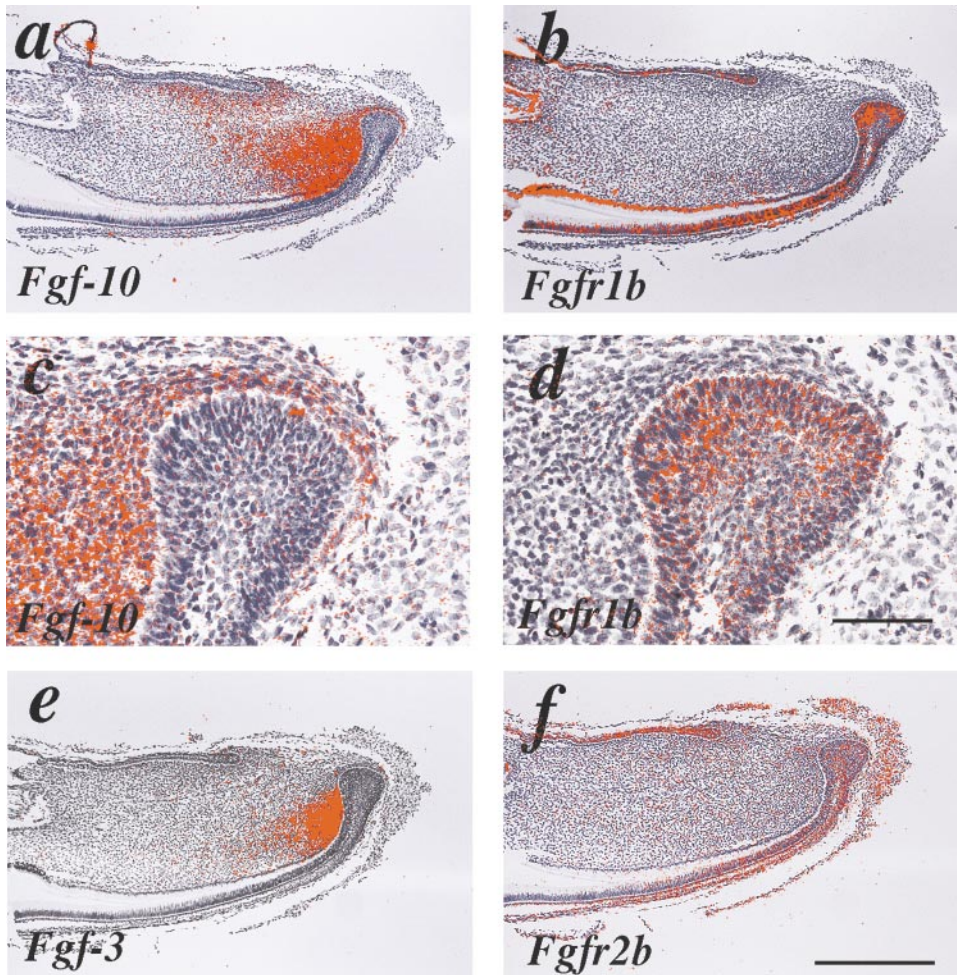


Figure 7. Localization of *Fgf-3* and *Fgf-10* and the receptors *Fgfr1b* and *Fgfr2b* (*Kgfr*) mRNA in the mandibular incisor of 2-d-old mice. (a and c) *Fgf-10* is intensely expressed in the dental mesenchyme adjacent to cervical loop and inner enamel epithelium. (b and d) *Fgfr1b* is expressed throughout cervical loop epithelium with high intensity at the border between basal epithelial cells and stellate reticulum. (e) *Fgf-3* is expressed in dental mesenchyme under the inner enamel epithelium in a more restricted area than *Fgf-10*. (f) *Fgfr2b* (*Kgfr*) is expressed in the cervical loop in similar locations as *Fgfr1b*. c and d show higher magnification of the cervical loop in a and b, respectively. Bars: (a, b, e, and f) 1 mm; (c and d) 200 μ m.

