Cessation of Fgf10 signaling, resulting in a defective dental epithelial stem cell compartment, leads to the transition from crown to root formation

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Mouse, rat and human molars begin to form root after the completion of crown formation. In these teeth, fibroblast growth factor (Fgf) 10 disappears in the transitional stage from crown formation to root. By contrast, rodent incisors and vole molars demonstrate continuous growth, owing to the formation and maintenance of a stem cell compartment by the constant expression of *Fgf10*. To clarify the relationship between root formation and disappearance of *Fgf10*, we carried out two experiments for the loss and gain of Fgf10 function. First, we examined postnatal growth in the incisors of *Fgf10*-deficient mice, which have the defect of a dental epithelial stem cell compartment referred to as 'apical bud', after implantation under the kidney capsule. The growth at the labial side in the mutant mice mimics the development of limited-growth teeth. 5′-Bromo-2′-deoxyuridine (BrdU) labeling and cytokeratin (CK) 14 and Notch2 immunostaining suggested that the inhibition of inner enamel epithelium growth and the more-active proliferation of the outer enamel epithelium and/or stellate reticulum result in Hertwig's epithelial root sheath formation. Second, we examined the effects of *Fgf10* overexpression in the transitional stage of molar germs, which led to the formation of apical bud involving in the inhibition of HERS formation. Taken together, these results suggest that the disappearance of Fgf10 signaling leads to the transition from crown to root formation, owing to the loss of a dental epithelial stem cell compartment.

KEY WORDS: Fibroblast growth factor 10, Apical bud, Inner enamel epithelium, Outer enamel epithelium, Hertwig's epithelial root sheath (HERS)

INTRODUCTION

Teeth are epithelial appendages located at the entrance to the digestive tract and possess a complex morphology consisting of different arrangements, shape and number of cusps, crown size, and a manner of growth that is evolutionarily dependent on dietary habits. The exquisitely functional form of a developing tooth is the result of precise coordination between the processes of cell proliferation, differentiation and death (Salazar-Ciudad et al., 2003; Tucker and Sharpe, 2004). These processes are regulated by the sequential and reciprocal interactions between the oral ectoderm and the neural crest-derived mesenchyme, as well as other ectodermal organs (Pispa and Thesleff, 2003). Though several signaling pathways and transcription factors have been implicated in the regulation of molar crown development, relatively little is known about the regulatory mechanisms of root development. It has recently been reported that NFI-C/CTF (nuclear factor I-C/CAAT-box transcription factor) is essential for root or root analog formation during tooth development (Steele-Perkins et al., 2003). *Nfic*-null mice exhibit the uniform lack of lower incisors. Interestingly, despite forming the crown of molar and the crown analog of upper incisors, the root and the root analog were defective in their teeth. Furthermore, it has been speculated based on gene expression studies during mouse molar root development that

some growth factors, including bone morphogenetic proteins (BMPs), epidermal growth factors (EGFs) and transcriptional factors (i.e. Msx1, Msx2 and Runx2), are related to the growth and differentiation of odontoblasts and/or cementoblasts, and the mineralization of dentin and/or cementum (Yamashiro et al., 2003). An investigation of tooth phenotypes in follistatin knockout mice and of transgenic mice overexpressing follistatin under the keratin 14 promoter demonstrated that follistatin regulates the decision between the crown and root analogs of incisors by asymmetrically inhibiting ameloblast differentiation by antagonizing BMP signaling (Wang et al., 2004a). It was not clear, however, whether follistatin regulates this process during molar development, as follistatin mRNA was not expressed in Hertwig's epithelial root sheath (HERS) during root development (Wang et al., 2004b).

In this study intended to elucidate the mechanism for the transition from crown morphogenesis to the initiation of root formation, we focus on the differential gene expression patterns of fibroblast growth factor (Fgf) 10 mRNA between continuously growing teeth and limited-growth teeth (Fig. 1A). The former tooth type has two continuously growing structures: the crown analog, consisting of enamel and dentin (the labial side of mouse incisors), and the root analog, consisting of dentin, cementum and the periodontal ligaments (lingual side). The growth at the lingual side mimics the root development of mouse molar germs (Fig. 1B,C). HERS and the fragmented epithelium are visible at the lingual side of the apical end, and periodontal ligaments are formed between the fragmented epithelium. In the teeth, Fgf10, which is continuously expressed at the apical end of mouse incisors (Fig. 1A) (Harada et al., 1999), plays a role in the maintenance of a dental epithelial stem cell compartment referred to as 'apical bud' (Harada et al., 2002b; Harada and Ohshima, 2004). The apical bud is an epithelial bulge consisting of a basal epithelium producing inner enamel epithelium (IEE), stellate

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DEVELOPMENT

reticulum and outer enamel epithelium (OEE). The border between the basal epithelium, which expresses lunatic fringe mRNA, and the stellate reticulum, which expresses *Notch1* mRNA in the apical bud, has been identified as the location of dental epithelial stem cells (Harada et al., 1999). Plural epithelial bulges show histological structures similar to the apical buds in guinea pig molars, which are continuously growing (Harada et al., 2002a; Ohshima et al., 2005). In a recent study, the constant expression of *Fgf10* was also observed at the apical ends of sibling vole molars, which are also continuously growing (Fig. 1A) (Tummers and Thesleff, 2003). However, in mouse, rat and human molars, the expression of *Fgf10* disappears when crown morphogenesis shifts to root formation (Fig. 1A) (Kettunen et al., 2000; Tummers and Thesleff, 2003). Neither histological structures

(e.g. an apical bud) nor gene expression patterns characteristic of a dental epithelial stem cell compartment is observed in HERS during root development. In mouse molar germs, the disappearance of Fgf10 signaling leads to the lack of a regulatory system for the formation and maintenance of a dental stem cell compartment. Hence, to clarify the relationship between root formation and the loss of a dental stem cell compartment, we attempted to observe the growth of *Fgf10*-deficient mouse incisors by transplanting them into the kidney capsule. We have also examined the effects of transient *Fgf10* overexpression during HERS formation in mouse molar germ cultures.

MATERIALS AND METHODS

Fgf10-deficient mice

The animal experiments were approved by the Committee on Animal Experiments, Osaka University Graduate School of Dentistry, Osaka, Japan. Heads from $Fgf10^{-/-}$ mice were obtained at the desired embryonic stages from intercrosses of heterozygous breeding pairs. Genotyping was performed as described previously (Sekine et al., 1999).

