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DEVELOPMENT AND DISEASE

FGF10 maintains stem cell compartment in developing mouse incisors

Hidemitsu Harada[1,*], Takashi Toyono[1], Kuniaki Toyoshima[1], Masahiro Yamasaki[2], Nobuyuki Itoh[2], Shigeaki Kato[3], Keisuke Sekine[3] and Hideyo Ohuchi[4]

[1]Second Department of Oral Anatomy and Cell Biology, Kyushu Dental College, 2-6-1, Manazuru, Kokurakita-ku, Kitakyushu, 803-8580, Japan
[2]Department of Genetic Biochemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-shimo-Adachi-cho, Sakyo-ku, Kyoto City, Kyoto, 606-8501, Japan
[3]Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo 113-0032, Japan
[4]Department of Biological Science and Technology, Faculty of Engineering, University of Tokushima, 2-1 Minami-Jyosanjima-cho, Tokushima City, Tokushima 770-8506, Japan
*Author for correspondence (e-mail: hide-h@mail.kyu-dent.ac.jp)

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SUMMARY

Mouse incisors are regenerative tissues that grow continuously throughout life. The renewal of dental epithelium-producing enamel matrix and/or induction of dentin formation by mesenchymal cells is performed by stem cells that reside in cervical loop of the incisor apex. However, little is known about the mechanisms of stem cell compartment formation. Recently, a mouse incisor was used as a model to show that fibroblast growth factor (FGF) 10 regulates mitogenesis and fate decision of adult stem cells. To further illustrate the role of FGF10 in the formation of the stem cell compartment during tooth organogenesis, we have analyzed incisor development in *Fgf10*-deficient mice and have examined the effects of neutralizing anti-FGF10 antibody on the developing incisors in organ cultures. The incisor germs of FGF10-null mice proceeded to cap stage normally. However, at a later stage, the cervical loop was not formed. We found that the absence of the cervical loop was due to a divergence in *Fgf10* and *Fgf3* expression patterns at E16. Furthermore, we estimated the growth of dental epithelium from incisor explants of FGF10-null mice by organ culture. The dental epithelium of FGF10-null mice showed limited growth, although the epithelium of wild-type mice appeared to grow normally. In other experiments, a functional disorder of FGF10, caused by a neutralizing anti-FGF10 antibody, induced apoptosis in the cervical loop of developing mouse incisor cultures. However, recombinant human FGF10 protein rescued the cervical loop from apoptosis. Taken together, these results suggest that FGF10 is a survival factor that maintains the stem cell population in developing incisor germs.

Key words: Stem cells, FGF10, Incisor development, Epithelial-mesenchymal interactions, Mouse

INTRODUCTION

During embryogenesis, a single fertilized oocyte gives rise to a multicellular organism whose cells and tissues adopt differentiated characteristics or fates to perform the specified functions of each organ of the body. Many tissues and organs maintain a process known as homeostasis: as cells die, either by apoptosis or by injury, they are replenished. Additionally, the epidermis, hair, small intestine and hematopoietic system are all examples of adult tissues that exist naturally in a state of dynamic flux. Even in the absence of injury, these structures continually give rise to new cells, and are able to divide transiently, terminally differentiate and die. These regenerative tissues are maintained by adult stem cells, which have both the capacity to self-renew, to divide and create additional stem cells, and also to differentiate along a specified molecular pathway. In recent years tremendous advances have been made in our understanding of identification of adult stem cells (reviewed by Fuchs and Segre, 2000). However, molecular mechanisms for establishing adult stem cell compartments during embryogenesis have not been examined in detail.

The mouse incisor tooth is an excellent model for analyzing certain aspects of stem cell regulation and function (Harada et al., 1999). The continuous eruption of mouse incisors throughout the lifetime of an animal is maintained by the division of germs localized in the cervical loop of the apical region. We have previously shown that stem cells divide rarely and asymmetrically, and one daughter cell remains as an undifferentiated stem cell in the cervical loop, whereas the other cell moves to the incisal end, giving rise to transit-amplifying cells. Dental epithelial stem cells in the cervical loop give rise to four cell-lineages – inner enamel epithelium (ameloblast cell-lineage), stratum intermedium, stellate reticulum and outer enamel epithelium.

The ability to select among multiple terminal differentiation pathways and to generate cells that can migrate in the selected direction are characteristics that parallel those of the hair follicle bulge or of crypt cells of the small intestine (Rochat et al., 1994; Fuchs and Segre, 2000). These stem cells seem to rely upon mesenchymal cues for their survival and differentiation (Korinek et al., 1998; DasGupta and Fuchs, 1999; Millar et al., 1999). Epithelial-mesenchymal interactions play an essential role in regulating a wide variety of developmental processes, including those of the teeth (Thesleff and Sharpe, 1997; Peters and Balling, 1999; Hogan, 1999). Tissue recombination experiments have shown that the mesenchymal tissue controls advancing morphogenesis after initiation of tooth development and regulates the continuous growth of epithelium in the mouse incisor (Koller and Baird, 1969). Fibroblast growth factors (FGFs) play a crucial role in the developing tooth (Kettunen and Thesleff, 1999). Our recent studies have shown that mouse incisor mesenchymal cells express *Fgf3* and *Fgf10*, and their receptors, *Fgfr1b* and *Fgfr2b*, are expressed throughout the dental epithelium (Kettunen et al., 1998; Harada et al., 1999). Furthermore, bead-implantation assays have shown that FGF10 signaling from the mesenchyme indirectly regulated cell division and fate decision of epithelial stem cells by modulating the Notch pathway in the cervical loop via stimulation of *lunatic fringe* expression. The involvement of FGF10 in tooth morphogenesis was further indicated by the hypoplastic tooth organ in FGF10 null mice (Ohuchi et al., 2000).

In the present study, which focuses on mesenchymal molecular cues for forming the stem cell compartment during organogenesis, we used the developing mouse incisor germ cell as a model of regenerative tissues. To further examine the role of FGF10 during incisor development, we have designed two loss-of-function experimental lines. Analysis of developing incisor germ of *Fgf10*-deficient mice showed that FGF10 is not involved in the early signaling networks that regulate tooth initiation at early morphogenesis, but is involved in the establishment of adults stem cells. Other experiments using neutralizing antibody provided direct evidence that FGF10 prevents apoptosis in the stem cell compartment. We propose a model that demonstrates the formation of adult stem cells during organogenesis and shows that stem cells are maintained as undifferentiated cells by mesenchymal signals.

MATERIALS AND METHODS

Fgf10-deficient mice

Heads from *Fgf10*$^{-/-}$ mice were obtained at desired embryonic stages from intercrosses of heterozygous breeding pairs. Genotyping was performed as described previously (Sekine et al., 1999). Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated in graded ethanol, embedded in paraffin and sectioned. The sections were stained with Hematoxylin and Eosin.

Immunohistochemistry

The sections were incubated with rabbit anti-keratin (L-1824, DAKO, Carpinteria, CA) as a primary antibody for 20 minutes at room temperature. The anti-rabbit Vectastain ABC-kit (PK-6101, Vector Laboratories) and the chromogen DAB (Vector Laboratories, Burlingame, CA) were used for antibody detection. The sections were then counterstained with Hematoxylin.

