Relationship between Enamel Knot and Cuspal Formation in Mouse Molar

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Hyun-A Lee

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ABSTRACT

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The enamel knot (EK), which is located in the center of bud and cap stage tooth germs, is a transitory cluster of non-dividing epithelial cells. The EK acts as a signaling center that provides positional information for tooth morphogenesis and regulates the growth of tooth cusps by inducing secondary EKs. The morphological, cellular and molecular events leading to the relationship between the primary and secondary EKs have not been described clearly. Therefore, this study investigated the relationship between the primary and secondary EKs in the mouse molar. I investigated the location of the primary EK and secondary EKs by chasing *Fgf4* expression patterns in tooth germ. To clarify the relationship between the primary EK and the buccal secondary EK, the primary EK cells were traced by the cell labeling method.

The present DiI-labeling experiment demonstrated that correctly DiI-labeled primary EK cells would not migrate during the 48 hours of culture, which correspond to the future paracone and protoconid respectively according to Osborn's terminology. Semithin and ultrathin sections of the cap stage enamel organ of molars demonstrated morphological structures such as the primary EK, enamel cord (septum), and enamel navel.

Overall, these results suggest that primary EK cells strictly contribute to form the paracone or protoconid, which are the main cusps of tooth in maxilla or mandible.

Key words: *tooth development, enamel knot, DiI-labeling, slice culture, cuspal patterning*

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I. INTRODUCTION

One of the crucial events for pattern formation during embryonic genesis is an interaction between epithelium (endoderm) and mesenchyme (mesoderm). Tooth bud, kidney, hair follicle and feather bud require specific and complex epithelial-mesenchymal interactions in order to develop completely as an organ. [1] These all ectodermal organs share similar signaling molecules and morphological processes during early development with each organ undergoing its own specific pattern of formation later in development.[2, 3] The tooth develops from the epithelium lining the oral region and from the ectomesenchymal derived from the caudal mesenphalic and rostral rhombencephalic neural crest.[4, 5] The tooth is an excellent model to study reciprocal tissue interaction that occur

between the oral epithelium and its underlying dental mesenchyme, which lead to cuspal pattern, cell differentiation and the synthesis of specialized mineralized matrices.[6] During mammalian tooth development, the oral ectoderm and mesenchyme coordinate their growth and differentiation to give rise to organs with precise shapes, sizes and functions.[7] From these reasons, tooth development would be often used as a model for studying the nature of epithelial-mesenchymal interactions controlling the morphogenesis, histogenesis, and cytodifferentiation as well.[4]

At embryonic day 11 (E11), oral epithelium thickening is the first indication of tooth morphogenesis in mice.[8] Subsequently, this thickening proliferates and invaginates into the underlying ectomesenchyme forming an epithelial tooth bud at E12.5 with the mesenchyme condensing around the dental lamina resulting in dental papilla.[8] During the bell stage (E14.5), the tooth shape is determined by epithelial folding, and the dentin and enamel forming odontoblasts and ameloblasts, respectively, were differentiated.[8, 9] A key event during tooth morphogenesis is the transition from bud to cap stage when the epithelial bud is divided into specific compartments distinguished by morphology as well as gene expression patterns.[10] The enamel knot (EK), a transient epithelial structure, appears at the onset of mammalian tooth shape development.[11] EK, located in the center of cap-stage tooth germs, is a

transitory cluster of non-dividing epithelial cells. It might act as a signaling center providing positional information for tooth morphogenesis.[12] The EK is considered to be the most important structure that determine the number of cusps and regulate the growth of tooth cusps as well as the shape of an individual tooth through the induction of secondary signaling.[8, 13] A characteristic feature of tooth development is the reiterated appearance of the enamel knot in the epithelium.

The first EK (primary EK) appears during the bud to cap stages of tooth germs and the subsequent EKs (secondary EKs) appear at the sites of epithelial folding that mark the cusp initiation sites.[14~17] The primary EK expresses several signals belonging to different families such as *Bmps*, *Fgfs*, *Wnts*, and *Shh*, which is proposed to act as a signaling center that regulates early tooth morphogenesis.[14, 18, 19] Secondary EKs share gene expression patterns with the primary EK. Among these signals, *Fgf4* and *Slit1* can be used as EK markers because they are observed in the primary and secondary EKs.[8, 18~21] The secondary EKs in the bell stage tooth germ have been suggested to determine the cusp sites and promote their growth.[8] Therefore, the patterning of secondary EKs can determine the cuspal patterning. Each tooth shows a specific cuspal pattern, by which the teeth can be classified into several categories such as incisors, canines, premolars, and molars, even though the number of each varies

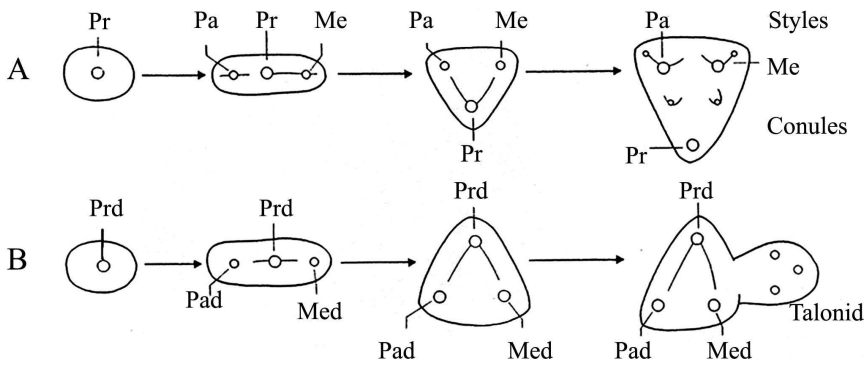
from one species to another and some categories may be absent. Studies on the signaling molecules have revealed associations of the gene expression pattern with tooth morphogenesis. The signaling pathways have been analyzed to determine the relationship between tooth evolution and the shape of teeth.[14]