epithelium (Kollar and Baird, 1969) including the continuous growth of the incisor epithelium (Jernvall, J., personal communication), and our recent studies on early tooth morphogenesis have implicated FGFs-3 and -10 as mesenchymal signals regulating the early morphogenesis of dental epithelium (Kettunen, P., N. Itoh, and I. Thesleff, manuscript submitted for publication). Hence, we analyzed the patterns of the expression of these signals as well as their receptors by in situ hybridization in the 2-d-old mouse incisors. Both Fgfs were intensely expressed in a restricted area of the dental mesenchyme in the apical end of the tooth. *Fgf-10* expressing cells surrounded the whole cervical loop epithelium and extended to the zone underlying inner enamel epithelium (Fig. 7, a and c). *Fgf-3* showed a more restricted pattern of expression. It overlapped with *Fgf-10* in the mesenchyme under the inner enamel epithelium, but was not expressed in the mesenchyme surrounding the cervical loop (Fig. 7 e). *Fgfr1b* was intensely expressed in the cervical loop epithelium including the basal epithelial cells and stratum intermedium, but was less intense in the stellate reticulum cells (Fig. 7, b and d), and *Fgfr2b* (*Kgfr*) showed a similar pattern in the cervical loop (Fig. 7 f). Hence, the patterns of the expression of *Fgf-3* and *Fgf-10* and their receptors were in line with the suggestion that these FGFs may be mesenchymal signals regulating the development of cervical loop epithelium.

FGF-10 Stimulates the Growth of Cervical Loop Epithelium

The effects of FGF-10 on the growth of cervical loop epithelium were analyzed in explant cultures of apical ends of the 2-d-old mouse incisors. FGF-10 recombinant protein was applied with beads on the explants, and the control explants received beads soaked in BSA. Two beads were placed on each explant in contact with the cervical loop epithelium, one at its apical end and one at the inner enamel epithelium. When the development of the explants with FGF-10 and BSA beads was compared during 48 h of culture, a clear acceleration of the growth of the epithelium was observed by FGF-10 (Fig. 8, a–f).

The effects of the FGF-10 and BSA beads on cell proliferation were analyzed by culturing them on isolated cervical loop epithelium. The beads were placed on different areas of the dental epithelium, and the explants were cultured in the presence of BrdU for 6 h, fixed, and processed for immunohistological analysis of BrdU incorporation. A clear zone of BrdU incorporating cells was evident around the FGF-10 beads that were placed on the inner enamel epithelium and cervical loop epithelium (Fig. 8, g and h), but not when placed on the zone of differentiated ameloblasts (not shown). No increase in BrdU incorporation was seen around the control BSA beads (Fig. 8, g and h).