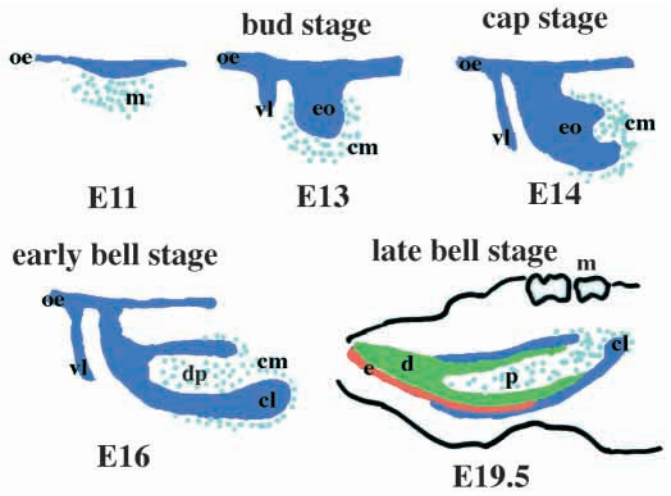

Fig. 1. The developing mouse incisor during the initiation (E11), bud (E13), cap (E14), early bell (E16) and late bell (E19.5) stages. The initial stages of morphogenesis are very similar in all teeth. The first morphological sign of incisor development is a thickening of the oral epithelium (E11), which subsequently buds into the underlying mesenchyme (E13, bud stage). At later stages, the developing incisor rotates anteroposteriorly (E14, cap stage) and becomes parallel to the long axis of the incisors (E16, early bell stage). At early bell stage (E16), the cervical loop is seen at the apical end of the labial epithelium. Only the labial epithelium gives rise to the enamel-forming ameloblasts (E19.5, late bell stage). Epithelium in dark blue, dental mesenchyme in light blue dots, enamel in red and dentin in green. cl, cervical loop; cm, condensed mesenchyme; d, dentine; dp, dental papilla; e, enamel; eo, enamel organ; m, molar; oe, oral epithelium; p, pulp; vl, vestibular lamina.

Organ culture experiments

Culture methods were as described previously (Harada et al., 1999). Dissected incisors from the mandible of *Fgf10*$^{+/+}$ or *Fgf10*$^{+/-}$ (n=8) and *Fgf10*$^{-/-}$ mice (n=7) at E19 were cultured for 20 days. All pictures were digitized and the growth length of dental epithelium was estimated at intervals of about 5 days by using NIH image 1.62 (public domain program, US National Institute of Health). Averaged growth rate was plotted using Microsoft Excel 98 (Microsoft Corporation, Redmond, WA).

For culture of the cervical loop, the mesenchyme or tissue recombinants the incisors were incubated for 4 hours in 2% collagenase (GibcoBRL) in DMEM at 4°C, and the epithelia were mechanically separated from mesenchyme in PBS. The cervical loops were cut from the epithelial sheets with 26G needle. On tissue recombination experiments the cervical loop was placed in contact with mesenchyme. These explants were cultured for 48 hours on the filters coated matrigel (BD Biosciences, MA). The cervical loops were cultured in the medium supplemented with human recombinant FGF10 (R&D systems Inc, Minneapolis, MN) varying in concentration (0-10 ng/ml). Heparin acrylic beads (Sigma) that released FGF10 (25 ng/μl) were placed in cultures of the cervical loop or the apical end of the mesenchyme. Control beads were incubated in bovine serum albumin (BSA, Sigma). The preparation of the beads has been described in detail (Kettunen et al., 1998). For antibody inoculation, anti-FGF3 antibody (SC-135, Santa Cruz Biotechnology, Santa Cruz, CA), anti-FGF10 antibody (SC-7375, Santa Cruz Biotechnology, Inc) and control goat IgG (SC-2028, Santa Cruz Biotechnology) were added to the culture medium at a concentration of 50 μg/ml.