Incisor germ transplantation

Incisor germs were dissected from the mandibles of $Fgf10^{+/+}$ or $^{+/-}$ and $Fgf10^{-/-}$ mice at embryonic day 19 (E19). For transplantation of the incisor germs, the apical end regions were mechanically separated from the germs using an 18G needle (Fig. 2). The regions were transplanted underneath the kidney capsules of female mice (C57BL/6, PN w6), allowed to incubate for 3 weeks and removed. Eighty percent of the explants (wild type, 24; mutant, 16) grew well. The tissues were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (pH 7.2), decalcified in 12.5% EDTA in PBS. For observation of translucent specimens, the samples were treated with graded ethanol and acetone-ethanol, repeatedly. For Hematoxylin and Eosin (HE) staining, the samples were dehydrated in graded ethanol, embedded in paraffin wax and sectioned. For ultrastructural analysis, the tissues were also fixed in 4% PFA + 2.5% glutaraldehyde in PBS (pH 7.2) and decalcified in 12.5% EDTA in PBS. The samples were subsequently postfixed in 1% OsO_4 reduced with 1.5% potassium ferrocyanide, dehydrated in an ascending series of ethanol and finally embedded in Epon 812 (Taab, Berkshire, UK). Semi-thin sections (1 μm in thickness) were stained with Toluidine Blue and ultra-thin sections (70 nm in thickness) were double-stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7100 transmission electron microscope. For comparison with normal root formation, the upper second molars of ICR mice at postnatal week three (PN w3) were used.
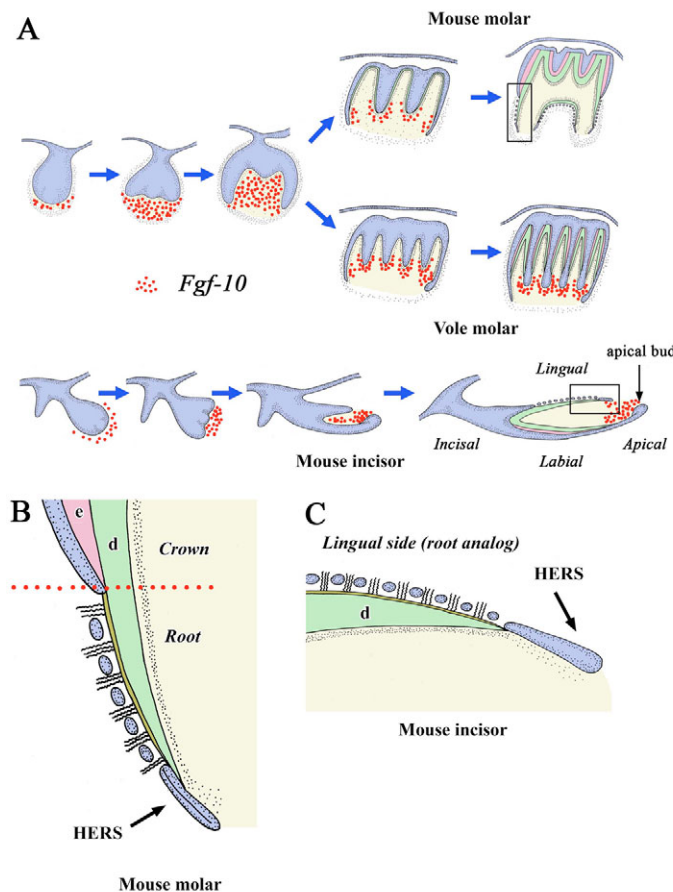

Fig. 1. Root development in mouse molar, mouse incisor and vole molar. (**A**) Differential gene expression of *Fgf10* during the development of mouse incisors and molars and sibling vole molars. In mouse molar development, *Fgf10* is expressed in the neighboring mesenchyme adjacent to the proliferating epithelium during crown morphogenesis, but disappears at the root formation stage. In the vole molar and the mouse incisor, *Fgf10* is expressed continuously only in the mesenchyme of the crown analog, but not in that of the root analog. However, slight expression of *Fgf10* is detectable around the tip of the lingual epithelium of mouse incisors. (**B**) Higher magnification of the boxed area in A during the root development of mouse molars. The broken red line indicates the border between the crown and root. (**C**) Higher magnification of the boxed area during root analog (lingual side) formation in mouse incisors. The histological features at the lingual side (C) mimic the root development of mouse molars (B). The HERS-like epithelial sheath and its fragmented epithelium, like Malassez epithelial rests, are visible at the apical end. Periodontal ligaments are formed between the fragmented epithelium. d, dentin; e, enamel.
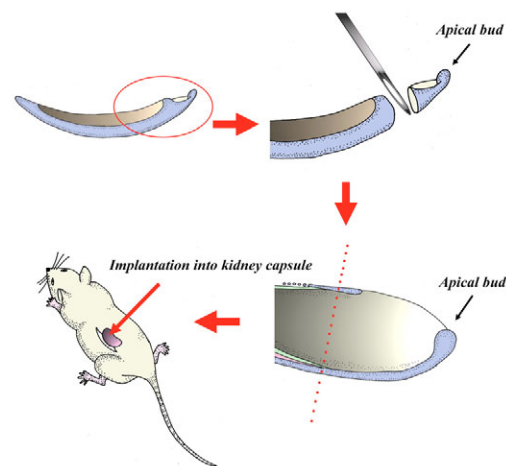

Fig. 2. Incisor germ transplantation. To observe the growth of the mutant incisors, we implanted the apical regions of mutant incisors under kidney capsules. Because a tooth germ is too large to implant under a kidney capsule, we separated the region surrounding the apical end from the tooth germ and implanted only the apical end. After 3 weeks of incubation, we removed the implants.

Molar germ organ culture and transfection of *Fgf10* cDNA

Organ culture was carried out as described previously (Harada et al., 1999). Molar germs (*n*=16) were dissected from the mandibles of ICR mice at postnatal day 1 (PN d1). The vector pIRES2-ZsGreen1 (BD Biosciences-Clontech, Palo Alto, CA, USA) containing the *mFgf10* cDNA (kind gift from Prof. Nobuyuki Itoh) was microinjected into the proximal dental papilla of the molar germs using a Transjector5246 (Eppendorf, Hamburg, Germany) or transfected using a BTX electroporation system T820 (voltage, 600 V; pulse, 30 μseconds; interval, 1 minute; three stimulations). The germs were cultured for 5 days. Cells expressing Fgf10 protein were identified by detecting ZsGreen1 fluorescence using a Keyence stereofluorescence microscope system VB-G25 (Keyence, Osaka, Japan). To observe molar germ development, we used samples (*n*=4) in which expression was restricted to the proximal dental papilla.