Cope and Osborn first developed the theory of mammalian molar evolution that is generally accepted today.[22] Osborn also proposed a nomenclature for the various tooth cusps. According to Osborn's terminology, the three major cusps are referred to as the protocone, paracone, and metacone in the maxilla, and the protoconid, paraconid, and metaconid in the mandible (Fig. 1). Trigon and trigonid refer to the triangular arrangement of these three major cusps of the upper and lower cheek teeth (premolars and molars), while the distal triangular regions are known as the talon (hypocone) or talonid (hypoconid, hypoconulid, and entoconid), respectively. This terminology for the various cusps has also been applied to the naming of the various secondary EKs according to their locations in the developing tooth germ (Fig. 2).[15, 23, 24]

The signals of the primary EK have been considered to be instructive for the formation of secondary EKs on the future cusp tips.[25] It was suggested that primary EK might have cellular continuity with the secondary EKs through the division of the surviving cells in the primary EK and their migration into the secondary EKs. In this case, some cells of the primary EK would have to escape

the apoptotic fate. In teeth such as the molars, in which multiple cusps are formed, secondary EKs develop at the tips of the future cusps.[8] In the other words, the primary EK could induce the formation of secondary EKs located at the tips of forming cusps and endowed with similar signaling activities.[25, 26] However, a recent study reported that no cells of the primary enamel knot were seen to move toward the developing secondary enamel knots. Although the primary and secondary enamel knots have a close molecular and functional relationship in molar development, they are not actually derived from the same cells.[27] The other paper probed the existence of primary EK in the gene expression and cell proliferation methods. They confirmed on many cases that primary EK is slanted toward the buccal away form the mandible in between the cap stage and the bell stage.[14, 23]

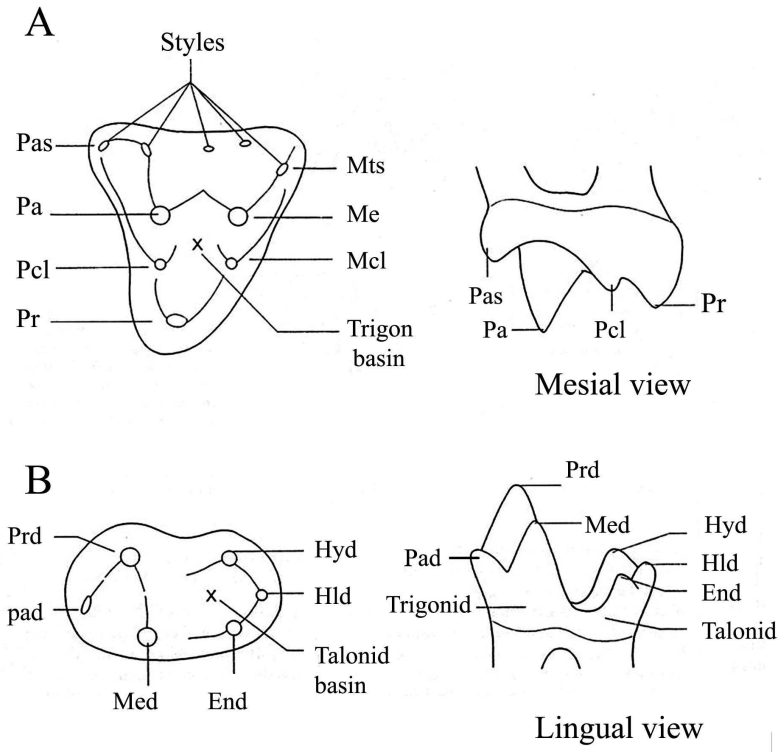
In this study, in order to elucidate the precise mechanisms in tooth cuspal formation, I investigated the relationship between the primary and secondary EKs was investigated from the evolutional and developmental biology points of view by analyzing the gene expression pattern and tracing cell migration in tooth development.



Osborn JW. Dental Anatomy and Embryology. eds. Rowe AHR, Johns RB. (Blackwell Scientific Publications), 1981; Vol.1, Book 2, p.340

Figure 1. The Cope-Osborn theory of molar evolution.

The theory of molar evolution on which is based the nomenclature (known as the Tritubercular theory). (A) In the upper, to the mesial of the protocone, the cusp that appeared is the paracone. Distal to the protocone is the metacone. The protocone displaced to the lingual with the base of the triangle. (B) In the lower the protoconid remained to the buccal with the base of the triangle. A cusp appeared earlier, which was that the protocone / protoconid being the 'first cusp'. (B) Note that the theory was essentially correct as regards the lower molars, (A) but not the upper molars. Pa; paracone, Pad; paraconid, Pr; protocone, Prd; protoconid, Me; metacone, Med; metaconid.



Osborn JW. Dental Anatomy and Embryology. eds. Rowe AHR, Johns RB. (Blackwell Scientific Publications), 1981; Vol.1, Book 2, p.341

Figure 2. Names of the cusps on tribosphenic molars.