Cell proliferation assays

Cell proliferation was localized by mapping the distribution of S-

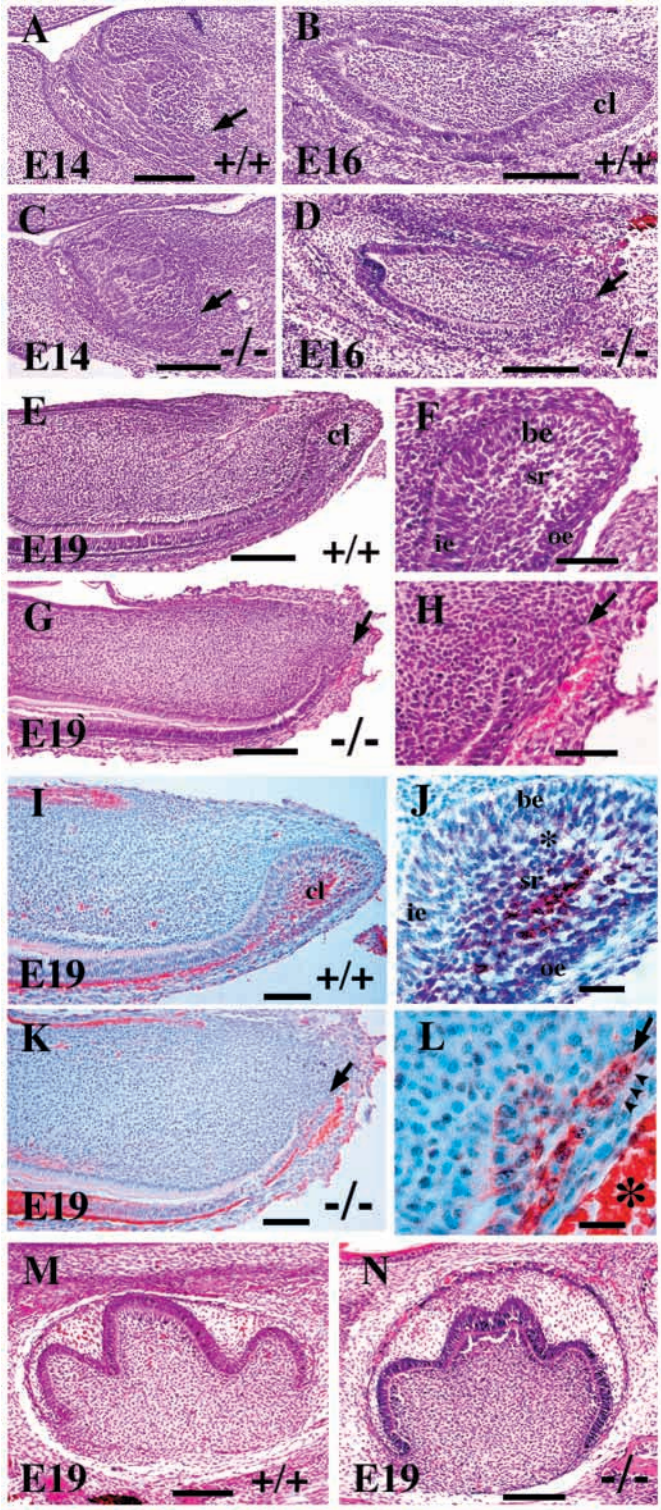

Fig. 2. Absence of cervical loop in the developing incisor germ of FGF null mice. These pictures show Hematoxylin and Eosin stained sections of lower incisor germs at cap (A,C), early bell (B,D) and late bell (E,G) stages in *Fgf10*$^{+/+}$ embryos (A,B,E,F) and *Fgf10*$^{-/-}$ embryos (B,D,G,H). At cap stage (E14), FGF10 null mice did not show clear morphological abnormalities in incisor development (C). Arrows show the end of labial epithelium. At early bell stage (E16), the epithelial bulge of FGF null mice was smaller than that of wild type (B,D, arrows). At late bell stage (E19), the cervical loop was missing in FGF10 null mice (G,H). F and H show higher magnification of E and G at the region of the apical end. (I-L) Immunostaining of cytokeratin (CK) at late bell stage (E19). At the apical end of wild type, the center region of the stellate reticulum in the cervical loop was strongly CK positive (J). The peripheral region of the stellate reticulum, found in close proximity to basal epithelium, was CK negative (J, smaller asterisk). In mutant incisor, CK-negative stellate reticulum cells were missing around strongly CK-positive cells shown by arrowheads (L). Arrows show the apical end of labial epithelium. The staining of erythrocytes was due to nonspecific binding of antibody (L, larger asterisk). Hematoxylin and Eosin staining of the lower first molar germ in *Fgf10*$^{+/+}$ (M) and *Fgf10*$^{-/-}$ (N) embryos. be, basal epithelium; cl, cervical loop; ie, inner enamel epithelium; oe, outer enamel epithelium; sr, stellate reticulum. Scale bars: 200 μm in A-D,E,G,I,K,M,N; 100 μm in F,H,J,L.

phase cells. BrdU (1.5 ml/100g, 5-bromo-2′-deoxyuridine, Roche Diagnostics, Mannheim, Germany) was administered to ether-treated mice, which were sacrificed 2 hours later, and incorporated BrdU was detected immunohistochemically by the BrdU Staining Kit (Oncogene Research Products, Darmstadt, Germany).

In situ hybridization

For in situ hybridization analysis, digoxigenin-labeled antisense riboprobes for *Fgf3* and *Fgf10* mRNA were synthesized according to the manufacturer's instructions (Roche Diagnostics). Paraffin sections were made as described previously (Kettunen and Thesleff, 1998; Kettunen et al., 1998). The chromogen 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Roche Diagnostics) was used for color detection.

Annexin V staining

We used Alexa 488-conjugated Annexin V (Molecular Probes, Eugene, OR), which binds to phosphatidylserine moieties that become exposed on the outer surface of the cell membrane at the onset of apoptosis, to detect apoptotic cells in the explants. The explants were incubated with Hepes buffer (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM MgCl, 1.8 mM $CaCl_2$) containing 1 μg/ml Annexin V-Alexa 488 for 20 minutes. After rinsing with Hepes buffer, fluorescence was detected by fluorescent microscopy.

RESULTS

Absence of *Fgf10* causes defect in stem cell compartment in developing mouse incisor

A mouse incisor exhibits special developmental systems that allow for regeneration, a capacity not seen in molars. The first morphological sign of incisor development is a thickening of the oral epithelium (Fig. 1, E11), which subsequently buds into the underlying mesenchyme (E13, bud stage). The mesenchymal cells condense around the bud, and during the following cap stage the tooth bud is lain down longitudinally in the mandible (E14, cap stage). The labial epithelium, which produces differentiated ameloblasts, grows longer in length than the lingual epithelium and the cervical loop, which is a region thought to contain a population of self-renewing epithelial stem cells (Harada et al., 1999) formed at the end of the epithelium (E16, early bell stage). Subsequently, unlike in the molar, the incisor germs continue to grow permanently without forming a root (E19.5, late bell stage). Elucidating the molecules that orchestrate specific developmental programs for stem cells is important for determining the key components that regulate stem cells.

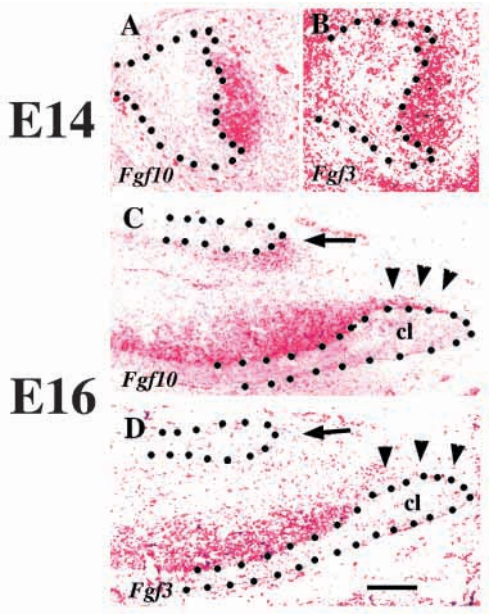

Fig. 3. Gene expression patterns of *Fgf3* and *Fgf10* in the developing lower incisor by in situ hybridization. Both *Fgf3* and *Fgf10* mRNA was co-expressed in the dental papilla at the cap stage (E14) (A,B). At early bell stage (E16), *Fgf3* mRNA was restricted to the mesenchyme underlying the inner enamel epithelium (D). *Fgf3* mRNA was not seen around the cervical loop (D, arrowheads) and lingual epithelium (arrow). Cells expressing *Fgf10* mRNA extended to the mesenchyme neighboring the inner enamel epithelium and covered the cervical loop (C, arrowheads). Scale bar: 200 μm. cl, cervical loop.

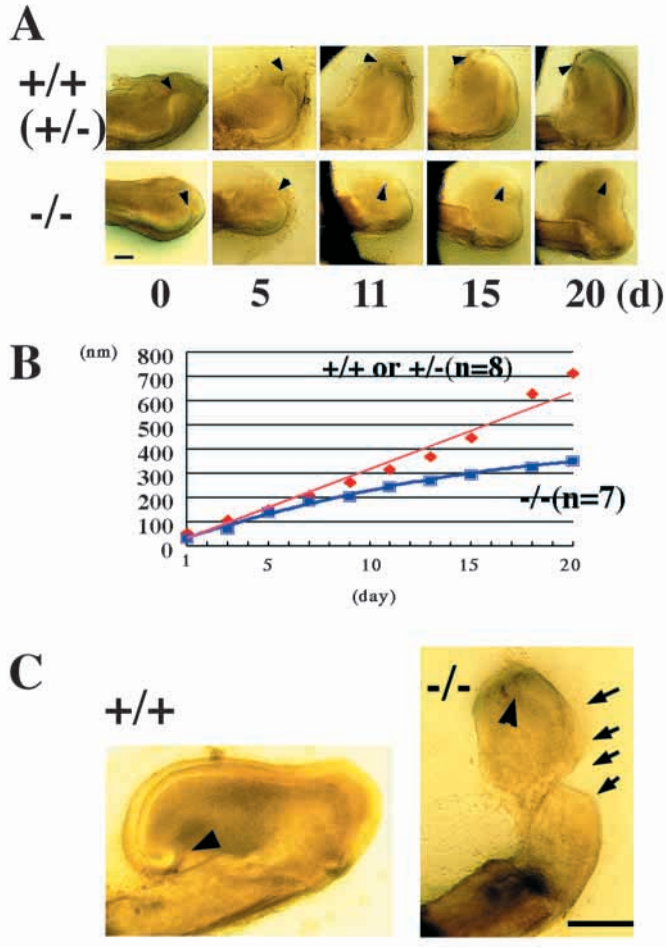

Fig. 4. Development of the apical end of incisors in $Fgf10^{+/+}$ and $Fgf10^{-/-}$ mice by organ culture and estimation of growing dental epithelium. (A) The dissected incisors from the mandible of $Fgf10^{+/+}$ or $Fgf10^{+/-}$ (n=8) and $Fgf10^{-/-}$ mice (n=7) at E19 were cultured for 20 days as described. (B) All pictures were digitized, and the growth length of dental epithelium was estimated using NIH image 1.62. The graph of average growth rate was created using Microsoft Excel 98. red line, $Fgf10^{+/+}$ or $Fgf10^{+/-}$; blue line, $Fgf10^{-/-}$. (C) These explants were cultured for 30 days. Arrows show disruption of the dental epithelium in the mutant. Arrowheads indicate apical end of the dental epithelium in the explants. Scale bars: 200 μm. *P<0.05.