Immunohistochemistry

We used unfixed 9 μm frozen sections for immunostaining. The sections were incubated with mouse monoclonal anti-cytokeratin 14 (CK14, LL002, NOVOCASTRA, Newcastle, UK) and rabbit anti-Notch2 (R&D Systems, Minneapolis, MN, USA) antibodies for 2 hours at room temperature. Anti-mouse VECTASTAIN ABC-kit (PK-6101, Vector Laboratories, Burlingame, CA, USA), donkey anti-mouse antibody directly conjugated to Alexa488 (Invitrogen, Carlsbad, CA, USA) and goat anti-rabbit antibody directly conjugated to Alexa546 (Invitrogen) were used as secondary antibodies.

Cell proliferation assays

We carried out 5′-bromo-2′-deoxyuridine (BrdU) labeling analyses to detect cellular proliferation. To label the mutant incisors growing under the kidney capsules, 1 mg/100 g (body weight) BrdU (Sigma-Aldrich, St Louis, MO, USA) was injected into the abdominal cavity of recipient mice (those transplanted with the mutant incisor germs), which were sacrificed 2 hours later, at which point the incisor immunostaining was carried out. In vitro cultures for observing root development of mouse molars were performed as previously described (Fujiwara et al., 2005). Explants from PN d3 and d5 mice were incubated for 1 day and then washed with Hanks' balanced salt solution in the presence of BrdU (0.5 mg/ml for 3 hours), embedded in paraffin, and serially sectioned into 5 μm sections. BrdU was detected on the sections using a BrdU staining kit (Calbiochem, Oncogene Research Products, Cambridge, MA, USA) according to the manufacturer's instructions, and visualized with streptavidin-HRP and DAB. BrdU-stained specimens were then weakly counterstained with Hematoxylin. To compare the mitotic activity between the inner and outer layers of the dental epithelium in the mutant incisors and between the IEE and OEE in cultured molar germs, we counted the number of BrdU-immunopositive cells and all cells in the inner and outer layers and the dental papilla, and then estimated the mitotic index of each. Dental epithelial cells were distinguished from mesenchymal cells by CK14 immunostaining of serial sections.

RESULTS

Incisor growth under kidney capsules

Because *Fgf10*$^{-/-}$ mice die immediately after birth (they lack lungs), it is impossible to observe incisor growth after that time. To solve this problem, we transplanted the apical region of the incisors into the kidney capsules. The adult stem cells of the dental epithelium and the mesenchyme are known to exist in the apical bud and the surrounding area of the incisors, respectively (Harada et al., 1999; Harada and Ohshima, 2004). Thus, even if the region is separated from the incisor germ, the snippets are able to grow into parent incisors under the kidney capsule. After the kidney was removed from the abdominal cavity of the recipient three weeks after implantation, both wild-type and mutant incisors were visible at the surface of the kidney (Fig. 3C). The apical regions of the teeth were covered by alveolar bone (Fig. 3C, asterisk), and their teeth erupted from sockets of bone. The mutant teeth were smaller than the wild-type teeth. To observe the incisor structure, we prepared translucent
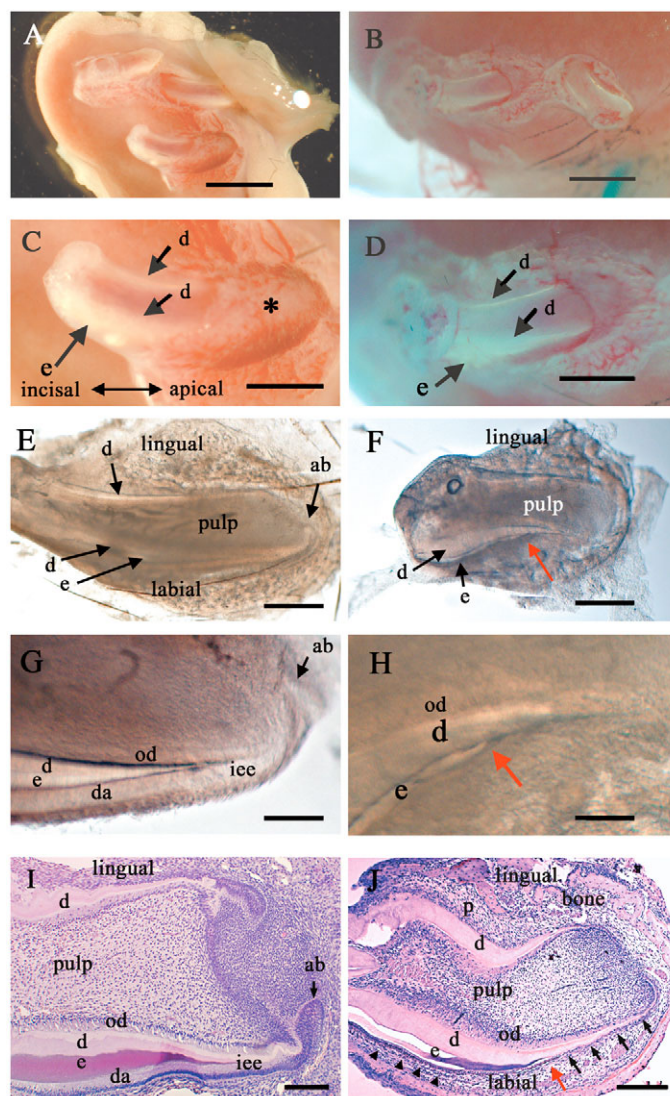