(A) upper molar, (B) lower molar. All living mammals, excepts the monotremes, have been derived from Cretaceous ancestors with tribosphenic molars. A new cusp (protocone) developed from the basal ridge (cingulum). The protocone pounded into the surface of the talonid which had developed as basin ringed by further cusps: the hypoconid buccally, the hypoconulid distally and the entoconid lingually. Thus the hypoconid/trigon and the

protocone/talonid are reciprocal cusp and basin surface of interaction. The mesial edge of the trigonid (protoconid-paraconid crest) sheared against the distal edge of the upper molar in front. The distal edge of the trigonid (protoconid-metaconid crest) sheared against the mesial edge of the upper molar. Meanwhile the metaconid sheared against the mesial crest of the protocone. Pa; paracone, Pad; paraconid, Pr; protocone, Prd; protoconid, Me; metacone, Med; metaconid, Pcl; paraconule, Mcl; metaconule, Pas; parastyle, Mts; metastyle, Hyd; hypoconid, End; entoconid, Hld; hypoconulid.

II. MATERIALS AND METHODS

1. Animals

Adult ICR mice were housed in a temperature-controlled room (22±1°C) under artificial illumination (lights on from 05:00 h to 17:00 h) and at 55% relative humidity, with free access to food and water. The embryos were obtained from time-mated pregnant mice. Embryonic day 0 (E0) was designated as the day a vaginal plug was confirmed. Embryos at each developmental stage (daily intervals from E13.5-E15.5) were used in this study.

2. Slice culture

The E13.5 embryos were placed in a petri-dish filled with a sterile physiological saline containing penicillin-streptomycin (100 U/ml; GIBCO). The embryos were rinsed in the sterile saline and remove extra embryonic membranes. The embryos were Transferred through three successive washes in sterile saline containing penicillin-streptomycin. After the last wash, maxillae and mandibles were got from dissected E13.5 mice. And they were embedded in 2% low melting agarose (Invitrogen, UltraPureTM Agarose, Carlsbad, CA, USA) made up in sterile physiological saline in a plastic boat of the type commonly used for paraffin or cryo embedding. The embryos were placed in

the boat once the agarose has sufficiently cooled. The embryos into the desired slicing orientation by use forceps to move. A block of agarose containing the embryo was mounted on the vibratome platform. Specimens were cut using a Vibratome (Vibratome Series 1000, TED PELLA system #10, TPI Inc., ST. Louis MO. USA) at a thickness of 250 μm. Slices were transferred to a petri dish containing culture medium. Slices can be kept in these dishes for several hours at 37°C and 5% CO_2 until transferred to their final culture dishes. The sliced tooth germ specimens showing a clear primary EK were *in vitro* cultured using the Trowell method. The culture medium consisted of 10% fetal bovine serum with DMEM, which had been supplemented with penicillin and streptomycin. The slices were cultured in this manner at 37°C and 5% CO_2 for 2 days (Fig. 3).

3. DiI-labeling

DiI (1,1-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchloride; Molecular Probes) is a vital dye and a member of the carbocyanine dye family. DiI is a strongly fluorescent lipophilic dye that labels the cell membrane and is widely used for examining cell fate. DiI, non-toxic reagent, is passed on to the progeny of labeled cells but does not leak to neighbouring cells.[28] DiI (3 mg/ml in dimethylformamide) was administered by a air pressure injection using a

rubber bulb (Microcaps, Drummond Scientific Co., USA) via a micropipette with a tip opening of 1 mm, which was made using a thin walled 10 µm diameter capillary pipette in a Flaming Brown Micropipette puller (Model P-97, Suter Instrument). This method labels approximately 300 cells. The sliced tooth germs were cultured for 48 hours, and the migration of the DiI-labeled cells was investigated each day (Fig. 3).

4. Whole mount *in situ* hybridization

Embryos were fixed in 4% (w/v) paraformaldehyde and processed in methanol for whole mount *in situ* hybridization as previously described.[29] Whole tissue was washed in PBST and was treated for 10-20 minutes with proteinase K (10 µg/ml in PBST). It was washed twice in PBST and refixed for 1 hour in 4% PFA. Embryos were then prehybridized for 2 hours at 60°C in hybridization buffer containing 50% deionized for formamide. Hybridization was performed overnight at 65°C in hybridization buffer containing 0.2-0.5 µg/ml riboprobe. Excess probe was removed by sequential washes in 2X standard saline citrate (SSC; three times at 60°C), 0.2X SSC (three times at 60°C), 1:1 of 0.2X SSC: 0.1 M phosphate buffer (PB), and PB(twice). Nonspecific binding in the tissue was blocked for 1-2 hours in 15% goat serum. After this treatment, the specimen was incubated overnight with

anti-digoxigenin antibody conjugated to alkaline phosphatase diluted 1:2,000 in blocking solution. Excess antibody was removed by washes in 0.1M PB, and the tissue was equilibrated with color buffer containing 100 mM Tris, pH 9.5; 50 mM $MgCl_2$ 100 mM NaCl; and 0.1% Tween 20. After *in situ* hybridization, specimens were cryosectioned at a thickness of 15 μm.

5. Electron microscopy

The dissected tooth germs were fixed in 4% (w/v) paraformaldehyde plus 2.5% glutaraldehyde in 0.05 M phosphate buffer for 3 to 4 hours at 4℃. They were then post-fixed in 1% osmium tetroxide for 1 hour, dehydrated through a graded series of ethanol, and embedded in LX-112. The frontal semi-thin sections of tooth germs (1 μm in thickness) were counter-stained with 0.03% methylene blue. Ultra-thin sections (70 nm in thickness) were double-stained with uranyl acetate and lead citrate, and examined using a Hitachi H-7100 transmission electron microscope.