We studied the role of FGF10 in mesenchymal signaling by analyzing the development of mouse incisors in Fgf10-deficient mice. Morphological defects were not found before E14 (Fig. 2A,C). Tooth germs in $Fgf10^{+/+}$, $Fgf10^{+/-}$ and $Fgf10^{-/-}$ mice developed normally until bud, cap and bell stage. However, at E16, the cervical loop of FGF10 null mice was smaller than that of wild type (Fig. 2B,D). At later stages, the cervical loop was missing (Fig. 2E-H). We examined the cervical loop defect in detail by immunostaining with anti-cytokeratin (CK) antibody. The cervical loop consists of a core of stellate reticulum cells surrounded by basal epithelial cells contacting the dental mesenchyme. Within the cervical loop of $Fgf10^{+/+}$ or $Fgf10^{+/-}$ mice, the center of the stellate reticulum was CK positive, whereas the remaining cells were CK negative. Notably, the peripheral stellate reticulum cells (putative stem cells), which are located in close proximity to basal epithelial cells, were CK negative (Fig. 2I,J, a smaller asterisk). However, in FGF10 null mice, although a CK-positive stellate reticulum remained (Fig. 2L, arrowheads), the basal epithelium and CK-negative stellate reticulum cells were missing, verifying the absence of the incisor stem cell population. However, a normal cell-differentiation gradient of enamel-forming ameloblasts and dentin-forming odontoblasts was observed in both incisors and molars of FGF10 null mice (Fig. 2E,G,M,N).

To characterize the timing of the morphological inconsistency between the mutant and wild type, we examined the gene expression pattern of *Fgf3* and *Fgf10* in the developing incisors by in situ hybridization. At E14, *Fgf3* and *Fgf10* mRNA were co-expressed in the dental papilla (Fig. 3A,B) suggesting a functional redundancy between the two FGFs in early incisor morphogenesis. After E16, however, cells expressing *Fgf10* mRNA spread from the zone neighboring the inner enamel epithelium to the apical end and surrounded the cervical loop, and this expression pattern appeared to correlate with the growth of labial epithelium (Fig. 3C). By contrast, cells expressing *Fgf3* mRNA remained only in dental papilla neighboring the inner enamel epithelium (Fig. 3D). *Fgf7* mRNA was weakly expressed in the dental follicle at the apical end and not seen in dental papilla (Kettunen et al., 2000). *Fgfr1b* and *Fgfr2b* are expressed throughout the dental epithelium (Kettunen et al., 1998; Harada et al., 1999). These characteristic patterns do not change after birth (Harada et al., 1999). Thus, the timing of cervical loop formation correlates well with the divergence of *Fgf10* and *Fgf3* gene expression patterns.

Dental epithelium lacking a cervical loop exhibits decreased growth rate

To determine the role of existing stem cells, we examined the growth of epithelium that lacked a stem cell compartment. As *Fgf10*-deficient mice die soon after birth, owing to an absence of lung development (Sekine et al., 1999), we cultured incisor germs dissected from the mandible of E19 mice. The dental epithelium of both mutant and wild-type mice seemed to grow steadily for 20 days (Fig. 4A). However, as shown in Fig. 4B, the epithelial growth rate in mutant mice began to decrease and reached a maximum of approximately 400 μm (Fig. 4B). By contrast, the epithelium of wild-type mice continued to grow. When these explants were cultured beyond 20 days, the explants of mutant type showed disruption of the dental epithelium. Consequently, the length could not be estimated precisely.

To investigate difference of epithelial growth pattern between wild type and mutant or between incisor and molar, BrdU labeling analysis was used to detect the dividing cells of dental epithelium in E18 mice (Fig. 5A) and the percentage of BrdU-positive cells was quantified (Fig. 5B). Labeled cells were detected in the dental epithelium of wild type mice in the cervical loop and extended into the inner enamel epithelial zone (Fig. 5A, parts a and b). The percentage of BrdU-positive cells was 24% on average in the cervical loop and 68% in the inner enamel epithelium (Fig. 5B). In the cervical loop the positive cells were located in peripheral stellate reticulum showing putative stem cell population (Harada et al., 1999). The characteristic scattering pattern of positive cells observed in the cervical loop was clearly distinct from the pattern of dense labeled cells in the inner enamel epithelium. In the mutant incisor, the percentage was high (63%) in the apical end of dental epithelium and was nearly identical (68%) to that in inner enamel epithelium of wild type. But, the labeling pattern seen in the cervical loop of the wild type was missing in the epithelium of mutant type (Fig. 5A, parts c and d). The inner enamel epithelium of molar germs showed high percentages (88%, 67.5%) in both wild type and mutant (Fig. 5B), and their patterns of labeled cells were dense (Fig. 5A, parts e and f). These data suggest that a stem cell population does not exist in the dental epithelium of FGF10 null mice and molar germs. As the growth of epithelium that lacks stem cells depends only on proliferation of transit-amplifying cells, which undergo terminal differentiation after several rounds of cell division, the growth of mutant incisors was expected to be limited (Fig. 4B). Furthermore, cell death in the dental epithelium of the mutant involved limit of cell division of transit-amplifying cells (Fig. 4C). Thus, continuous growth would depend on stem cell division induced by mesenchymal FGF10.