Fig. 3. Incisor growth under kidney capsules. Wild-type and mutant incisors growing under kidney capsules. (**C,D**) Higher magnification of wild-type (**A**) and mutant (**B**) incisors, respectively. (**E-H**) Translucent wild-type (E,G) and mutant (F,H) specimens. (G,H) Higher magnification of the apical region at the labial side of wild-type (E) and mutant (F) incisors, respectively. (**I,J**) HE staining of wild-type (I) and mutant (J) incisors. The apical regions of wild-type mouse incisors grew under the kidney capsules similar to naturally growing incisors in the mandible. The apical region is covered by alveolar bone (C, asterisk), and incisors erupt from the sockets of the bones. In the wild type, the enamel, the apical bud, the inner enamel epithelium, differentiated ameloblasts and odontoblasts are clearly recognizable at the labial side. The lingual side is root analog consisting of dentin, cementum and periodontal tissues. These features are consistent with incisors growing in the mandible. However, at the labial side of the mutant incisor, thin enamel is visible, along with enamel formation over half the developing tooth. Red arrows indicate the border between the crown and root (F,H,J). Neither the apical bud nor the dental epithelium at the surface of the dentin is recognizable. ab, apical bud; d, dentin; da, differentiated ameloblast; e, enamel; iee, inner enamel epithelium; od, odontoblasts; p, periodontal ligament. Scale bars: 2 mm in A; 1 mm in B,C; 500 μm in D,E; 300 μm in F; 100 μm in G-I; 150 μm in J.

specimens (Fig. 3E-H) and HE stained 4 μm sections (Fig. 3I,J). The translucent specimens were useful for three-dimensional structural observations. In wild-type incisors, enamel and dentin were clearly formed at the labial side. The apical bud, IEE and the related differentiated ameloblasts were visible in the translucent specimens. The histological structure of the wild-type incisors was similar to the parent incisor germs in the mandibles of E19 mice. The differentiation of ameloblasts and odontoblasts was normal. Enamel matrix was formed at the labial side, and dentin was visible at both the labial and the lingual side. The related dental epithelium, consisting of IEE, stratum intermedium, stellate reticulum and OEE, was visible at the apical end. However, in the mutant incisors growing under the kidney capsules, enamel was seen only in incisal end of the labial side (Fig. 3F). The rest was composed only of dentin. The epithelial layers of ameloblasts, IEE and the apical bud were not observed at the surface of the enamel and dentin (Fig. 3H,J). The features of the labial side resembled the transitional region from crown to root as seen in the mice molar. The border between crown and root was marked clearly, like the cervical margin of molar teeth (Fig. 3H,J, red arrows). The surface of the enamel was covered with dental epithelium consisting of cuboidal cells (Fig. 3J, arrowheads); this differed from secretory ameloblasts, which exhibit a tall and columnar shape and produce enamel matrix. The epithelial layer was not clearly identifiable at the surface of the dentin (Fig. 3J, arrows). Dentin formation and odontoblast differentiation were normal.

Immunohistochemical analysis

To analyze the structure of mutant incisors growing in the kidney capsules, we examined the expression of CK14 and Notch2 by immunohistochemistry (Fig. 4). The wild-type labial dental epithelium was continuously connected from the apical to the incisal end (Fig. 3I). However, in mutant incisors stained by HE, no labial epithelium was observed at the surface of the dentin (Fig. 3J). Immunostaining for CK14, however, clearly showed the localization of dental epithelial cells that were unrecognizable in the HE-stained sections (Fig. 4). The labial epithelium was visible as epithelial fragments similar to epithelial rests of Malassez at the dentin surface (Fig. 4A arrowheads), and epithelial sheaths at the apical end consisted of two or three layers of cells with a histological structure similar to HERS, which was apparent during root formation of the molar germ. Because HERS had been reported to express *Notch2* mRNA (Tummers and Thesleff, 2003), we double stained for CK14 and Notch2 in the mutant incisors (Fig. 4). The results indicated that the epithelium expressed both CK14 and Notch2. Immunostaining for Notch1, which is expressed by putative stem cells within the apical bud and stratum intermedium, was not observed in the mutant incisors (data not shown). Hence, it was likely that the epithelium was made up of HERS and involved in the induction of root dentin formation. Interestingly, Notch2 staining was observed in lingual epithelium (Fig. 4J, larger arrow), as well as in OEE and the stellate reticulum of the labial epithelium in wild-type incisors (Fig. 4J, smaller arrows).

Localization of proliferating cells at the initiation of HERS formation

To reconfirm the hypothesis that HERS originated from the stellate reticulum and/or OEE, we examined the localization of the proliferating cells at the initiation of HERS formation (Fig. 5D). To capture the precise environment surrounding the start of HERS formation and its subsequent growth, we used an in vitro root culture system (Fujiwara et al., 2005). In vitro cultures are effective for
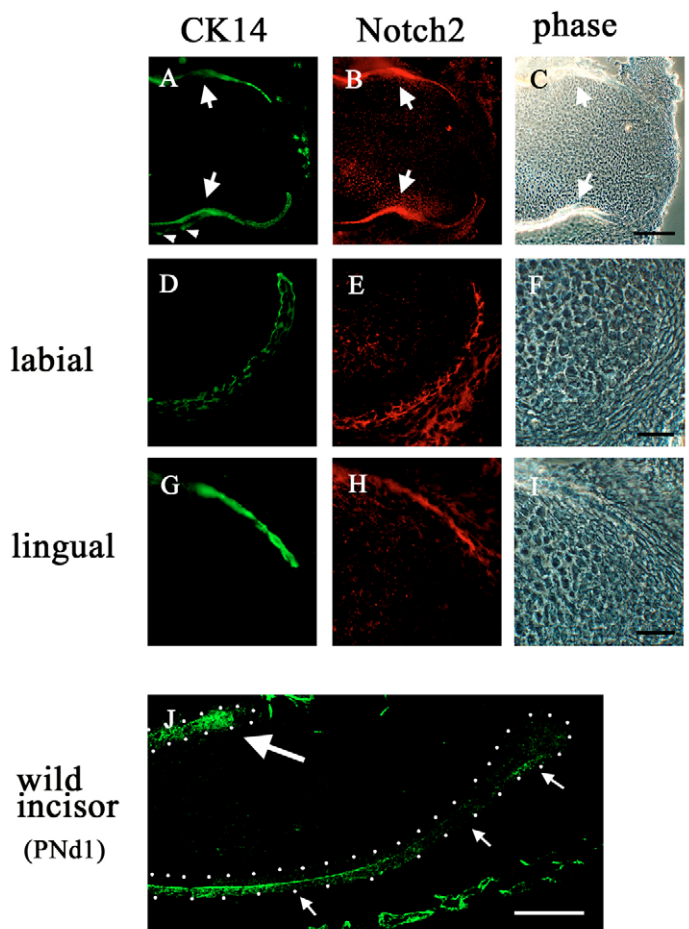

Fig. 4. Immunohistochemical analysis. Double immunostaining of CK14 (A,D,G) and Notch2 (B,E,H) in the epithelial sheaths of mutant incisors grown under kidney capsules (A-I), and staining for Notch2 in lower incisors of wild-type mice at PN d1 (J). (**A-I**) Both labial and lingual HERS are strongly labeled by anti-CK14 and Notch2 antibodies. Fragmentation of the dental epithelium (arrowheads), similar to Malassez epithelial rests, is also recognizable at the dentin surface. Arrows indicate non-specific staining against calcified dentin. (D-F,G-I) Higher magnifications of the labial HERS and of the lingual HERS, , respectively, in A-C. (C,F,I) Phase pictures of A,B, D,E and G,H, respectively. (**J**) Immunostaining for Notch2 at the apical end of PN d1 mouse lower incisors. Notch2 is expressed in the OEE and the stellate reticulum at the labial side (J, smaller arrows), and in the epithelial sheath at the lingual side (J, larger arrow). Scale bars: 25 μm in A-C; 10 μm in D-I; 200 μm in J.