6. Histological analysis

Cell count correction factors for the quantitative histological analysis of the tooth development. The number of nuclei visible in a microtome section can easily be counted. But not all of the objects thus counted are whole nuclei.

Some must be fragments of nuclei, because some nuclei lie partly within the section examined, partly within an adjacent section. A nuclear-point cannot overlap two adjacent sections, and the number of nuclear-points in a section can therefore be extrapolated to the number in any volume of tissue without error, and can serve for exact comparison of nuclear population in different tissues. Although all cell counts are presented as relative values rather than absolute values, Abercrombie's correction factor was used for the purpose of calculating enamel organ cell density: $P=A[M/(L+M)]$, where P is estimated cell number, A is nuclear count, M is section thickness in micrometers (1µm for this analysis) and L is average nuclear diameter.

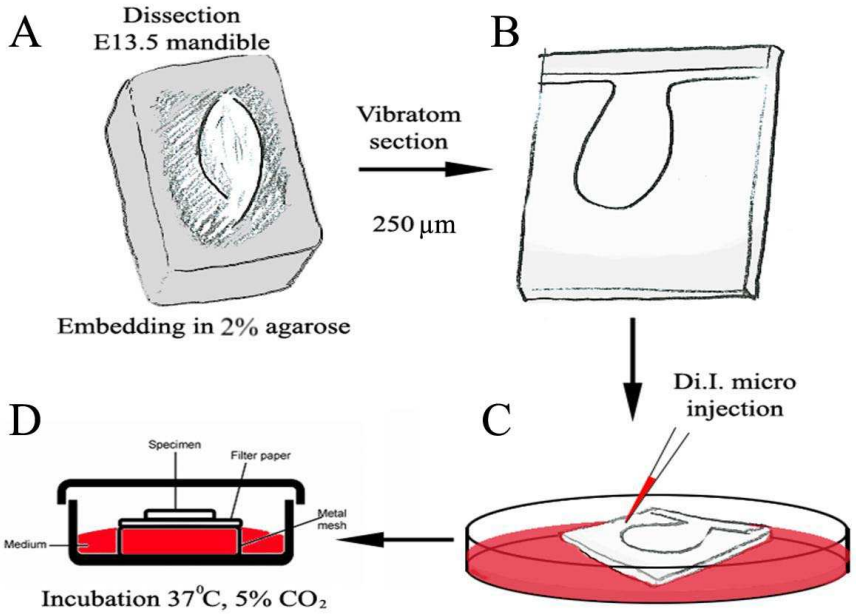


Figure 3. Schematic diagram of cutting and culturing embryo slice.

(A) At E13.5, mice tooth germs are dissected from mandible. And obtained tooth germs are embedded in 2% low melting agarose made up in sterile physiological saline in a plastic boat of the type commonly used for paraffin or cryo embedding. The embedded embryos are placed in the boat once the agarose has sufficiently cooled. (B) A block of agarose containing the embryo are mounted on the vibratome platform. These agar blocks are sectioned with 250 µm thickness. (C) The sliced tooth germ specimens are showed a clear

primary EK, therefore theses sliced tooth germ are easy to be micro-injected. (D) After DiI micro-injection, slices are transferred to a petri-dish containing culture medium. The culture medium consisted of 10% fetal bovine serum with DMEM, which had been supplemented with penicillin and streptomycin. Specimens are cultured specimen by modified Trowell's culture method at 37°C and 5% CO_2 for 2 days.

III. RESULTS

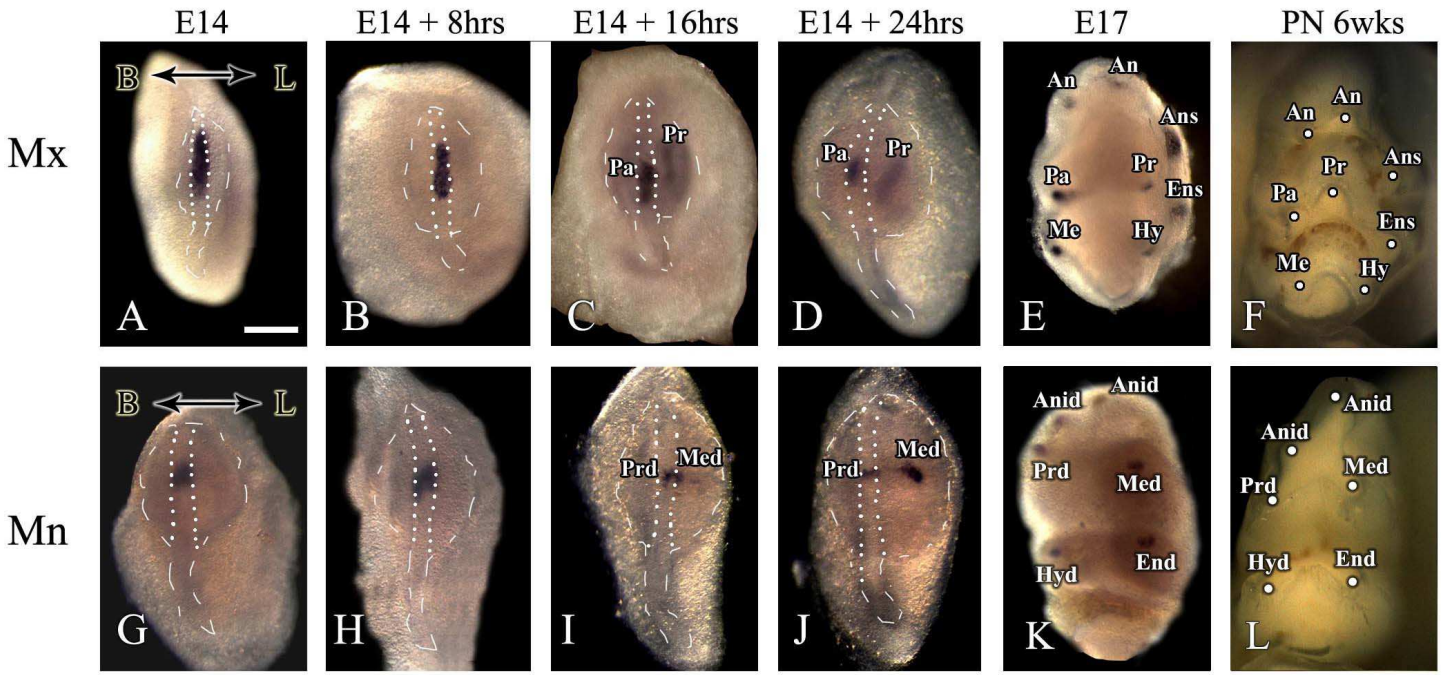
1. Sequential appearance of the EK in the developing molar of maxilla and mandible

