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Cell dynamics in cervical loop epithelium during transition from crown to root: implications for Hertwig's epithelial root sheath formation

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Background and Objective: Some clinical cases of hypoplastic tooth root are congenital. Because the formation of Hertwig's epithelial root sheath (HERS) is an important event for root development and growth, we have considered that understanding the HERS developmental mechanism contributes to elucidate the causal factors of the disease. To find integrant factors and phenomenon for HERS development and growth, we studied the proliferation and mobility of the cervical loop (CL).

Material and Methods: We observed the cell movement of CL by the DiI labeling and organ culture system. To examine cell proliferation, we carried out immunostaining of CL and HERS using anti-Ki67 antibody. Cell motility in CL was observed by tooth germ slice organ culture using green fluorescent protein mouse. We also examined the expression of paxillin associated with cell movement.

Results: Imaging using DiI labeling showed that, at the apex of CL, the epithelium elongated in tandem with the growth of outer enamel epithelium (OEE). Cell proliferation assay using Ki67 immunostaining showed that OEE divided more actively than inner enamel epithelium (IEE) at the onset of HERS formation. Live imaging suggested that mobility of the OEE and cells in the apex of CL were more active than in IEE. The expression of paxillin was observed strongly in OEE and the apex of CL.

Conclusion: The more active growth and movement of OEE cells contributed to HERS formation after reduction of the growth of IEE. The expression pattern of paxillin was involved in the active movement of OEE and HERS. The results will contribute to understand the HERS formation mechanism and elucidate the cause of anomaly root.

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Some rare clinical cases of hypoplastic tooth root are congenital (1–3). Because it has been known that root development is induced by the growth of Hertwig's epithelial root sheath (HERS), the disease might be related closely with HERS development and growth. However, the mechanism for HERS formation has not been clearly elucidated. We considered that the study on mechanisms of HERS development contributes to discover the cause of the hypoplastic root. An experiment using *fibroblast growth factor (FGF)* -deficient mice or voles showed that the cessation of *FGF-3* and *-10* expression results in a shift from crown morphogenesis to root formation (4,5). Klein *et al.* (6) showed that *FGF* signaling regulates the fate of crown or root analogs in the *sprouty* conditional knockout mouse. Wang *et al.* (7) showed that in *follistatin* knockout mice, enamel formation is observed in the lingual epithelium of the incisors, which is the root analog. In contrast, the labial side of the incisor changes from crown analog to root analog in mice over-expressing follistatin. Fujiwara *et al.* (8,9) further reported that downregulation of epidermal growth factor and upregulation of insulin-like growth factor (IGF) are important events for the transition from crown to root.

At the onset of root formation, HERS develops. Though it has been proposed that alternation of growth factor signaling triggers the transition from crown to root, we still cannot clearly explain how HERS is formed. According to the classic theory of HERS formation, both the inner enamel epithelium (IEE) and outer enamel epithelium (OEE) proliferate and fuse to form HERS in the cervical loop (CL) (10). However, although this theory has been widely accepted, it is not extensively reported that the origin of HERS has not been explored. Recently, it was reported that the expression patterns of several genes and proteins are similar or identical between HERS and OEE (4,5,11,12). In particular, Notch2, a regulatory factor for the decision of cell fate, is expressed in both OEE and HERS (5). On the other hand, IEE cells express Jagged2 (13). In addition, by performing Ki67 staining and a bromodeoxyuridine labeling chase assay, Fujiwara *et al.* (14) suggested that the outer cells of HERS move into inner layers after cell division at the transitional stage. In light of these findings, we hypothesized that the OEE makes a more significant contribution to HERS formation than does the IEE. This model is distinct from the conventional one in which the OEE and IEE make comparable contributions.