BrdU labeling during a strict time frame, because there is almost no time lag between the time of BrdU addition and cellular uptake of the BrdU. The initiation of HERS formation was visible after 1 day in the molar cultures of PN d3 mice, at which time the BrdU labeling was carried out for 3 hours. BrdU-labeled cells were more apparent in the OEE than in the IEE (Fig. 5D,G). The results were similar in the HERS of the cultures of PN d5 mice (Fig. 5E,G). These results showed that although IEE cells had nearly finished proliferating, the OEE proliferated more actively. We were unsuccessful in our attempts to find a similar situation in the mutant incisors growing under kidney capsules, because the growth rate of the transplants
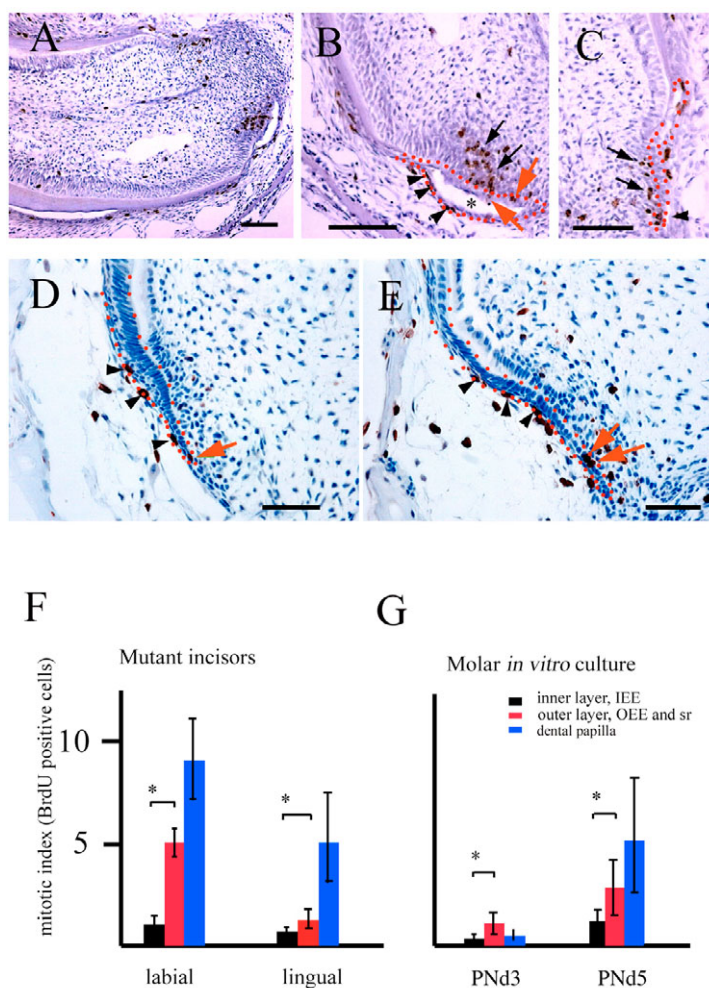
Fig. 5. Localization of proliferating cells at the initiation of HERS formation. BrdU labeling analysis of mutant incisors grown under kidney capsules (A-C) and of the formation of HERS in in vitro cultures (D,E). (**A-C**) Small amounts of labels are detected in the dental epithelium and in the surrounding mesenchyme at the apical end. (B,C) Higher magnification of BrdU immunostaining at the labial and lingual sides. BrdU-positive cells are visible in the outer layer (arrowheads) and in the inner layer (red arrows) of the epithelial sheath. Black arrows indicate BrdU-positive papilla cells. An asterisk indicates artificial space separating the inner and outer layers of the epithelial sheath formed when making sections. (**D,E**) BrdU staining of the mouse molar germ cultures at PN d3 and d5. The number of BrdU-positive cells (red arrows) in the IEE and the inner layer of HERS is much smaller than the number of positive cells in the OEE and the outer layer (arrowheads). (**F,G**) BrdU-labeled cells in the inner and outer layers and dental papilla cells in mutant incisors (F), and in vitro cultures of molar germs of PN d3 and d5 (G). More BrdU-positive cells are visible in the outer layer than in the inner layer on either side (F). Comparing the OEE/stellate reticulum (sr) and the IEE in molar germ at PN d3, or comparing the outer and inner layers at PN d5, showed similar results as those found in the mutant incisors. The total number of BrdU-positive cells increased at PN d5. Data are presented as mean±s.d. Significant differences in the mitotic indices between two groups (with error bars) were calculated using a Student's t-test (asterisks indicate $P<0.01$). The broken red line indicates the border between the epithelium and the mesenchyme, which is identified by the CK14 immunostaining of serial sections. Scale bars: 100 μm in A; 50 μm in B-E.

was unstable and impossible to control. However, BrdU labeling analyses in the epithelial sheaths of mutant incisors also showed greater numbers of positive cells in the outer epithelium than in the inner epithelium (Fig. 5A-C,F). These results were identical to those of a previous paper demonstrating the proliferating cells of mouse molar HERS in in vitro cultures (Fujiwara et al., 2005).