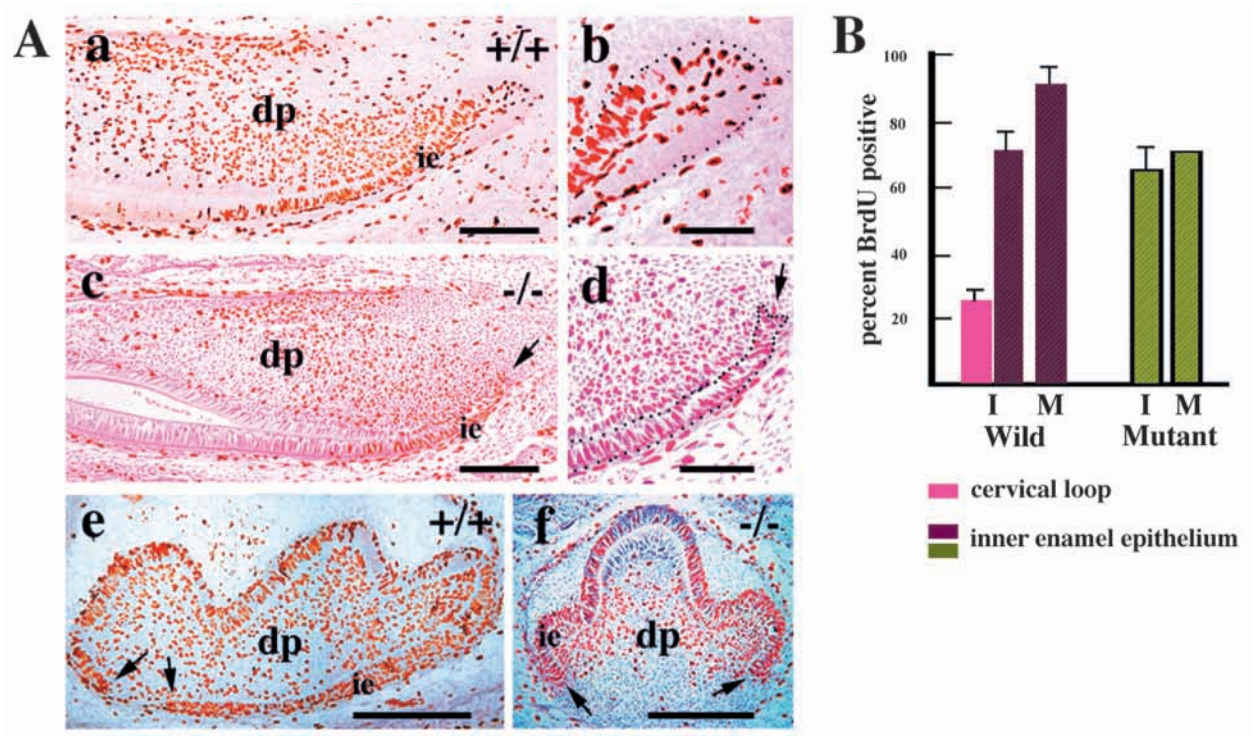

Fig. 5. Distribution of dividing cells in apical end of dental epithelium. (A) Representative longitudinal section of lower incisors and first molars in *Fgf10*$^{+/+}$ and *Fgf10*$^{-/-}$ embryos at E18 as shown by BrdU labeling. In lower incisor of *Fgf10*$^{+/+}$ embryos (a,b), BrdU-incorporating cells are sparsely distributed in the cervical loop (b) and densely distributed in inner enamel epithelium. In lower incisor of *Fgf10*$^{-/-}$ embryos (c,d), the labeling cells were seen densely at the edge of dental epithelium. The sparse pattern seen in the cervical loop of the *Fgf10*$^{+/+}$ embryo was not observed (arrows in c,d). b and d show higher magnification of a and c at the apical end of the dental epithelium. Black dots outline the dental epithelium. In molar germs of *Fgf10*$^{+/+}$ and *Fgf10*$^{-/-}$ embryos (e,f), BrdU is densely incorporated into the inner dental epithelium. Arrows show the apical end of dental epithelium. (B) Quantification of percentage of BrdU-positive cells at the apical region of dental epithelium in incisors and molars of wild type and mutant. The percentage of BrdU-posisitve cells in the cervical loop in wild type is significantly lower than the percentage in the inner enamel epithelium. Each bar presents data from three samples. Error bars represent standard deviation. ie, inner enamel epithelium; dp, dental papilla; I, incisor; M, molar. Scale bars: 200 μm in a,c,e,f; 100 μm in b,d.

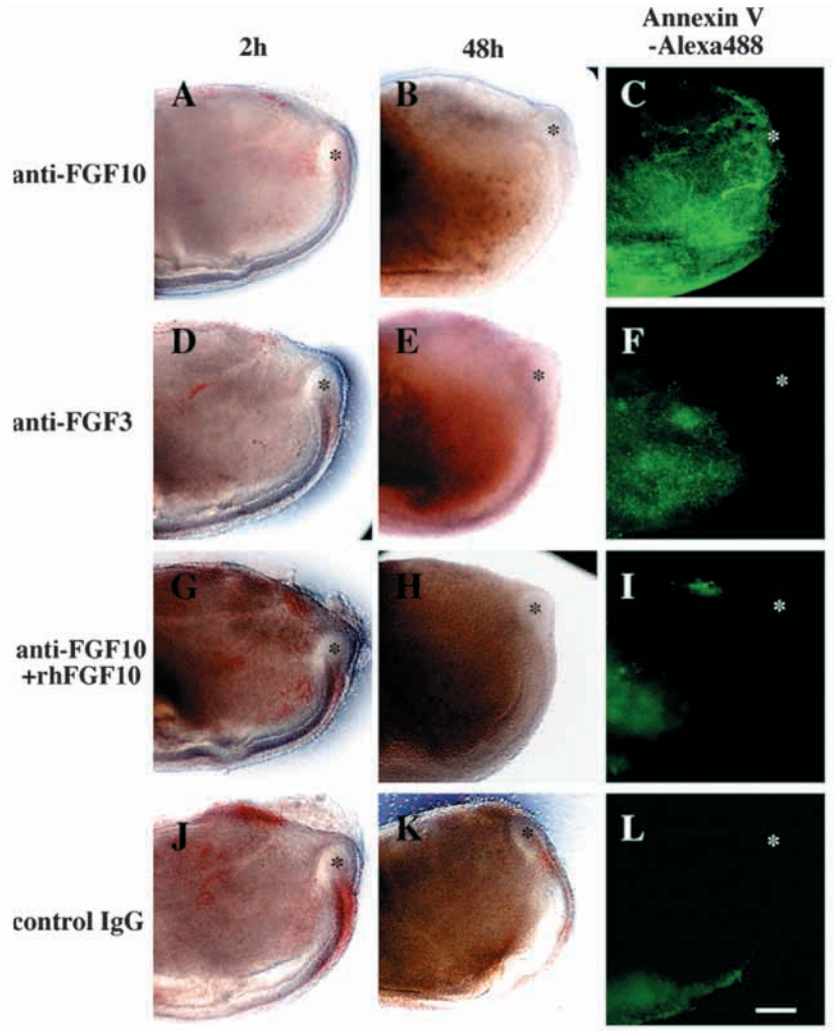

Fig. 6. Effect of anti-FGF10 neutralizing antibody on cultured incisor explants. The explants were cultured for 24 hours in the presence of anti-FGF10 antibody (A-C), anti-FGF3 antibody (D-F), anti-FGF10 antibody and human recombinant FGF10 (G-I), or normal goat IgG (J-L). Apoptotic cells were detected by Annexin V staining. Anti-FGF10 antibody disrupted the cervical loop epithelium (A,B) and caused apoptosis in the cervical loop cells (C). In the presence of anti-FGF3 antibody (D,E) or anti-FGF10 antibody and human recombinant FGF10 (G,H), the cervical loop was recognized. Anti-FGF3 antibody caused partly apoptosis in the inner enamel epithelium and the neighboring mesenchyme, but apoptotic cells were rarely seen in the cervical loop and the surrounding mesenchyme. In the presence of control IgG, few apoptotic cells were detected (K,L). Asterisks show the cervical loop. Scale bar: 200 μm.