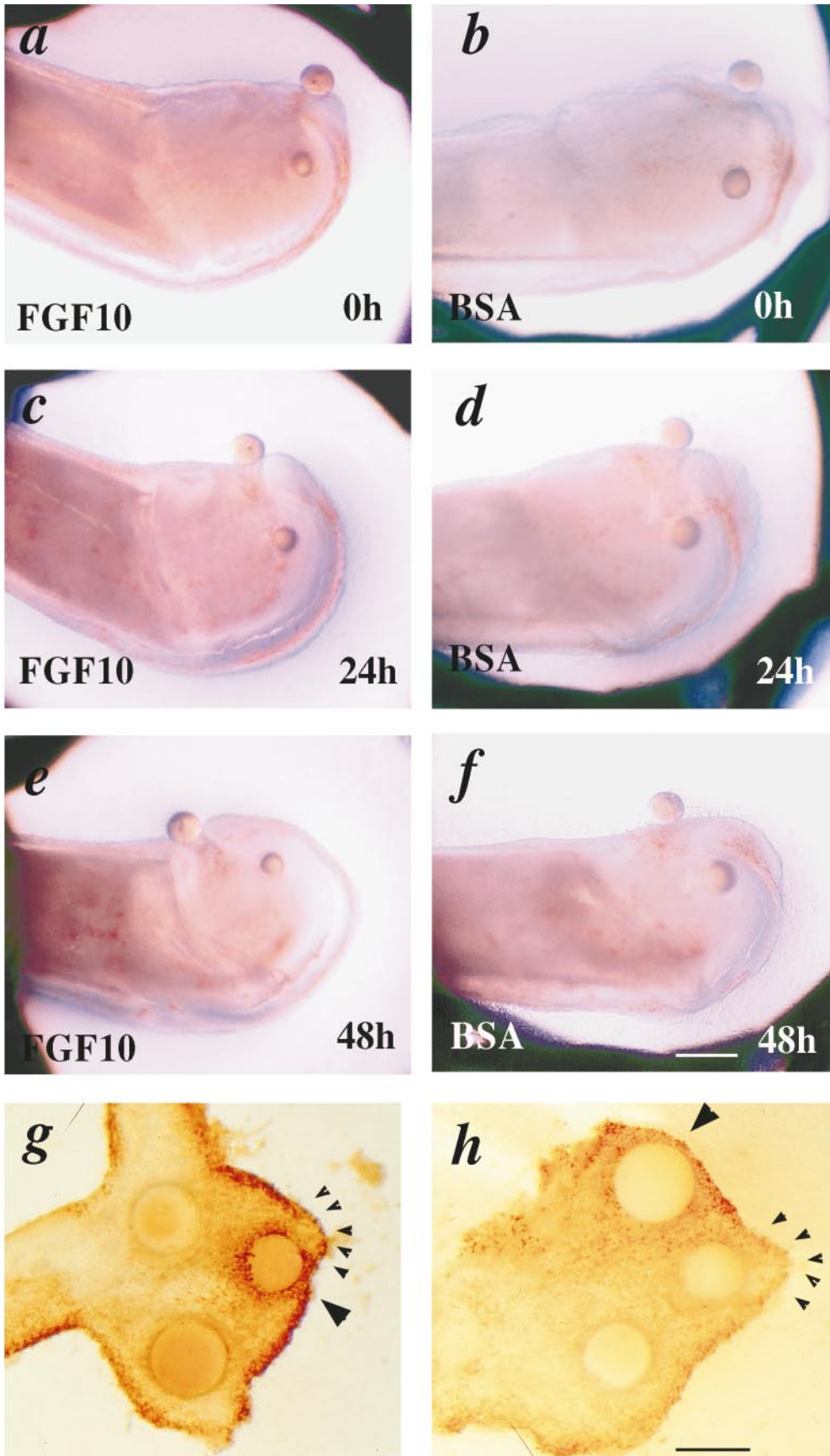


Figure 8. Effect of FGF10 on the growth of cervical loop epithelium. Beads releasing FGF-10 (a, c, and e) and BSA (b, d, and f) were placed in contact with cervical loop and inner enamel epithelium, and the explants were cultured for 48 h. A stimulation of growth of the cervical loop epithelium by FGF-10 beads is evident. (g and h) FGF-10 beads (large arrowheads) stimulated BrdU incorporation when placed on epithelium isolated from the apex of the incisor. The control BSA beads (no arrows) did not affect cell division. The stimulatory effect of FGF-10 was more pronounced when beads were placed on cervical loop epithelium (g, small arrowheads) than on inner enamel epithelium (h). Bars: (a–f) 250 μm; (g and h) 200 μm.

***Lunatic Fringe* Expression in Cervical Loop Epithelium Is Stimulated by the Dental Mesenchyme and FGF-10**

The expression of *lunatic fringe* in the basal epithelial cells of the cervical loop suggested that it may be regulated by the underlying mesenchyme, and the close correlation of *Fgf-10* and *lunatic fringe* expression in mesenchyme and epithelium was suggestive of a role for FGF-10 in the regulation of *lunatic fringe*. We examined these possibilities in vitro in isolated epithelial and mesenchymal tissues from the apical ends of 2-d-old mouse incisors. When the isolated epithelium and mesenchyme were placed in contact on the filter and cultured for 24 h, intense expression of *lunatic fringe* was seen in the basal epithelial cells of the cervical loop and in the inner enamel epithelium contacting the mesenchyme (Fig. 9 a). No expression was seen in the epithelium of the differentiated zone contacting mesenchyme. When the epithelium was cultured alone, *lunatic fringe* expression was not seen, indicating that expression had been downregulated in the absence of mesenchyme. When FGF-10 releasing beads were placed on the cervical loop epithelium and inner enamel epithelium, they caused an upregulation of *lunatic fringe* expression in the contacting epithelium (Fig. 9 b). When FGF-10 beads were placed on the ameloblast zone, the beads had no effect (Fig. 9 c). Control BSA beads had no effect on expression. Hence, these experiments demonstrated that the maintenance of *lunatic fringe* in the cervical loop epithelium depends on mesenchymal signals, and that this effect is mimicked by FGF-10.