The dynamic transformation of the EK during tooth development in maxilla and mandible was investigated by determining the location of the *Fgf4*-expressing spots in developing tooth germs at E14 and E17, as well as in the E14 tooth germs in culture for 8, 16, and 24 hours (Fig. 4). At E14 maxillary molars, the primary EK expressing *Fgf4* showed a spindle shape in the center of the tooth germs (Fig. 4A). After 8 hours *in vitro* culture, the *Fgf4* expression domain was smaller than that at E14 (Fig. 4B). After 16 hours, in two restricted domains of *Fgf4* expression were found detected. They would be considered as the secondary EKs. The secondary EK on buccal side was located in the center of the tooth germs, whereas the other secondary EK appeared on the lingual side from the center of the tooth germs (Fig. 4C). After 24 hours in culture, *Fgf4* was expressed in two clear domains. Interestingly, the buccal secondary EK, which was smaller than the lingual secondary EK, might correspond to the prospective paracone (Fig. 4D).

At E14 mandibular molars, the primary EK expressing *Fgf4* appeared as an

oval shape in the center of the tooth germs (Fig. 4G). After 8 hours *in vitro* culture, the size of the primary EK was similar to that in the previous stage (Fig. 4H). After 16 hours, the tooth germs showed two *Fgf4*-expressing EKs which connected to each other. The size of the buccal secondary EK was smaller than that of the lingual secondary EK. The buccal secondary EK located in the center of the tooth germs might correspond to the prospective protoconid (Fig. 4I). After 24 hours, the two secondary EKs, the *Fgf4* expressions were clearly distinguished. The buccal secondary EK was maintained its position in the center of the tooth germs. The lingual-positioned secondary EK separated from the center of the tooth germs could be regarded as the metaconid (Fig. 4J). At E17, eight *Fgf4*-expressing spots were observed in the upper molars, which corresponded to a pair of putative anterocones, a protocone, paracone, metacone, hypocone, anterostyle, and enterostyle (Fig. 4E). Six spots were observed in the lower molars, which corresponded to a pair of putative anteroconids (some would consider them as paraconid), a protoconid, metaconid, entoconid, and hypoconid (Fig. 4K).

On the day of postnatal 6 weeks, eight upper molar cusps (Fig. 4F) and six lower molar cusps (Fig. 4L) were recognizable in each erupted tooth.


E14
E14 + 8hrs
E14 + 16hrs
E14 + 24hrs
E17
PN 6wks
Mx
Mn
B
L
A
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E
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H
I
J
K
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Pr
Pa
An
Ans
Ens
Me
Hy
Anid
Prd
Med
End
Hyd

Figure 4. *Fgf4* gene expression patterns in tooth germ.

(A and G) At E14, the primary EK showing *Fgf4* expressions are clearly identified along the dental lamina (surrounded by dotted lines) in the center of the tooth germs in both the maxilla and mandible. (B and H) After 8 hours of culture, the size of *Fgf4*-expressing domain is slightly smaller or equal to that at E14. (C and I) After 16 hours, another *Fgf4*-expression domain appears, (C) resulting in two small *Fgf4*-expression domains (secondary EKs) corresponding to the future paracone and protocone forming regions in the maxilla, (I) and the protoconid and metaconid in the mandible. (D and J) After 24 hours, the *Fgf4* domain on the buccal side is smaller than on the lingual side, and the two spots separate from each other in both maxilla and mandible. (E and K) At E17, eight *Fgf4*-expressing domains are recognizable in the upper molar and (K) six domains appear in the lower molar. (F and L) Each cusp position of the erupted teeth on postnatal day 6 weeks is identical to each secondary EK position with an *Fgf4*-expression domain in the prenatal tooth germs. An; anterocone, Anid; anteroconid, Ans; anterostyle, End; entoconid, Ens; enterostyle, Hy; hypocone, Hyd; hypoconid, Me; metacone, Med; metaconid, Mn; mandible, Mx; maxilla, Pa; paracone, Pad; paraconid, Pr; protocone, Prd; protoconid. White dotted lines indicate the dental lamina, white dash lines indicate the tooth boundary (scale bar; 100 μm).