Recently, the OEE was reported to have the capacity to serve as stem cells (15,16). Additionally, HERS and the epithelial rests of Malassez possibly function as stem cells, and exhibit the phenotype alteration associated with the epithelial–mesenchymal transition (17,18). Therefore, studies on the relationship between OEE and HERS are significant. Furthermore, we do not know the reason why cells in the inner layer of HERS do not differentiate into ameloblasts in mouse or human. If the inner layer of HERS is originated from IEE, the layer should form enamel at the surface of root dentin. To find the clue to answer such questions, we think that exploration of the origin of HERS is very important. Studies on cell movement and behavior by use of live cell imaging in a variety of tissues such as brain and bone marrow have given us much information (19). In this present study, to examine the mechanism of HERS formation, we observed the cell dynamics of CL in detail by preparing organ cultures and performing DiI labeling. Further, we designed a novel real-time imaging system for observation of the CL during the transition from crown to root. These procedures allowed us to observe the behavior of living CL cells during organogenesis *ex vivo*. The results will contribute to elucidate the HERS formation mechanisms and be helpful in understanding the anomaly root.

Material and methods

All experiments were conducted in accordance with the Protocols for the Humane Treatment of Animals of Iwate Medical University.

Tooth-germ organ culture and DiI labeling

Lower molars were carefully dissected mechanically from the mandibular bone of ddy (Japan SLC, Hamamatsu, Japan) or green fluorescent protein (GFP) an embryonic day 15 (E15) mouse. Lower molar germs were isolated and cultured on 0.1-μm pore-size filters (Millipore, Billerica, MA, USA) supported by metal grids

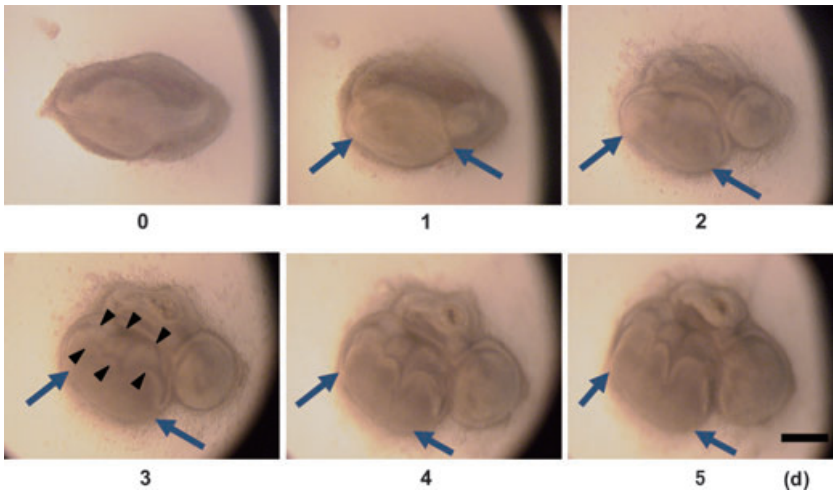

Fig. 1. Observation of tooth germ during crown morphogenesis. Lower molar germs of an E16 mouse were cultured in an incubation chamber under stereomicroscopic observation over the course of 5 d. The images were taken at the indicated time points (days). Arrows indicate apexes of the cervical loop. Arrowheads indicate apexes of cusps. Bar = 500 μm.

bathed in Dulbecco's Modified Essential Medium (Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (Invitrogen), penicillin/streptomycin, and 100 μg/mL ascorbic acid (Sigma, Tokyo, Japan). The cultures were kept in a humidified atmosphere of 5% CO_2 in air at 37°C. DiI [1,1′-dioctadecyl-6,6-di(4-sulfophenyl)-3,3,3′,3′-tetramethylindocarbocyanine;] (Invitrogen) diluted in 0.2% w/v in DMSO was injected into the dissected molars by using a microinjector (FemtoJet Express; Eppendorf, Tokyo, Japan), and the molars were subsequently cultured as described above. The time-lapse bright field and fluorescent images were taken using a real-time stereomicroscopy system (Leica, Tokyo, Japan).

Live cell imaging of dental epithelial cells in organ-slice cultures

This system consists of a laser microscope and a custom-made incubation chamber. For organ-slice cultures, lower first molars from a postnatal day 0 (PN0) GFP mouse were embedded in 5% low-melting point agarose (Invitrogen). The embedded samples were then sliced at a 200-μm thickness by using a microslicer (D.S.K., Kyoto, Japan) and transferred to the culture chamber for confocal microscopy (FV 300; Olympus, Tokyo, Japan). The sliced sample was maintained in culture medium (DMEM/F12, 10% fetal bovine serum and 1% of antibiotic mixture) at 37°C and exposed to moisturized 5% CO_2 gas. Data analysis and creation of the movies were performed with Metamorph software (Molecular Devices, Tokyo, Japan.).