FGF10 functional disorder causes apoptosis of cervical loop cells

To clarify the role of FGF10 in the dental epithelium of incisors, we designed an in vitro loss-of-function experiment using anti-FGF10 neutralizing antibody. When the incisor explants were cultured for 48 hours in the presence of anti-FGF10 antibody, destruction of the cervical loop was observed (Fig. 6A,B). By contrast, in the culture with anti-FGF3 antibody, anti-FGF10 antibody and human recombinant FGF10, control goat IgG at an equal concentration to anti-FGF10 antibody, the cervical loop epithelium maintained a morphological feature (Fig. 6E,H,K). To identify the extent of cell death in the explants, we visualized apoptotic cells using whole-mount Annexin V staining. In the culture with anti-FGF10 antibody, most epithelial cells in the cervical loop reacted with Annexin V (Fig. 6C). Interestingly, apoptotic cells were observed in the neighboring mesenchyme, suggesting that the survival of mesenchymal cells was associated with that of epithelial cells. In the presence of anti-FGF3 antibody, apoptosis occurred in the inner enamel epithelial and the neighboring mesenchymal cells (Fig. 6F). Notably, few apoptotic cells were seen in the cervical loop or the surrounding mesenchyme. Furthermore, human recombinant FGF10 rescued the explants from the destruction of the cervical loop, which was caused by the FGF10 neutralizing antibody (Fig. 6G-I). In the presence of control goat IgG, apoptotic cells were rarely seen (Fig. 6L).

We also determined whether FGF10 protein could prevent cell death in the cervical loop epithelium. The isolated cervical loops were cultured in the medium supplemented with recombinant human FGF protein varying concentration (0-10 ng/ml). The cervical loops were disrupted within 48 hours (Fig. 7B,F) in a concentration of 0.1 ng/ml or less and Annexin V bound to many of the cells (Fig. 7B′,F′). By contrast, in the culture supplemented with FGF10 (1 or 10 ng/ml), the epithelium maintained its tight appearance (Fig. 7D,G) and few cells bound Annexin V (Fig. 7D′,G′). FGF10-releasing beads also rescued the destruction of the cervical loop epithelium (Fig. 7H′). Control beads had no effect on maintaining the morphology or escaping apoptosis (Fig. 7I′). The neutralizing anti-FGF10 antibody (50 ng/ml) disturbed the effects of FGF10 protein (1 ng/ml) as a survival factor. These results indicated that FGF10 plays a role in the maintenance of the stem cell compartment in the developing incisors.

The apoptosis of some mesenchymal cells in the presence of anti-FGF10 antibody suggested that it might be due to absence of epithelial signaling (Fig. 6C). To examine whether absence of the cervical loop cause apoptosis of the neighboring mesenchyme, we had the tissue recombination experiments and the culture of mesenchyme in absence of the dental epithelium. When the isolated mesenchyme and cervical loop were placed in contact on the filter and cultured for 2 days, apoptotic cells were rarely seen in the mesenchyme around the cervical loop (Fig. 7L, asterisk). However, the mesenchymal cells distant from the cervical loop underwent apoptosis. A few apoptotic cells were also seen in the cervical loop. When the isolated mesenchyme was cultured, a large number of apoptotic cells were seen (Fig. 7N,N′). FGF10-releasing beads were not effective in preventing the isolated mesenchyme from undergoing apoptosis (Fig. 7O,O′).

DISCUSSION

Mouse incisors erupt throughout the animal's lifetime through the renewal of dental epithelium produced from the cervical loop located in the tooth apex. Slowly and asymmetrically dividing *Notch1*-expressing stellate reticulum cells, located near the *lunatic fringe*-expressing basal epithelial cells, have previously been identified as putative stem cells. As molecular signaling during development has been well studied (http://bite-it.helsinki.fi), the incisor presents an excellent