2. Cell lineage of the primary EK from the bud to bell stages

The primary EK cells were labeled with DiI on each tooth germ in the maxilla and mandible at E13.5 (Figs. 5A and D). It was able to identify whether the primary EK cells contributed to the secondary EK by chasing the DiI-labeled primary EK cells for 48 hours with *in vitro* slice culture.

In the maxilla, after 24 hours of *in vitro* culture, the shape of the tooth germs transformed into a cap shape, and the labeled cells did not migrate far from the center of the tooth germs (Fig. 5B). After 48 hours, the shape of the tooth germs developed into the bell shape, and the labeled cells were located in the buccal secondary EK, which corresponded to the paracone (Fig. 5C).

In the mandible, after 24 hours, the shape of the tooth germs transformed from a bud to a cap shape, and the labeled primary EK cells did not move far away from the center, thereby remaining in the primary EK (Fig. 5E). After 48 hours, the shape of tooth germs changed into the bell shape, and the labeled cells were located just within the buccal secondary EK, which corresponded to the protoconid (Fig. 5F). Even when the above tooth germs of maxilla and mandible were cultured for 60 hours *in vitro*, DiI-labeled cells kept their position within the buccal secondary EKs, which corresponded to the paracone in the maxilla and the protoconid in the mandible (data not shown).

On the other hand, DiI tracking of the EK around position area demonstrates different cell migration pattern (Figs. 5G-L). DiI-labeled cells in a slightly away from the primary EK (Fig. 5G) moved to the lingual side and dispersed after 24 hours, when the tooth germs transformed into the cap shape (Fig. 5H). After 48 hours, the tooth germs transformed into the bell shape, and the DiI-labeled cells moved around the lingual secondary EK corresponding to the protocone, and scattered to a greater extent than those in the cap stage (Fig. 5I).

The DiI-labeled cells located above the primary EK at E14.5 also showed the different cell migration pattern from DiI-labeled cells on primary EK. (Fig. 5J). After 24 hours, the tooth germs developed into a late cap stage and DiI-labeled cells were observed in slightly above primary EK to occlusal direction. (Fig. 5K). After 48 hours, the tooth germs transformed to the bell shape, and the DiI-labeled cells were located on the occlusal side of the buccal secondary EK, which corresponded to the protoconid (Fig. 5L).