Cell proliferation assay *in vivo*

Lower first tooth germs of PN1, PN3, and PN5 mice were fixed in phosphate-buffered 4% paraformaldehyde, decalcified in EDTA, embedded in paraffin, and serially sectioned into 6-μm sections. The antigens on the sections were activated by autoclave treatment (120°C for 10 min). Ki67 was detected on the sections by using anti-Ki67 antibody (Dako Japan, Tokyo, Japan), and visualized with diaminobenzidine. Ki67-stained specimens were then weakly counterstained with hematoxylin. To compare the mitotic activity between IEE and OEE of the CL, and/or the inner and outer layers of HERS, we counted the number of Ki67-immunopositive cells and all cells in their epithelium, and then after statistical processing, estimated the mitotic index of each.

Immunostaining

Lower first tooth germs of PN4 mice were fixed in phosphate-buffered 4% paraformaldehyde, decalcified in EDTA, embedded in OCT compound, and serially sectioned into 14-μm sections. Paxillin was detected on the sections by using anti-Paxillin antibody (Abcom Japan, Tokyo, Japan), and visualized with diaminobenzidine. Paxillin-stained specimens were then weakly counterstained with hematoxylin.

Results

Imaging of crown morphogenesis

First, we used stereomicroscopy over a time course to observe cell movement in the stage of crown morphogenesis preceding HERS formation. Cultured tooth germs from E16 mouse were

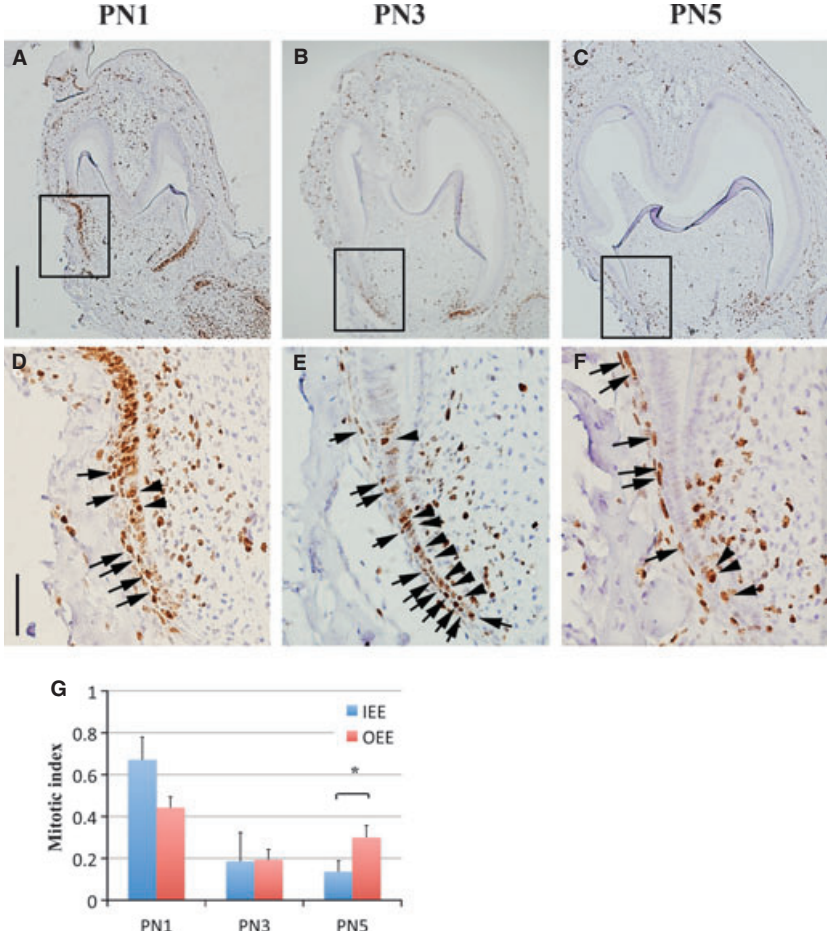

Fig. 2. Cell proliferation assay. Lower first molar tooth germs of postnatal day (PN)1 (A, D), PN3 (B,E), and PN5 (C,F) mice were stained with anti-Ki67 antibody. (D,E,F) Higher magnification of the box in (A,B,C), respectively. Arrows indicated Ki67-positive cells in outer enamel epithelium (OEE) and outer layer of Hertwig's epithelial root sheath and Arrowheads indicated that of inner enamel epithelium (IEE) and inner layer of Hertwig's epithelial root sheath. The number of Ki67-immunopositive cells and all cells in their epithelium were counted, and then the mitotic index of each was estimated, after statistical processing (G). *$p < 0.001$. Bar = 400 μm (A–C). Bar = 80 μm (D–F).