Discussion

The incisors of rodents erupt throughout the lifetime of the animals as the wear at the incisal edge is compensated for by renewal in the apex of the tooth located deep in the jawbone. The generation of the highly specialized dental tissues including the epithelially derived enamel and mesenchymal dentin involves proliferation of progenitor cells and their differentiation, matrix deposition, and subsequent mineralization. This process is seen as an increasing gradient of cell differentiation starting from the germinative apical area and extending towards incisal direction. Although it is generally assumed that there are stem cells in the apical end of the tooth, this problem has not been actively studied and the identity of the stem cells has remained enigmatic. We present evidence that epithelial stem cells possibly reside in a specific location in the cervical loop at the apex of the tooth, and that they produce a transit-amplifying cell population that gives rise to the differentiated dental epithelial cells, in particular, the enamel-producing ameloblasts. We also show data suggesting that Notch and FGF signaling are associated with specification of these stem cells and present a putative molecular mechanism, which may be applicable to the proliferation and determination of cell fate in stem cells also in other vertebrate tissues.

Putative Dental Epithelial Stem Cells Reside in the Cervical Loop, Divide Slowly, and Give Rise to Transit-amplifying Cells Differentiating into Ameloblasts

Our in vitro model system for the culture of the apical end of the mouse incisor supported the growth and differentia-

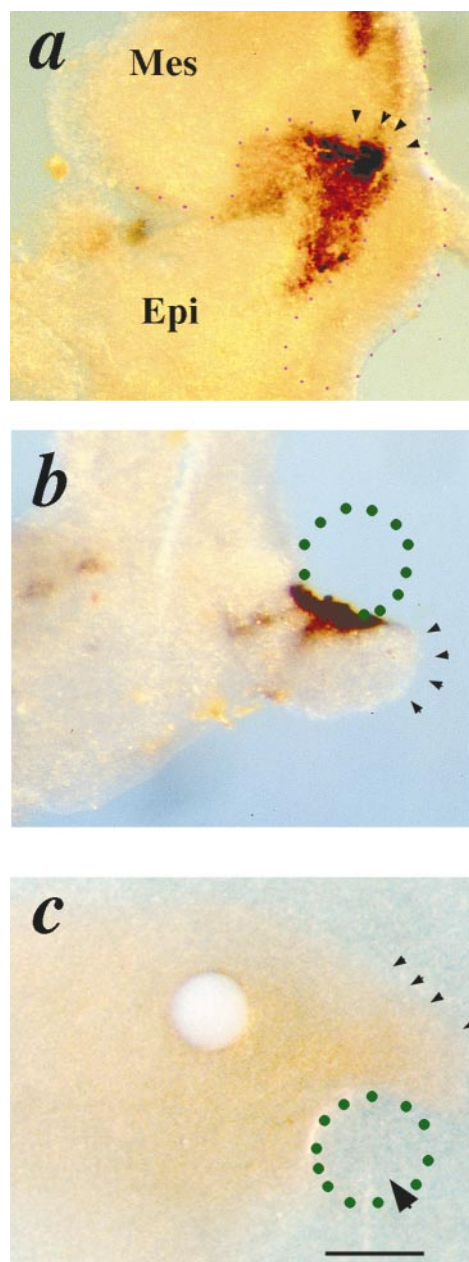


Figure 9. *Lunatic fringe* mRNA expression in dental epithelium is stimulated by dental mesenchyme and by FGF-10. (a) The epithelium and mesenchyme from the apical end of the 2-d-old mouse incisor were separated and cultured as recombinants for 24 h. Stimulation of *lunatic fringe* expression was seen in the cervical loop epithelium and inner enamel epithelium in close contact with mesenchyme. (b) FGF-10 beads stimulated *lunatic fringe* expression when placed adjacent to cervical loop epithelium. (c) FGF-10 beads did not induce *lunatic fringe* expression when placed on the zone of differentiated ameloblasts. No specific expression was observed around BSA beads (large arrowhead). Red points in a represent the border of epithelial tissue. Green dots in b and c represent beads (the beads were detached during processing). Small arrowheads point to cervical loop epithelium. Bar, 200 μ m.