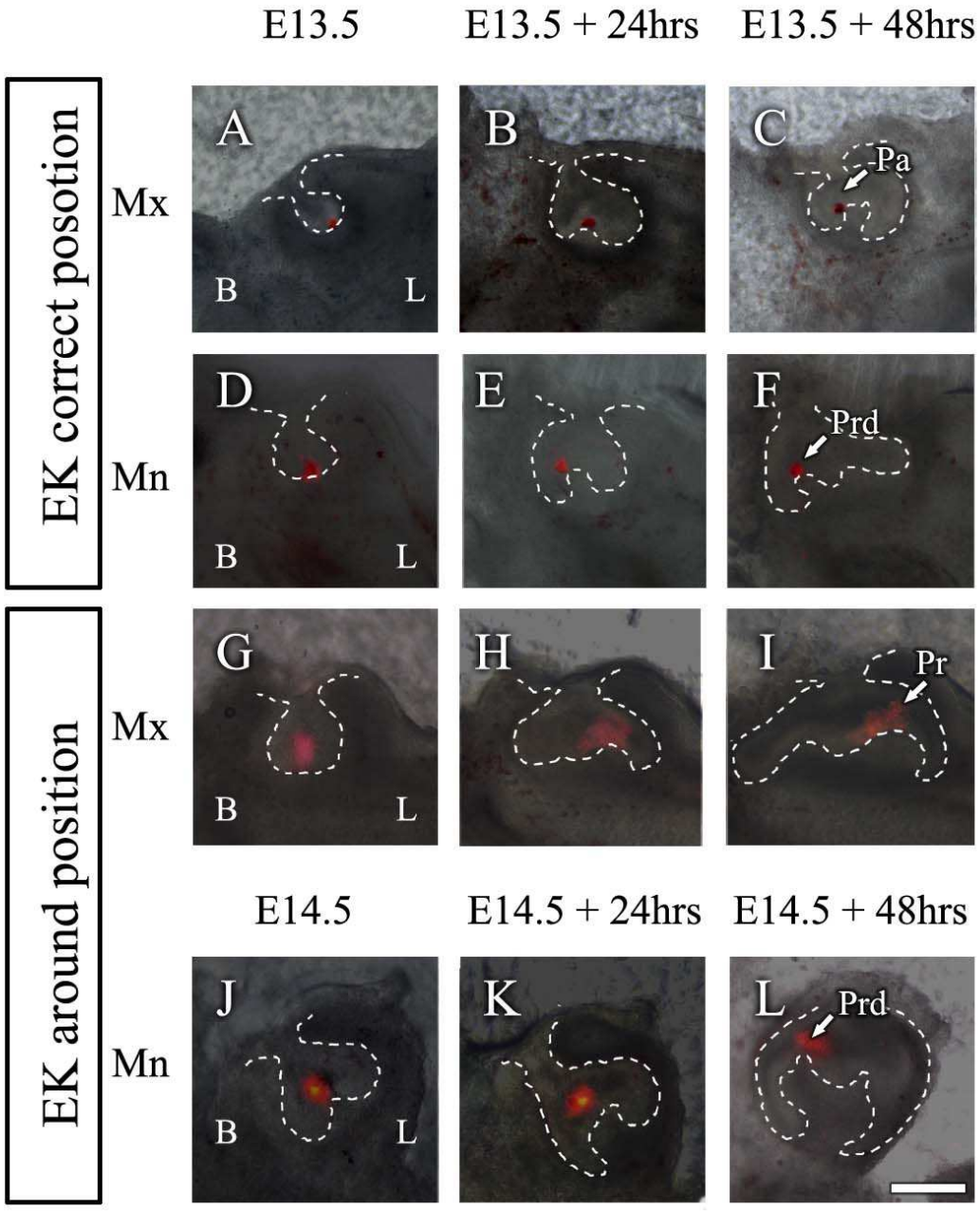

E13.5
E13.5 + 24hrs
E13.5 + 48hrs
EK correct posotion
Mx
Mn
A
B
C
D
E
F
Pa
Prd
B
L
EK around position
G
H
I
Pr
E14.5
E14.5 + 24hrs
E14.5 + 48hrs
J
K
L
Prd

Figure 5. Spatial relationship between the primary and secondary EK.

(A and D) DiI is injected into the primary EK at E13.5 (late bud stage) of upper and lower molars. (B and E) After 24 hours of culture (cap stage), DiI-labeled cells are located in the center of the tooth germs. (C and F) After 48 hours (bell stage), DiI-labeled cells are observed in the buccal secondary EK corresponding to the paracone in the maxilla and the protoconid in the mandible. (G) DiI is injected around the primary EK at E13.5 (late bud stage). (H) After 24 hours of culture, DiI-labeled cells are dispersed throughout in the secondary EK on the lingual cusp. (I) After 48 hours (bell stage), DiI-labelled cells are observed around the secondary EK with broader localization pattern than those cultured after 24 hours. Most the labelled cells migrate to the lingual cusp forming region. (J) DiI is injected into the primary EK at the cap stage (E14.5). (K) After 24 hours of culture, DiI-labeled cells are monitored in slightly above from primary EK to occlusal direction. (L) After 48 hours, DiI-labeled cells are located on the occlusal side of the buccal secondary EK. EK; enamel knot, Mn; mandible, Mx; maxilla, Pa; paracone, Pr; protocone, Prd; protoconid. White dotted lines indicate the basal layer (scale bar; 100 μm).

3. Cell lineage of enamel organ in the buccal and lingual region from the bud to bell stages

Enamel organ cells were DiI-labeled on buccal and lingual region from EK axis at bud stage tooth germ (E13.5) (Figs. 6A and D). After 24 hours *in vitro* culture, the DiI-labeled cells on the lingual region migrated to the center area and the area of labelled cells widened about 3 times than that at the moment of injection (Fig. 6B). After 48 hours *in vitro* culture, the DiI-labeled cells distributed half of lingual area at tooth germ (Fig. 6C). The buccal DiI-labeled cells on tooth germ, migrated to the buccal area lengthwise included inner dental epithelium after 24 hours (Fig. 6E). DiI-labeled cells were detected in the all buccal region of tooth germ after 48 hours (Fig. 6F). In order to define the precise fate of cell on both buccal and lingual regions of tooth germ, cells on both lingual and buccal sides near EK region in each specimens were labelled by DiI at E13.5 (Figs. 6G-L). After 24 hours culture, few DiI- labeled cells in the buccal region were away from the initial injection point (Fig. 6H). Whereas the DiI-labeled cells on the lingual region migrated and diffused to distribute of tooth germ widely (Fig. 6K). After 48 hours culture, the DiI-labeled cells in the buccal region showed similar pattern to culture for 24 hours, however the DiI-labeled cells on the lingual region were observed with weak color of DiI on lingual region (Figs. 6I and L).