incubated in a culture chamber, and images were collected every 30 min for 6 d. At the start, the center of the enamel organ was slightly raised, and the germ resembled a rugby ball. At 3 d culture, six bulges predictive of the cusp (Fig. 1, arrowheads) were seen at the upper face of the first molar tooth germ. These bulges gradually rose and swelled, and the growing cusps came to resemble mountain chains thrust upward by tectonic activity. Next, the IEE cells lined up for the formation of the cusp enamel (Fig. 1 and see Movie S1). These results showed that both IEE and OEE grew during crown morphogenesis.

Cell proliferation assay

To examine the difference in growth between OEE and IEE at the transitional stage, we carried out a cell proliferation assay using lower first tooth germs of PN1, PN3, and PN5 mice and Ki67 immunostaining (Fig. 2). At PN1, Ki67-positive cells were more numerous in IEE than in OEE (Fig. 2A, 2D and 2G). However, Ki67-positive cells are few at the tip of IEE in CL (Fig. 2D). At PN3 and PN5, the number of Ki67-positive cells in the total IEE decreased dramatically. On the other hand, the number of Ki67-positive cells in OEE and/or the outer layer of HERS decreased gradually, but was more than that of IEE at PN3 (Fig. 2D, 2E and 2G). At PN5, Ki67-positive cells in OEE and/or the outer layer of HERS are much more than that of IEE (Fig. 2F and 2G). These results indicate that OEE cells proliferated more actively than IEE ones at the onset of HERS formation.

Chase experiment on outer enamel epithelial cells by DiI labeling and organ culturing

Next, to examine cell movement during the process of HERS formation, we carried out DiI labeling and chase analysis in the stage following crown morphogenesis. First, a lower first molar germ from an E15 mouse was cultured for 5 d. After the crown outline was decided at that time, we labeled a point within the OEE and the adjacent dental follicle at the distal side of the cultured tooth germ using DiI. To chase the movement of DiI, we cultured the germ for > 10 d (Fig. 3A). The DiI label in the OEE expanded upward and downward in the 8-d culture. On the other hand, almost no label was seen in the IEE between the injection spot and CL (Fig. 3A, yellow arrowheads). Interestingly, in the culture at 11 d, DiI-labeled cells were also seen at the tip of the IEE of the CL, suggesting that OEE cells moved more actively than IEE cells and overhung the tip of IEE. Further, in the 15-d culture, HERS could be seen clearly as two layers; DiI was also seen in both the inner and outer layer of HERS (Fig. 3A, white arrowheads). Some DiI also remained in the IEE of the CL (Fig. 3A, a white arrow). Furthermore, using laser microscopy, we observed directly the movement of the OEE cells at the distal side of the lower first molar germ from a PN3 GFP mouse (Fig. 3B). The dental epithelium was distinguished from the dental mesenchyme by the strong GFP fluorescence of the dental epithelium, and OEE cells in the outer layer of the epithelium were labeled with DiI (Fig. 3B, arrows). The DiI fluorescence migrated to the CL apex and expanded with time. After 18 h, the DiI fluorescence further elongated toward and overran the CL apex (Fig. 3B, arrowheads). These results suggest that after the reduction in IEE growth, the growth of OEE in the direction of the CL apex made the