tion of the germinative cells of the tooth. Like in vivo, the cervical loop epithelium proliferated, differentiated into ameloblasts, and produced enamel matrix, which underwent calcification in organ culture. A special advantage of the system was that the stereomicroscopic examination of the advancing development was easy during culture. As the cervical loop and enamel-producing cells are located at the labial side of the incisor, and the explants were oriented so that the labial surface was on one side of the explant, the growth of the cervical loop epithelium and the gradient of differentiation and matrix deposition could be visualized almost two dimensionally.

Using the in vitro culture of the incisor apex, we produced two lines of evidence indicating that the epithelial stem cells reside within the cervical loop epithelium. First, when cells in the center of the cervical loop were labeled with the fluorescent dye DiI and cultured in vitro, the dye was seen in the differentiating cells after 1 d and in the ameloblasts after 2 d of culture, indicating that the ameloblast lineage starts in the cervical loop. This was supported by the analysis of BrdU incorporation during extended time periods showing that the labeled cells extended progressively more incisally and occupied the zone of postmitotic mature ameloblasts. Second, when the cervical loop was removed mechanically from the apex of the tooth, the remaining epithelium could not regenerate the cervical loop in vitro. In these explants, all epithelial cells differentiated and produced mineralized matrix forming a stunt end with no indication of new cervical loop formation. In the opposite experiment where the differentiated epithelium was removed, the remaining cervical loop generated new epithelium differentiating into secretory ameloblasts, thus, indicating that it contains a pool of immature cells competent to regenerate the dental epithelium.

We identified the slowly dividing putative stem cells in organ culture experiments by labeling the cells of the incisor apex with BrdU for 3 h, followed by a 7-d chase period. Labeled cells were detected in the cervical loop among the stellate reticulum cells in close vicinity to the basal epithelium. This location is consistent with the observed strong expression of the adenovirus receptor CVADR, a putative stem cell marker in brain cells (Johansson et al., 1999). We suggest that the BrdU-labeled cells represent the daughter cells of stem cells that had divided during the 3-h labeling period and had remained in the stem cell pool and not divided thereafter. We suggest that the other daughter cell had entered the pathway of cell differentiation, and that the cell division therefore was asymmetric. This is supported by the finding that the BrdU-labeled cells did not have labeled neighboring cells (unlike the labeled ameloblasts, which appeared in clusters as they presumably had been generated by inner enamel epithelium after the last division). Asymmetric cell division of stem cells has been demonstrated in invertebrates (Hawkins and Garriga, 1998; Jan and Jan, 1998), and in vertebrates there is evidence that neural stem cells undergo asymmetric cell division (Chenn and McConnell, 1995; Johansson et al., 1999).

Association of Notch Signaling Pathway with Dental Epithelial Stem Cells

The Notch signaling pathway is an evolutionarily con-

served cell interaction mechanism that controls fundamental aspects of cell determination during development (Artavanis-Tsakonas and Simpson, 1991; Artavanis-Tsakonas et al., 1995; Lewis, 1998), and it has been suggested that Notch signaling may provide a microenvironment where the stem cells are maintained. Although Notch has been shown to function in *Caenorhabditis elegans* and in *Drosophila* in the maintenance of the potential of stem cells and their undifferentiated state, respectively (Fortini and Artavanis-Tsakonas; 1993; Bray, 1998), definitive roles for Notch have so far not been demonstrated in vertebrate stem cells (Carlesso et al., 1999). The expression of Notch1 in skin keratinocytes and in immature neural cells including neural stem cells has been suggested to be associated with stem cell functions (Powell et al., 1998; Johansson et al., 1999). Furthermore, asymmetric distribution of Notch1 has been observed in immature neural cells before their cell division, suggesting a role in regulation of different cell fates in the daughter cells, i.e., whether they are maintained as stem cells or give rise to differentiated progeny (Chenn and McDonnell, 1995).