Analysis of semi-thin cross-sections and electron microscopy

To address a suspicion that these sections were not representative of the long axes, we made serial semi-thin cross-sections from the apical to the incisal ends. The cross-sections clearly showed the root formation of the mutant incisors. The dentin was visible as a squared circle, and dental follicle cells and the cementum matrix deposits were visible over the entire outer surface of the dentin (Fig. 6A). Furthermore, a small number of dental epithelial cells remained at the surface in patches (Fig. 6B,C, arrowheads). No deposition of an enamel matrix was detectable. To observe the formation of the periodontal tissue in detail, we examined the sections by electron microscopy. Cementoblast-like cells bundling collagen fibers, which are involved in the formation of periodontal ligament, were seen at the labial side of the mutant incisors (Fig. 6D, asterisk). The deposition of the cementum matrix and fragmented epithelial cells (Fig. 6D, arrowhead) were also visible. These results indicate that even on the labial side, the periodontal tissues appeared to form around the root dentin. This situation is similar to the root formation of mouse molars (Fig. 6E,F).

Overexpression of *Fgf10* leads to the formation of apical buds in molar germs

Finally, to determine whether Fgf10 expression inhibits the transition from crown to root formation, we transiently overexpressed Fgf10 protein in the lower first molar germs at PN d1. We monitored Fgf10 expression in the culture of molar germs by the expression of green fluorescence protein ZsGreen1. Before the experiments, we transfected the Fgf10 expression vector into HAT-7 cells (dental epithelial cell line), which do not express *Fgf10*, and confirmed the co-expression of Fgf10 and ZsGreen1 by reverse transcription PCR (data not shown). Next, the vector was transfected into the proximal dental papilla cells of molar germs and the germs were cultured for 5 days. Cells expressing Fgf10 protein were observed by green fluorescence only at the proximal dental papilla 2 days after the transfection (Fig. 7A, arrow), and further extended along with the growth of the distal epithelium 3 days later (Fig. 7B). The proximal dental epithelium expanded to a greater extent than the distal epithelium (Fig. 7C, arrow). Frozen sections clearly showed the formation of epithelial bulges like apical buds of mouse incisors (Fig. 7D, larger arrow) and an expanded inter-cuspal epithelium (Fig. 7D, arrowheads) following Fgf10 expression. However, the distal epithelium (control side) formed the HERS, consisting of two or three epithelial cell layers (Fig. 7D, smaller arrow). Because the transient expression of Fgf10 did not last beyond 1 week (data not shown), we could not observe the effects on root formation of molar germs in vitro or under the kidney capsule.
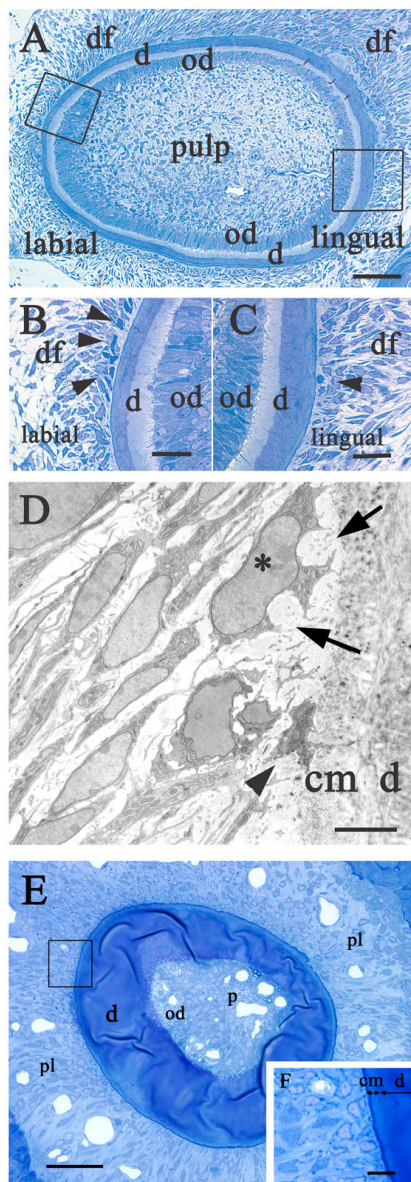
Fig. 6. Analysis of semi-thin cross-sections and electron microscopy. Transverse sections of the apical regions of mutant incisors grown under kidney capsules (**A-C**), and electron microscopic observation of a labial region (crown analog side) at an apical end (**D**). (**E,F**) Transverse section of upper second molar mesio-buccal root of PN w3 mice. (B,C,F) Higher magnifications of boxes in A and E. (A) This transverse section is very similar to that showing root (E,F) in a mouse molar. The ring is made of dentin, and the inside is dental pulp composed of odontoblasts and dental pulp cells. At the outer surface of dentin, the epithelial cell rests (B,C, arrowheads) and the invading dental follicle cells were apparent. (D) Electron microscopic analysis of the labial region of a mutant incisor. Cementum matrix is deposited at all dentin surfaces. Cementoblast-like cells (asterisk) are observed at all outer dentin surfaces. These cells have well-developed organelles and retain thick bundles of collagen fibers for periodontal ligament (arrows). A fragmented epithelial cell rest is also recognizable (D, arrowhead). cm, cementum matrix; d, dentin; od, odontoblasts; df, dental follicle; p, pulp; pl, periodontal ligament. Scale bars: 100 μm in A; 25 μm in B,C; 5 μm in D; 50 μm in E; 10 μm in F.
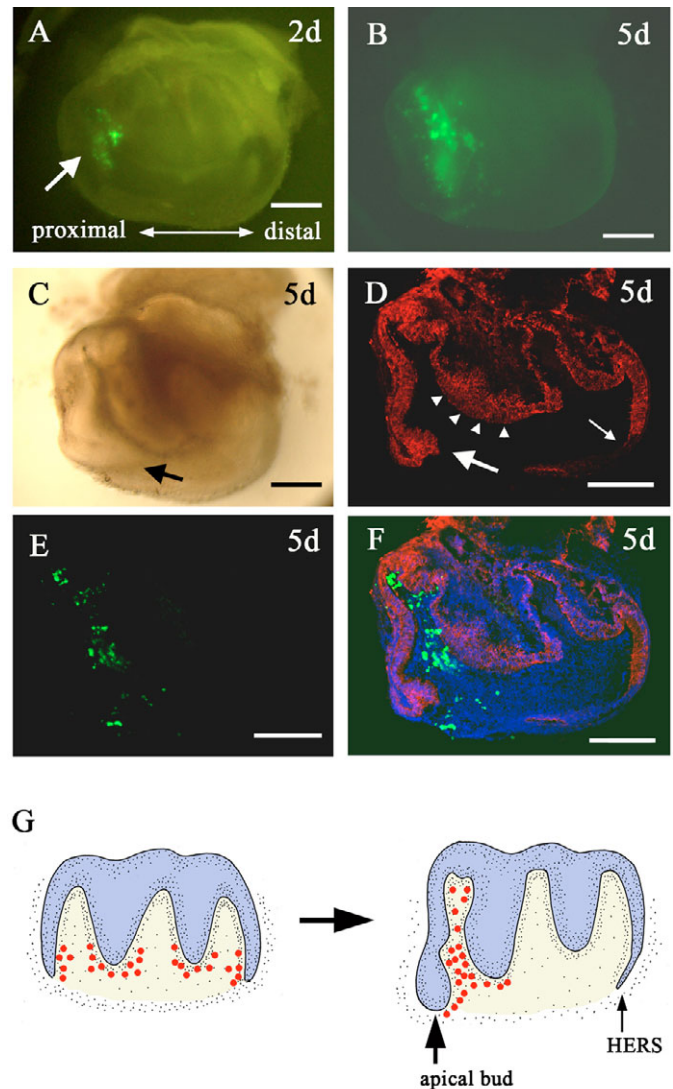