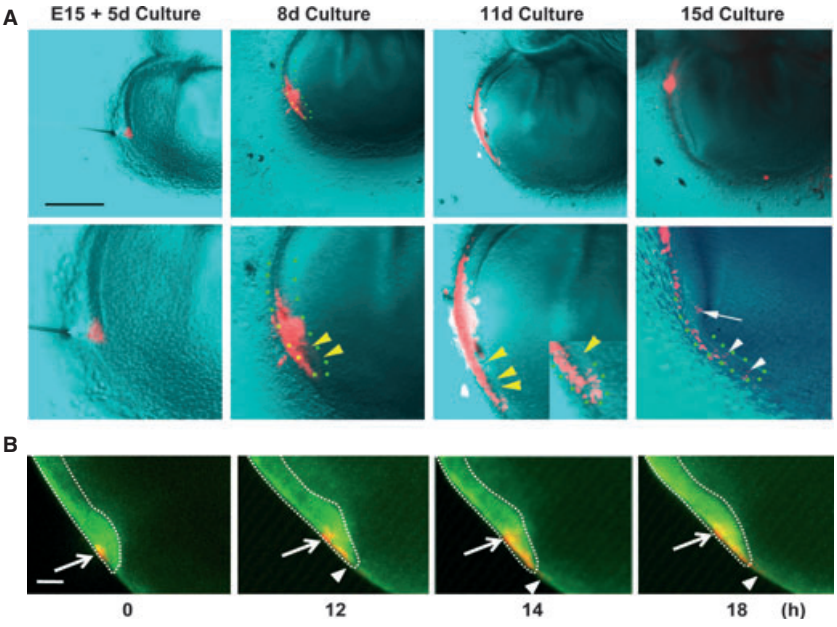

Fig. 3. Observation of movement of outer enamel epithelium (OEE) cells during the stage of Hertwig's epithelial root sheath formation. (A) After lower first molar germs (E15) were cultured for 5 d, the OEE and adjacent dental follicle at the distal side of the toothgerm were marked by DiI, and the growth of tooth germ and movement of DiI were observed by time-lapse stereomicroscopy for 10 d. The DiI of the OEE expanded over the cervical loop apex in synchrony with the movement of dental follicle cells around the OEE cells. Yellow arrowheads indicate DiI-negative cells in the inner enamel epithelium. White arrow and arrowheads indicate DiI in inner enamel epithelium and outer layer of Hertwig's epithelial root sheath, respectively. (B) The OEE at the distal side of the lower first molar of a postnatal day 3 green fluorescent protein mouse was marked by DiI, and the growth of tooth germ and movement of DiI were observed by time-lapse laser microscopy over 18 h. The dotted line indicates the shape of the cervical loop. Arrows indicate the positions where DiI was injected. Arrowheads indicate the tip of DiI movement. Bar = 400 μm (A, upper panels), 200 μm (A, lower panels), 20 μm (B).

major contribution to the formation of HERS.

Observation of cell dynamics during the transition from crown to root

To observe the cellular dynamic of CL at the transitional stage from crown to root directly, we designed an organ-slice culture system for the tooth germ of a GFP mouse. Because it was technically difficult to slice calcified tooth germ after PN1, we selected the tooth germ at PN0. However, because the outline of the crown was finished at PN0 and proliferation of the IEE cells was confined to the tip of the CL, we considered that the CL cells was preparing the onset of HERS formation. Time-lapse imaging was performed with laser confocal microscopy for 6 h (Fig. 4A). Interestingly, the experiments also showed that cells in the OEE and CL apex dynamically changed their cell morphology and locomotion (Fig. 4A, arrows and B, see Movie S2). On the other hand, most IEE cells were stable at the same position. To study cell mobility in the CL at PN4, we examined the expression of paxillin, a focal adhesion protein associated with the reorganization of actin cytoskeleton (20,21). The expression was observed strongly at OEE and the apex of CL (Fig.4C and 4D). The results suggested that the OEE cells and apex of CL actively proliferate and move.

Discussion

Shift of cell proliferation from inner to outer enamel epithelium during Hertwig's epithelial root sheath formation

Recent studies showed that expression of the epidermal growth factor receptor, IGF receptors, bone morphogenetic protein, and FGF and their inhibitors also play an important role in the transition from crown to root (8,9,14,22,23). However, we cannot explain how the change in expression of these genes would result in the morphological change from crown to root. Here, we focused on the difference in epithelial growth between IEE and OEE during the transition from crown to root. As was seen in Fig. 2, in the stage of crown morphogenesis, IEE cells actively proliferated. After that, the proliferation of IEE cells was downregulated, whereas the OEE cells continued to proliferate, suggesting that OEE had much more capacity for HERS formation than IEE. Actually, after cessation of epidermal growth factor and FGF in dental papilla, IGF and hepatocyte growth factor expressed by dental follicle induce the proliferation of OEE and HERS (8,12).