During tooth development, Notch signaling has been associated with the differentiation of dental epithelial and mesenchymal cells. *Notch1*, *-2*, and *-3* are downregulated in presumptive dental epithelium already during tooth initiation and, thereafter, these genes are not expressed in the ameloblast cell lineage, but they are intensely expressed in the other dental epithelial cells, suggesting roles in the determination and maintenance of cell differentiation of the ameloblast cell lineage (Mitsiadis et al., 1997, 1998). Therefore, these previous studies suggested to us that Notch signaling may be involved in the regulation of the stem cells in the continuously growing incisor. Interestingly, the slowly dividing potential stem cells in the cervical loop epithelium were located within the *Notch1* and *Notch2* expressing cells facing basal epithelial cells not expressing *Notch*, which is in line with the possibility that these Notch receptors regulate fate of the stem cells at this border.

The possible role of Notch signaling in the regulation of the dental stem cells is further strengthened by the expression pattern of *lunatic fringe*, a gene encoding a secretory molecule resembling a glycosyltransferase that modulates Notch signaling in *Drosophila* (Yuan et al., 1997). Three vertebrate *fringe* genes are known and of these *lunatic fringe* has a developmentally regulated expression pattern during early tooth morphogenesis (Mustonen, T., unpublished results). We localized *lunatic fringe* expression to the cervical loop epithelial cells neighboring Notch expressing cells. Such expression borders are characteristic to molecules in the Notch signaling pathway in *Drosophila* tissues, and have been shown to be sites of specific signaling activities (Irvine and Wieschaus, 1994; Johnston et al., 1997).

An important piece of evidence supporting a role for the Notch pathway in the regulation of stem cells is the finding that the *lunatic fringe* expressing basal epithelial cells that contact *Notch1* expressing stem cells on one side are facing the dental mesenchyme at the opposite side, and that the mesenchyme regulated *lunatic fringe* expression. This is significant because the dental mesenchyme controls the epithelial morphogenesis in teeth, including the type of tooth formed, i.e., whether it develops to a molar with roots or