Fig. 7. Overexpression of Fgf10 leads to the formation of apical bud in molar germs. Stereomicroscopic observation (A-C) and frozen sections (D-F) of cultured molar germs transfected with *Fgf10* cDNA. (**A**) The molar germ was cultured for 2 days after the transfection of *Fgf10* cDNA expression vector. Fgf10 expression was identified by expression of green fluorescence protein using a fluorescence stereomicroscope and was restricted to the proximal dental papilla cells (arrow). (**B**) The molar germ was cultured for 5 days. The expression expands along with the growth of the dental epithelium. (**C**) Stereomicroscopic observation of the cultured molar germ. The proximal dental epithelium (arrow) elongates and extends to a greater extent than the distal dental epithelium (control side). (**D**) CK14 immunostaining (red) of a frozen section clearly shows the formation of an apical bud (larger arrow) and an expanded inter cuspal epithelium (arrowheads) following *Fgf10* overexpression. However, at the control (distal) side, the epithelium forming the HERS consists of two or three epithelial cell layers (smaller arrow). (**E**) The expression of Fgf10 (green) is seen in the proximal dental papilla cells between the proximal dental epithelium and inter-cuspal epithelium. (**F**) The section was counterstained with DAPI (blue) and the three colors were merged. (**G**) Schematic illustration of the effects of *Fgf10* overexpression. After the morphogenesis of crown, *Fgf10* mRNA disappears and the formation of HERS starts (Fig. 1A). However, at the proximal side, ectopic overexpression of Fgf10 protein leads to apical bud formation. Conversely, at the distal side (control), HERS forms as Fgf10 disappeared. Scale bars: 20 μm.
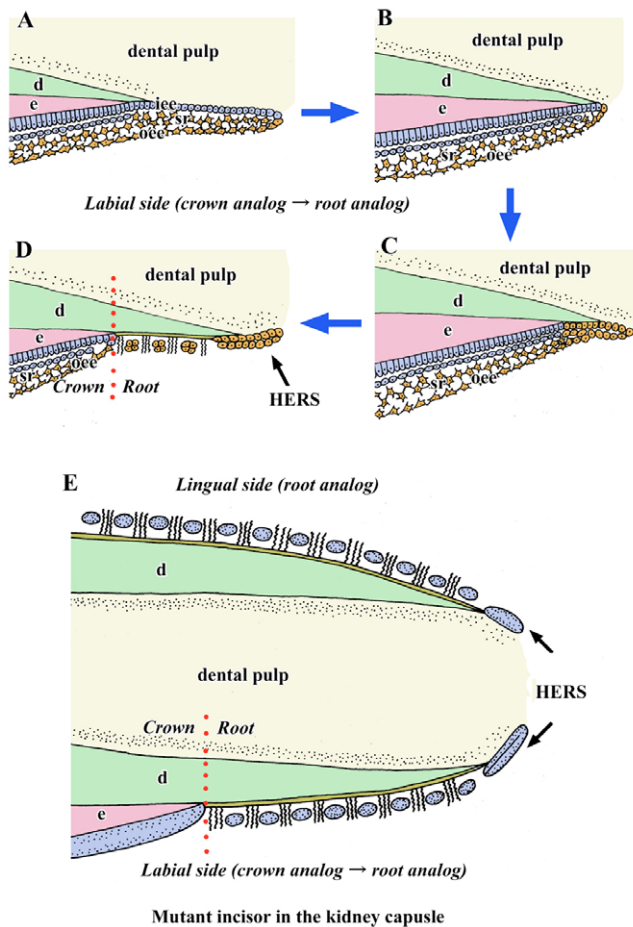
Fig. 8. Hypothesis of HERS formation. Schematic illustration of our hypothesis of the process of HERS formation (**A-D**) and of mutant incisors grown under kidney capsules (**E**). The more active proliferation of OEE and stellate reticulum elongates beyond the IEE after cessation of crown formation, leading to the formation of HERS. OEE, stellate reticulum and HERS expressed both Notch2 and CK14 with strong intensity (A-C, dark-yellow cells). The epithelial sheaths proliferate, and fragmentation occurs at the surface of the dentin. Dental follicle cells migrate among the fragmented epithelium and make up the periodontal tissues (D). (E) In mutant incisors, the histological features at the labial side mimic the development of mouse molar germ. A broken red line indicates the border between the crown analog and the root analog at the labial side. d, dentin; e, enamel; iee, inner enamel epithelium; oee, outer enamel epithelium; sr, stellate reticulum.

DISCUSSION

In this study, we suggest a mechanism for the transition from crown formation to root development. Based on comparisons of *Fgf10* gene expression patterns in developmental processes between continuously growing teeth, such as mouse incisors and vole molars, and limited-growth teeth, such as mouse molars (Fig. 1), we conclude that in continuously growing teeth, *Fgf10* mRNA expression is constant, while in limited-growth teeth, *Fgf10* disappears after the initiation of root development. Here, we show that the incisors of *Fgf10* deficient mice, grown under kidney capsules, begin to form roots on the labial side following the completion of crown formation. However, the transient overexpression of Fgf10 at the beginning of root formation during molar development resulted in the formation of epithelial

bulges resembling apical buds. Hence, the disappearance of *Fgf10* is an important event in the transition from crown morphogenesis to root formation, and is one of the molecular switches instigating the initiation of root formation.