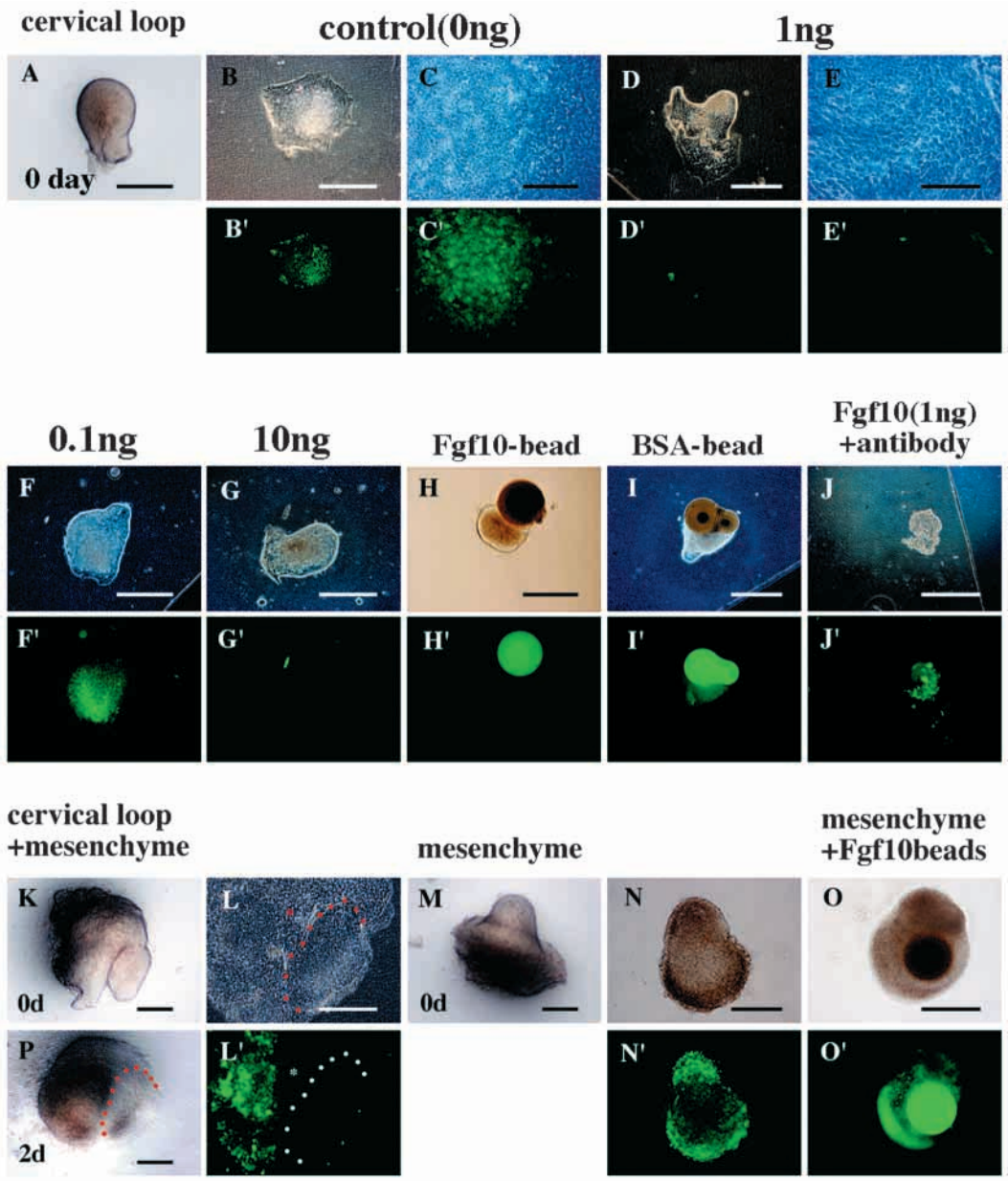

Fig. 7. FGF10 rescued the isolated cervical loop epithelium from apoptosis. The cervical loop epithelium was separated from the mesenchyme (A) and cultured for 48 hours (B,D,F-J) in the medium supplemented with recombinant human FGF10 at a concentration of 0.1 ng/ml (F,F′), 1.0 ng/ml (D,D′,E,E′), 10 ng/ml (G,G′), with bovine serum albumin (B,B′,C,C′), with FGF10-releasing beads (H,H′), with bovine serum albumin (BSA) -soaked beads (I,I′), or with FGF10 (1 ng/ml) and FGF10 neutralizing antibody (50 μg/ml) (J,J′). Apoptotic cells were detected using Annexin V staining (B′-J′,L′,N′,O′). In the presence of FGF10 (1 ng/ml and 10 ng/ml), the outline of the isolated epithelium was clearly maintained (D,G) and apoptotic cells bound to Annexin V were sparse (D′,E′,G′). Higher magnification (E) shows that the cell morphologies of the basal epithelium and the peripheral stellate reticulum (putative stem cells) in the explant were clearly maintained. The explants cultured with BSA, BSA beads or FGF10 (1 ng/ml) and FGF10 neutralizing antibody (50 μg/ml) showed disrupted epithelium (B,C,I,J) and many apoptotic cells (B′,C′,I′,J′). The shape of cells in the disrupted cervical loop epithelium was not clearly recognized (C) and a large number of cells were bound to Annexin V (C′). The explant in 0.1 ng/ml FGF10 showed the outline clearly (F), but many of these cells underwent apoptosis (F′). (K,P) The isolated mesenchyme (M) was placed in contact with the isolated cervical loop epithelium (A) and cultured for 2 days. In the mesenchyme adherent to the epithelium, apoptosis was rarely seen (L,L′). Dots outline the border between the epithelium and the mesenchyme (L,L′). In the isolated mesenchyme, several apoptotic cells were seen in the presence (O,O′) and absence (N,N′) of FGF10 beads. Scale bars: 200 μm in A,B,D,F-O; 40 μm in C,E.

model for the study of stem cell niche formation, stem cell division and fate decision mechanisms.

The cervical loop, including putative stem cells, is not formed in developing incisors of FGF10-null mice. The present study indicates that mutant incisors lacking the cervical loop lose the ability to grow continuously. In situ hybridization of developing incisor germs has revealed differential expression of *Fgf10* and *Fgf3*. After E16 and in conjunction with the growth of dental epithelium, cells expressing *Fgf10* mRNA spread out from a region underlying the inner enamel epithelium to the apical end of the tooth germ and overlaid the cervical loop. By contrast, cells expressing *Fgf3* remained restricted to the regions adjacent to the inner enamel epithelium. Thus, FGF10 rather than FGF3 is responsible for the formation of the cervical loop. Furthermore, our loss-of-function experiments using neutralizing antibodies verify that FGF10 plays a critical role in formation and maintenance of the stem cell population during organogenesis of mouse incisors.

Formation of cervical loop is crucial for regeneration of incisors

In contrast to molars, mouse incisors exhibit specific developmental systems that allow for regeneration. The putative stem cell compartment of incisors, the cervical loop, is not found in molar germs. In molars, the dental epithelium (Hertwig's epithelial root sheath) migrates downwards to the root base. At this point, the dental epithelium loses the ability to grow (Kaneko et al., 1999). By contrast, the continuously growing molars of rabbits show small epithelial bulges containing cells that behave like stem cells at the apical end of the germs (Starkey, 1963). Our experiments on incisor explants showed that epithelium-lacking stem cells exhibited limited growth. Taken together, these results suggest that formation of the cervical loop during incisor morphogenesis is crucial for developing the capacity to regenerate.

FGF10 is involved in the formation of incisor cervical loop in a non-redundant manner with FGF3

Because the expression pattern of *Fgf10* mRNA is identical to that of *Fgf3* during molar development (Kettunen et al., 2000), FGF10 null mice do not exhibit striking molar abnormalities. Likewise, *Fgf3*-deficient mice do not exhibit gross morphological changes in their teeth (Mansour et al., 1993). These data support the results from our loss-of-function experiments in which antibodies that neutralize FGF3 did not affect incisor explants perniciously. Considering that *Fgfr1b* and *Fgfr2b* are expressed throughout dental epithelium (Kettunen et al., 1998; Harada et al., 1999) (http://bite-it.helsinki.fi), these results suggest that the FGFs function somewhat redundantly. As expected from the phenotype of *Fgfr2(IIIb)*-deficient mice (Moerlooze et al., 2000), in *Fgf3* and *Fgf10*-double knockout mice, tooth germs would fail to progress beyond the bud stage. In the present study, in situ hybridization in the incisor germs revealed that after E16, the *Fgf10*-expression zone differed from that of *Fgf3*. Expression of *Fgf3* mRNA was restricted to the mesenchyme underlying the inner enamel epithelium, but cells expressing FGF10 extended to the zone covering the cervical loop. *Fgf7* mRNA was weakly expressed in the dental follicle at outside of the apical end, but not in the dental papilla (Kettunen et al., 2000), suggesting that FGF7 is associated with growth of outer enamel epithelium. Consequently, only FGF10 appears to function in maintaining cervical loop. Kettunen et al. (Kettunen et al., 2000) have found that *Fgf10*, *Fgf3* and *Fgf7* mRNA are not expressed in the mesenchyme after the later bell stage of molar development. These results suggest that continuous expression of *Fgf10* in the mesenchyme of the apical end is crucial for the formation of stem cells during incisor development.

Fgf10 may be an essential mesenchymal factor for reciprocal interaction between epithelial and mesenchymal stem cells in developing incisor germs

FGF signaling plays an important role in the mediation of epithelial-mesenchymal interactions in a variety of organs. Our previous studies of *Fgf10* expression patterns and in vitro application of FGF10-soaked beads suggested that FGF10 acts as a regulatory signal from the mesenchyme to the epithelium during tooth development (Harada et al., 1999; Kettunen et al., 2000). In the present study, when FGF10 neutralizing antibodies caused apoptosis in the cervical loop, some neighboring mesenchymal cells were also apoptotic (Fig. 6C). Furthermore, many of cells in the isolated mesenchyme from the epithelium were apoptotic regardless of the presence of FGF10 protein (Fig. 7N,N′,O,O′). Thus, the mesenchymal cell death appears to be attributable to the absence of signaling from the cervical loop. The development of tooth germs is governed by reciprocal epithelial-mesenchymal interaction and the molecular mechanism is very similar to that seen in limbs and hair (Tickle, 1995). The growth of limbs and genital tubercles is governed by the reciprocal interaction between FGF8 and FGF10 (Ohuchi et al., 1997; Xu et al., 1998; Haraguchi et al., 2000). Although *Fgf8* mRNA was observed in the dental epithelium from its thickening to early bud stage (E12), it was not seen in the developing incisor at a later stage (Kettunen and Thesleff, 1998). Kettunen et al. (Kettunen et al., 2000) have shown that the expression of both *Fgf3* and *Fgf10* in the mesenchyme depends on dental epithelium, and that FGF4 and FGF8 induce *Fgf3* expression in the mesenchyme. In addition, Bei and Maas (Bei and Maas, 1998) have also shown that FGFs (FGF1, FGF2 and FGF8), but not bone morphogenetic protein 4 (BMP4), regulate *Fgf3* expression in the dental mesenchyme. The supernatant from an odontogenic epithelial stem cell line originating from the incisor cervical loop stimulates cell proliferation and maintains expression of FGF10 in primary culture of odontogenic mesenchyme (S. Kawano, personal communication). However, FGFs, sonic hedgehog, BMP2, transforming growth factor β1 and Wnt6 expressed in the early dental epithelium did not stimulate *Fgf10* expression in the dental mesenchyme. Although the epithelial signals regulating *Fgf10* expression in teeth are still unknown, we speculate that a regulatory cascade exists between putative cervical loop molecules and FGF10 expressed in the mesenchyme. Adult stem cells in the mouse incisor seem to maintain an undifferentiated early embryonic stage by epithelial-mesenchymal interaction.