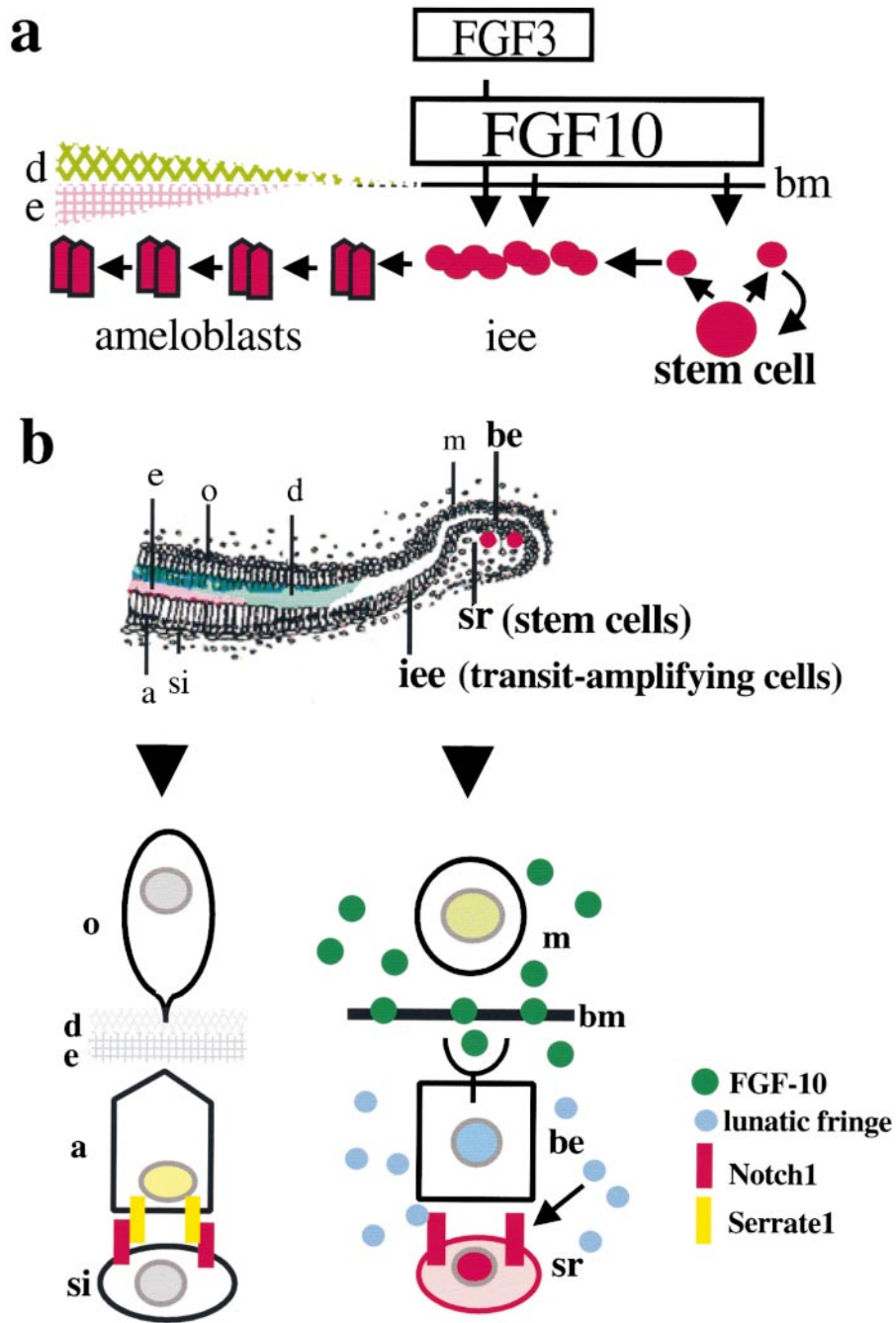


Figure 10. A model for the molecular mechanisms involved in the generation of the ameloblast cell lineage from stem cells. (a) Stem cell kinetics. The stem cell divides slowly and gives rise to one daughter cell remaining in the stem cell pool in the cervical loop, whereas the other daughter cell enters the zone of rapidly dividing inner enamel epithelial cells (transit-amplifying cell population). During rounds of divisions, these cells move toward the incisal direction and differentiate into ameloblasts forming enamel matrix. They are in close contact with dental mesenchyme expressing FGF-10, which stimulates the division of both stem cells and transit-amplifying cells (inner enamel epithelial cells), and FGF-3 stimulating division of transit-amplifying cells only. (b) Signaling pathways for cell fate specification in ameloblast cell lineage. Mesenchymal FGF-10 (green dots) stimulates *lunatic fringe* (blue dots) expression in the basal epithelium. The stem cells are located in the stellate reticulum expressing Notch1 (red stripes). Lunatic fringe modulates Notch signaling in the stem cells. When the daughter of a stem cell enters the zone of *lunatic fringe* expression in basal epithelium it will be incorporated to the ameloblast cell lineage interacting with adjacent mesenchymal cells. In the zone of differentiated cells (left), the Notch signaling pathway regulates interactions between ameloblasts expressing Serrate1 and stratum intermedium cells expressing Notch1. Abbreviations: a, ameloblast; be, basal epithelial cell; bm, basement membrane; d, dentin; e, enamel; iee, inner enamel epithelial cell; m, mesenchymal cell; o, odontoblast; si, stratum intermedium; and sr, stellate reticulum.

to an incisor that grows continuously (Kollar and Baird, 1969; Jernvall, J., personal communication). Hence, our demonstration that the mesenchyme controls epithelial *lunatic fringe* expression is in line with the possibility that the mesenchymal control of the continuous epithelial growth involves modulation of the Notch pathway affecting fate determination of the stem cells.