The stem cell compartment disturbs the transition from crown to root

The histological structure at the apical end of the labial dental epithelium in mutant incisors is very similar to that of the cervical loop of the molar germ, consisting of the IEE, stratum intermedium, stellate reticulum and OEE. Our previous data from in vitro cultures suggested that incisors, having no dental epithelial stem cells, lose the capacity for continuous growth throughout life. However, we could not observe the in vitro formation of dentin or periodontal tissues. Implantation into kidney capsules makes it possible to mimic the growth of the mutant incisor germ in vivo. Our data show that the mutant incisor germs form roots after completing crown formation at the labial side. Accordingly, the *Fgf10* deficiency causes the defective apical bud and the formation of structures mimicking the cervical loop of mouse molar germs, consequently leading to root formation. We previously presented another example exhibiting continuous formation of a crown analog (Harada, 2002a; Ohshima et al., 2005). Guinea pig molars also had plural epithelial bulges consisting of basal epithelium and stellate reticulum at the apical end. Taken together, the apical bud-like structure of the stem cell compartment has the capacity for constant production of ameloblasts, disturbing the transition from crown to root.

Is Fgf10 a stimulating factor for crown formation and/or a negative regulator of root formation?

During molar development, when the dental epithelium initiates root formation following the completion of crown morphogenesis, the expression of *Fgf10* in the dental papilla disappears (Kettunen et al., 2000; Tummers and Thesleff, 2003). In incisor germ and vole molar germ, the expression of *Fgf10* is not observed at the region of root formation. However, continuous expression of *Fgf10* leads to continuous formation of crown analog in the continuous growing teeth. Additionally, in our in vitro experiments, overexpression of *Fgf10* resulted in the formation of epithelial bulges resembling apical buds and the expansion of the inter-cuspal epithelium during molar development. Based on these data, *Fgf10* expression is closely associated with the formation of the epithelial stem cell compartment. Furthermore, it has been reported that Fgf10 plays a role in the production of the stratum intermedium through the induction of proliferation and the differentiation of the IEE (Kawano et al., 2004). The stratum intermedium is seen only at the proximal side of the IEE and differentiated ameloblasts during crown formation, but not in the root analog. These results suggest that Fgf10 signaling is required for both the formation of the tooth crown and the maintenance of the stem cell compartment. Conversely, it could be that Fgf10 inhibits the transition from crown formation to root development. However, two events related to these processes are paradoxical. First, why, despite Fgf10 expression around the HERS-like epithelial sheath at the lingual side, is crown analog not formed at the lingual side of mouse incisors? Wang and colleagues addressed this problem using follistatin knockout mice and transgenic mice overexpressing follistatin under the keratin 14 promoter (Wang et al., 2004b). Follistatin, when expressed in the lingual epithelium, inhibits the differentiation of ameloblasts by antagonizing BMPs and disturbing enamel formation. Follistatin may also be a negative regulatory factor in the selection of crown formation. Second, why does Fgf3, when expressed in the

mesenchyme adherent to the labial dental epithelium, not rescue the continuous formation of crown analog in mutant incisors? The deficiency of Fgf10 signaling leads to the lack of a stem cell compartment, because expression of *Fgf3* is not seen in the mesenchyme around the apical bud (Harada et al., 2002b). Accordingly, as previously described, even if the IEE can proliferate and differentiate in response to Fgf3 before birth, the IEE exhibits limited growth, as it is composed of transit amplifying cells. It follows that the root formation of mutant incisors is due to the lack of a dental epithelial stem cell compartment. During molar development, the disappearance of *Fgf10* causes the cessation of crown formation without the formation of a stem cell compartment.

Transition processes between crown morphogenesis and root development

Based on these results, we consider mechanisms for the transition from crown morphogenesis to root formation during the following three processes in mutant incisors: (1) cessation of IEE growth, (2) formation of HERS, and (3) fragmentation of dental epithelium. Previous studies using in vitro cultures have shown that the growth of mutant incisors decreases gradually and then stops (Harada et al., 2002b). Therefore, the IEE cells stop proliferating after several cell divisions and become differentiated ameloblasts. During molar development, ameloblasts do not exhibit proliferative activity after the IEE molds the crown morphology. Taken together, the termination of cell division and differentiation of IEE cells is one of the most important events in the transition from crown to root development.

Second, how is the HERS of molars or mutant incisors produced? The IEE and OEE have been thought to fuse below the level of the crown cervical margin. However, little evidence has supported this hypothesis. Fig. 5 shows that BrdU-labeled cells are observed in the OEE to a much greater degree than in the IEE at the initiation of HERS formation. During the following stage, the outer layer of the HERS preferentially proliferates in both the mouse molars and the mutant incisors. A recent paper reported that insulin-like growth factor (IGF) 1, a signaling molecule produced by dental follicles, induces the proliferation of HERS during root development (Fujiwara et al., 2005); the dental follicle cells then migrate downwards in coordination with the elongation of the OEE (Diekwisch, 2002). Furthermore, *Notch2* is expressed in the OEE and stellate reticulum during crown development of molars and incisors, and in the HERS during molar root development (Mitsiadis et al., 1995; Harada et al., 1999; Tummers and Thesleff, 2003). In this study, we show that the lingual epithelium of wild-type incisors and the HERS of mutant incisors express Notch2 receptors (Fig. 4). Taken together, we hypothesize that the OEE proliferates more actively than the IEE and elongates downwards below the crown cervical margin, producing a bilayered epithelial sheath referred to as the HERS.

Third, immunostaining for CK14 shows that the fragmentation of the dental epithelial sheath, an important event in root development, occurs at the labial side in mutant mice. Additionally, electron microscopy shows cementum matrix deposition at the surface of the dentin and the formation of thick collagen bundles for the periodontal ligament. Consequently, these events during root development are also caused by the deficiency in Fgf10 signaling.

Based on our data and previous work, the disappearance of Fgf10 signaling is key to the cessation of crown formation and the initiation of root development. It is crucial to elucidate the molecular mechanisms regulating the formation of both crown and root, as these mechanisms will contribute greatly to our understanding of the evolution of tooth morphology and growth patterns. The variety of *Fgf10* expression patterns is thought to facilitate the diversification of teeth: size, number of cusps and whether the teeth are of continuous or limited growth. Recently, a cis-element was identified that regulates *Fgf10* expression during inner ear development (Ohuchi et al., 2005). However, the molecular mechanisms controlling the expression of *Fgf10* in teeth have not yet been elucidated and remain the subject of future studies.

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