Does FGF10 control stem cell survival?

The persistence of stem cell populations through adulthood probably depends on the survival of quiescent cells, as well as

on the ability of cycling cells to self-renew. Quiescent stem cells have been identified in the liver, brain, bone marrow (reviewed by Morrison et al., 1997) and hair bulge (Taylor et al., 2000). However, it is not clear whether these apparently quiescent cells are in G_0 or whether they are moving very slowly through G_1. We have previously identified slow-cycling cells of the incisor cervical loop as putative stem cells in the dental epithelium (Harada et al., 1999). In the present study, BrdU-labeled cells in the cervical loop showed a sparse distribution pattern. Cells escaping from the cell cycle appear to behave like quiescent cells. Although previously we have shown that FGF10 stimulates division of stem cells, we do not yet know whether FGF10 plays a role in the survival of quiescent cells.

Increased apoptotic cells are found in hair follicles of FGF10 null mice (Ohuchi et al., 2000). These data are consistent with the present study, which demonstrates that the FGF10-neutralizing antibodies cause apoptosis in the incisor cervical loop epithelium. Furthermore, recombinant human FGF10 could rescue the isolated cervical loop epithelium from apoptosis. The evidence indicates that FGF10 plays a role in stem cell survival and stimulates cell-proliferation; however, other, more complex regulatory mechanisms in the stem cell compartment remain to be studied.

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REFERENCES

Bei, M and Maas, R. (1998). FGFs and BMP4 induce both Msx-1 independent and Msx1-dependent signaling pathways in early tooth development. *Development* **125**, 4325-4333.

DasGupta, R. and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* **126**, 4557-4568.

Fuchs, E. and Segre, J. A. (2000). Stem cells: a new lease on life. *Cell* **100**, 143-155.

Harada, H., Kettunen, P., Jung, H. S., Mustonen, T., Wang, Y. A. and Thesleff, I. (1999). Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *J. Cell Biol.* **147**, 105-120.

Haraguchi, R., Suzuki, K., Murakami, R., Sakai, M., Kamikawa, M., Kengaku, M., Sekine, K., Kawano, H., Kato, S., Ueno, N. and Yamada, G. (2000). Molecular analysis of external genitalia formation: the role of *fibroblast growth factor* (*Fgf*) genes during genital tubercle formation. *Development* **127**, 2471-2479.

Hogan, B. L. M. (1999). Morphogenesis. *Cell* **96**, 225-233.

Kaneko, H., Hashimoto, S., Enokiya, Y., Ogiuchi, H. and Shimono, M. (1999). Cell proliferation and death of Hertwig's epithelial root sheath in the rat. *Cell Tissue Res.* **298**, 95-103.

Kettunen, P., Karavanova, I. and Thesleff, I. (1998). Responsiveness of developing dental tissues to fibroblast growth factors: expression of splicing alternatives of FGFR1, -2, -3, and of FGFR4; and stimulation of cell proliferation by FGF-2, -4, -8, and -9. *Dev. Genet.* **22**, 374-385.

Kettunen, P., Laurikkala, J., Itaranta, P., Vainio, S., Itoh, N. and Thesleff, I. (2000). Associations of FGF-3 and FGF-10 with signaling networks regulating tooth morphogenesis. *Dev. Dyn.* **219**, 322-332.

Kettunen, P. and Thesleff, I. (1998). Expression and function of FGFs-4, -8, and -9 suggest functional redundancy and repetitive use as epithelial signals during tooth morphogenesis. *Dev. Dyn.* **211**, 256-268.

Koller, E. L. and Baird, G. R. (1969). The influence of the dental papilla on the development of tooth shape in embryonic mouse tooth germs. *J. Embryol. Exp. Morphol.* **21**, 131-148.

Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. and Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat. Genet.* **19**, 379-383.

Mansour, S. L., Goddard, J. M. and Capecchi, M. R. (1993). Mice homozygous for a targeted disruption of the proto-oncogene int-2 have development defects in the tail and inner ear. *Development* **117**, 13-28.

Millar, S. E., Willert, K., Salinas, P. C., Roelink, H., Nusse, R., Sussman, D. J. and Barsh, G. S. (1999). Wnt signaling in the control of hair growth and structure. *Dev. Biol.* **207**, 133-149.

Morrison, S. J., Shah, N. M. and Anderson, D. A. (1997). Regulatory mechanisms in stem cell biology. *Cell* **88**, 287-298.

Moerlooze, L. D., Spencer-Dene, B., Revest, J.-M., Hajihosseini, M., Rosewell, I. and Dickson, C. (2000). An important role for the lllb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signaling during mouse organogenesis. *Development* **127**, 483-492.

Ohuchi, H., Hori, Y., Yamasaki, M., Harada, H., Sekine, K., Kato, S. and Itoh, N. (2000). FGF10 acts as a major ligand for FGF receptor 2IIIb in mouse multi-organ development. *Biochem. Biophys. Res. Commun.* **277**, 643-649.

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M., Itoh, N. and Noji, S. (1997). The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* **124**, 2235-2244.

Peters, H. and Balling, R. (1999). Teeth. Where and how to make them. *Trends Genet.* **15**, 59-65.

Rochat, A., Kobayashi, K. and Barrandon, Y. (1994). Location of stem cells of human hair follicles by clonal analysis. *Cell* **76**, 1063-1073.

Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N. and Kato, S. (1999). Fgf10 is essential for limb and lung formation. *Nat. Genet.* **21**, 138-141.

Starkey, W. E. (1963). The migration and renewal of tritium labeled cells in the developing enamel organ of rabbits. *Br. Dent. J.* **115**, 143-153.

Taylor, G., Lehrer, M. S., Jensen, P. J., Sun, T. T. and Lavker, R. M. (2000). Involvement of follicular stem cells in forming not only the follicle but also the epidermis. *Cell* **102**, 451-461.

Thesleff, I. and Sharpe, P. (1997). Signaling networks regulating dental development. *Mech. Dev.* **67**, 111-123.

Tickle, C. (1995) Vertebrate limb development. *Curr. Opin. Genet. Dev.* **5**, 478-484.

Xu, X., Weinstein, M., Li, C., Naski, M., Cohen, R. I., Ornitz, D. M., Leder, P. and Deng, C. (1998). Fibroblast-growth-factor-receptor-2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development* **125**, 753-765.