Activity of cell behavior of outer enamel epithelium during the formation of Hertwig's epithelial root sheath

To observe cell behavior in the preparation of HERS formation, we performed live cell imaging of the CL in the lower first molar germ of a PN0 mouse (Fig. 4). The live imaging showed that cells in the OEE and apex of CL actively changed their morphology, whereas many cells in IEE stayed fairly constant. Actively migrating cells, such as cancer cells, always change their shape by repeatedly elongating and contracting their cell processes, which are accompanied by remodeling of the actin filaments (24–26). It has been reported that some cancer cells, which are likely to become metastatic, increase the expression of paxillin, a focal adhesion associated protein (27). Previous data reported that IGF contribute to HERS growth (8). In addition, it has been reported that IGF-I promotes the motility of some cell types and the IGF signaling is associated with actin filament disassembly and tyrosine dephosphorylation of FAK, Cas, and paxillin (28). Because immunostaining of paxillin was observed strongly in OEE and the apex of CL, the increase of paxillin expression might be needed for the reorganization of

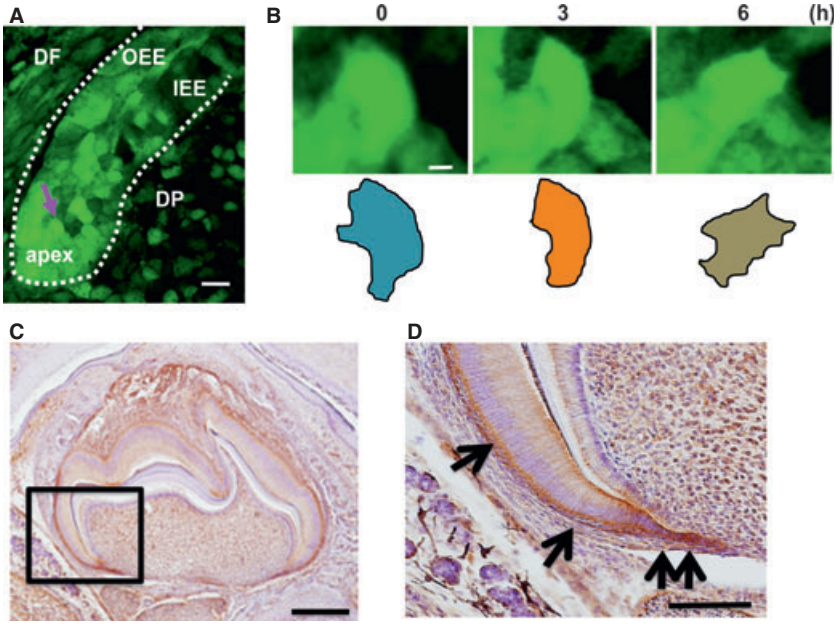

Fig. 4. Observation of cellular dynamics in CL. (A) Green fluorescent protein image shows the appearance of the CL in sliced first molar from a postnatal day 0 green fluorescent protein mouse. Bar = 10 μm. (B) Images show the active morphological change of the single cell indicated by the arrow in (A). Lower panels represent the manually traced cell shapes at the indicated time. Bar = 2 μm. (C) The immunostaining of paxillin of lower first molar at postnatal day 4. Bar = 200 μm. (D) Higher magnification of the box in (C). Arrows indicate the strong expression of paxillin in OEE and Hertwig's epithelial root sheath. Bar = 100 μm.DF, dental follicle; DP, dental papilla; IEE, inner enamel epithelium; OEE, outer enamel epithelium.

actin filaments in OEE and CL cells during HERS growth and development. These studies suggested that cells in the OEE and apex of the CL moved more actively than IEE cells. In addition, because it has been considered that growth of OEE and HERS is associated with the migration of dental follicle cells (29), the movement of OEE and HERS might be regulated by dental follicle. Taken together, our data suggested that OEE cell proliferation and mobility are very important in the process of HERS formation, and the deficient cell mobility in the epithelium might cause hypoplastic root and short root anomaly.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Movie S1 Time-lapse movie of tooth germ during crown morphogenesis.

Movie S2 Time-lapse movie of CL.

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