FGFs May Be Mesenchymal Factors, which Couple the Proliferation and Determination of Epithelial Stem Cells by Stimulating Cell Division and Lunatic Fringe Expression

It is not clear whether the maintenance and differentiation

of stem cells and their cell division are regulated independently by distinct signals or whether there are signals that couple the different processes. It is also not known how similar the molecular mechanisms are between stem cells in different tissues, although there is evidence suggesting that there may be significant similarities (Morrison et al., 1997). Of the various regenerating tissues in vertebrates, the tooth resembles developmentally other derivatives of the epithelium such as skin, gut and, in particular, hair follicles. In all these tissues, the growth and differentiation of the regenerating epithelium is controlled by interactions with the underlying mesenchymal cells (Thesleff et al., 1995). The continuously growing incisor bears significant similarities histologically to the hair follicles as in both or-

gans the regenerating epithelium encompasses a mesenchymal papilla, which is necessary for the growth of the epithelium. Stem cells have been located in skin keratinocytes (Jones et al., 1995) and hair follicle epithelium (Cotsarelis et al., 1990), but possible mesenchymal factors involved in their regulation have not been examined. Interestingly, the stem cells in skin and hair follicle epithelium as well as in the gut are located among the basal epithelial cells and are associated with the basement membrane, whereas the putative dental stem cells were located in the stellate reticulum in close vicinity to the suprabasal aspect of the basal epithelial cells and, hence, did not contact the basement membrane.

Our localization of the expression of *Fgf-10* and *Fgf-3*, two signal molecules that we have recently identified as proliferative signals from mesenchyme to epithelium during early tooth morphogenesis (Kettunen, P., N. Itoh, and I. Thesleff, manuscript submitted for publication), suggested that they might function in the regulation of the continuous growth of the incisor epithelium. The expression of both *Fgfs* was restricted to the mesenchyme underlying the rapidly proliferating inner enamel epithelium. In addition, *Fgf-10* expression extended more apically and surrounded the entire cervical loop. Furthermore, the FGF receptors that are known to bind these particular FGFs, namely the IIIb splice forms of FGFR1 and FGFR2 were expressed in the cervical loop epithelium, *Fgfr1b* mRNA being specifically intense in the basal epithelial cells facing the mesenchyme. These findings supported roles for FGF-3 and FGF-10 as signals mediating the effects of mesenchyme on epithelium, and our bead implantation experiments showed that FGF-10, in fact, stimulated proliferation in isolated cervical loop epithelium in vitro. It is apparent that they stimulated cell division in the transit-amplifying cell population in the cervical loop, and we also suggest that they stimulated proliferation of the stem cells (Fig. 10 a).

Interestingly, FGF-10 also stimulated *lunatic fringe* expression in the isolated cervical loop epithelium and, thereby, mimicked the effect of the dental mesenchyme in recombination cultures. We have recently shown that FGF-10 has a similar stimulatory effect on dental epithelial *lunatic fringe* expression during early budding morphogenesis of mouse teeth (Mustonen, T., unpublished results). As discussed above, the regulation of *lunatic fringe* expression likely affects Notch signaling (Cohen et al., 1997; Fleming et al., 1997; Panin et al., 1997; Klein and Arias, 1998) and, thereby, perhaps the cell fate of the stem cells in the cervical loop (Fig. 10 b). We propose that FGFs may be regulatory signals for stem cells in the tooth and perhaps also in other tissues. FGF stimulates cell division in neural stem cells, whereas removal of FGF permits their differentiation to neurons (Gritti et al., 1996). The expression of *Fgf-3* and *Fgf-10* in the dental mesenchyme was downregulated exactly in the region where *lunatic fringe* was downregulated in the inner enamel epithelium differentiating into ameloblasts, and it is possible that this arrest in FGF signaling contributes to terminal differentiation of the cells.

In conclusion, we suggest that the Notch 1 expressing stellate reticulum cells in the cervical loop of the continuously erupting mouse incisor are stem cells that have the

capacity to self-renew and to generate progeny that are fated to differentiate into the various cell types of the dental epithelium, most importantly the enamel-producing ameloblasts. Based on the localization of slowly dividing cells among the peripheral stellate reticulum cells, the expression of genes in the Notch and FGF pathways, and the findings that FGF-10 stimulates both cell proliferation and expression of *lunatic fringe* in the cervical loop epithelium, we propose a model for the molecular mechanism of the putative dental stem cell lineage (Fig. 10 b). We propose that Notch receptors influence the maintenance of the stem cell state, and that the dental mesenchyme controls their proliferation and differentiation by FGF signals directly stimulating stem cell division and regulating the Notch pathway via stimulation of *lunatic fringe* expression.

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