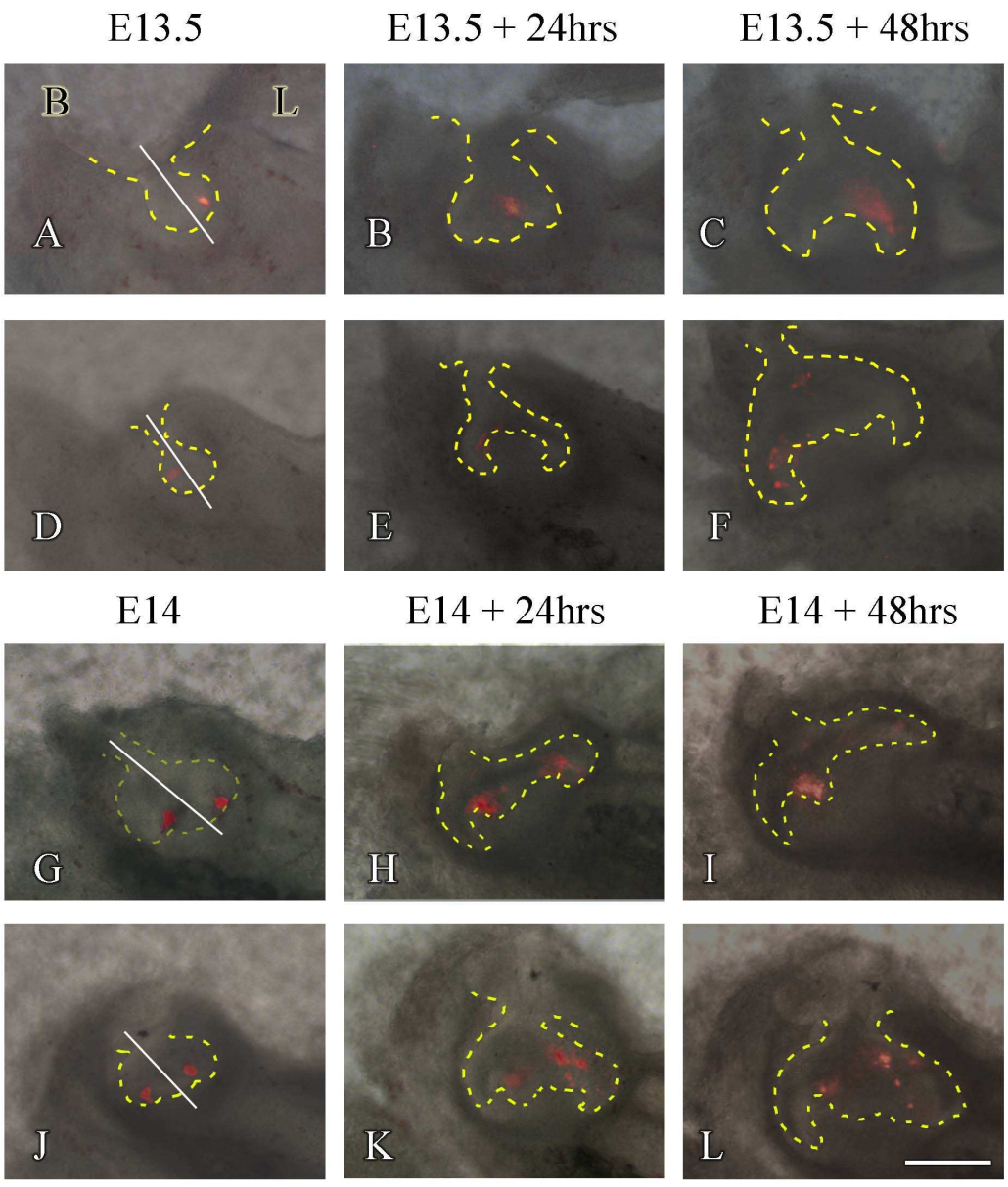

E13.5
E13.5 + 24hrs
E13.5 + 48hrs
B
L
A
B
C
D
E
F
E14
E14 + 24hrs
E14 + 48hrs
G
H
I
J
K
L

Figure 6. The lineage of enamel organ cells from the bud to bell stages on mandible.

The enamel organ cells are labeled with DiI (A) in the buccal region, (D) the lingual region tooth germ at E13.5 base on EK axis. (B) After 24 hours *in vitro* culture, the labelled cells are observed in the center of tooth germ. (C) After 48 hours *in vitro* culture, the DiI-labeled cells extend to the lingual part. (E) After 24 hours culture, the DiI-labeled cells in the buccal region of tooth germ migrate to the buccal area lengthwise include inner dental epithelium. (F) After 48 hours *in vitro* culture, the DiI-labeled cells in the buccal region migrate all of buccal tooth germ. (G and J) The two region (buccal and lingual) are labeled DiI at early cap stage (E14). (H and K) After 24 hours culture, DiI-labelled cells in buccal region are observed away from the initial injection point. While the DiI-labeled cells on the lingual part migrated and diffused to distribute of tooth germ widely. (I and L) After 48 hours culture, similar pattern of DiI-labeled cells is formed as that after 24 hour culture. Yellow dotted lines demarcate the basal layer. White lines indicate EK axis, which divides the tooth germ into lingual and buccal parts (scale bar; 100 μm).

4. Expression patterns of signals related with the EK

The strongest expression for *Fgf4* domain was observed in the tooth germs at the late bud stage or early cap stage (Figs. 7A and J). After the cap stage, the range and intensity of the *Fgf4* expression domain were reduced and weakened than those in the previous stage (Figs. 7B and K), and intense expression was detected in the secondary EKs at the bell stage (Figs. 7C and L).

At the bud stage, *Bmp4* expression, was detected at the primary EK and was detected condensed dental mesenchyme around the epithelial bud with a somewhat stronger expression at the buccal side of the bud in both maxilla and mandible (Figs. 7D and M). *Msx2* was intensely expressed on the buccal side of the dental epithelium, which did not reach the primary EK area at the bud stage (Figs. 7G and P). At the cap stage, intense *Bmp4* expression was found continuously in the dental papilla mesenchyme, and slight *Bmp4* expression was observed throughout the dental epithelium including the EK (Figs. 7E and N). *Msx2* expression was observed in the primary EK area and at the buccal side of the dental epithelium (Figs. 7H and Q). At the bell stage, strong *Bmp4* expression was localized in the cuspal area of the dental papilla (Figs. 7F and O). The dental epithelium showed a moderate *Msx2* expression. Weak *Msx2* expression was observed around the dental papilla (Figs. 7I and R). Taken

together, *Bmp4* and *Msx2* were expressed mainly on the buccal side, but showed an opposite pattern to each other. *Bmp4* was most predominantly expressed in the EK and mesenchyme but *Msx2* was expressed in the buccal side of the epithelium at the bud stage. These key signaling molecules showed a similar pattern in both the upper and lower molars.

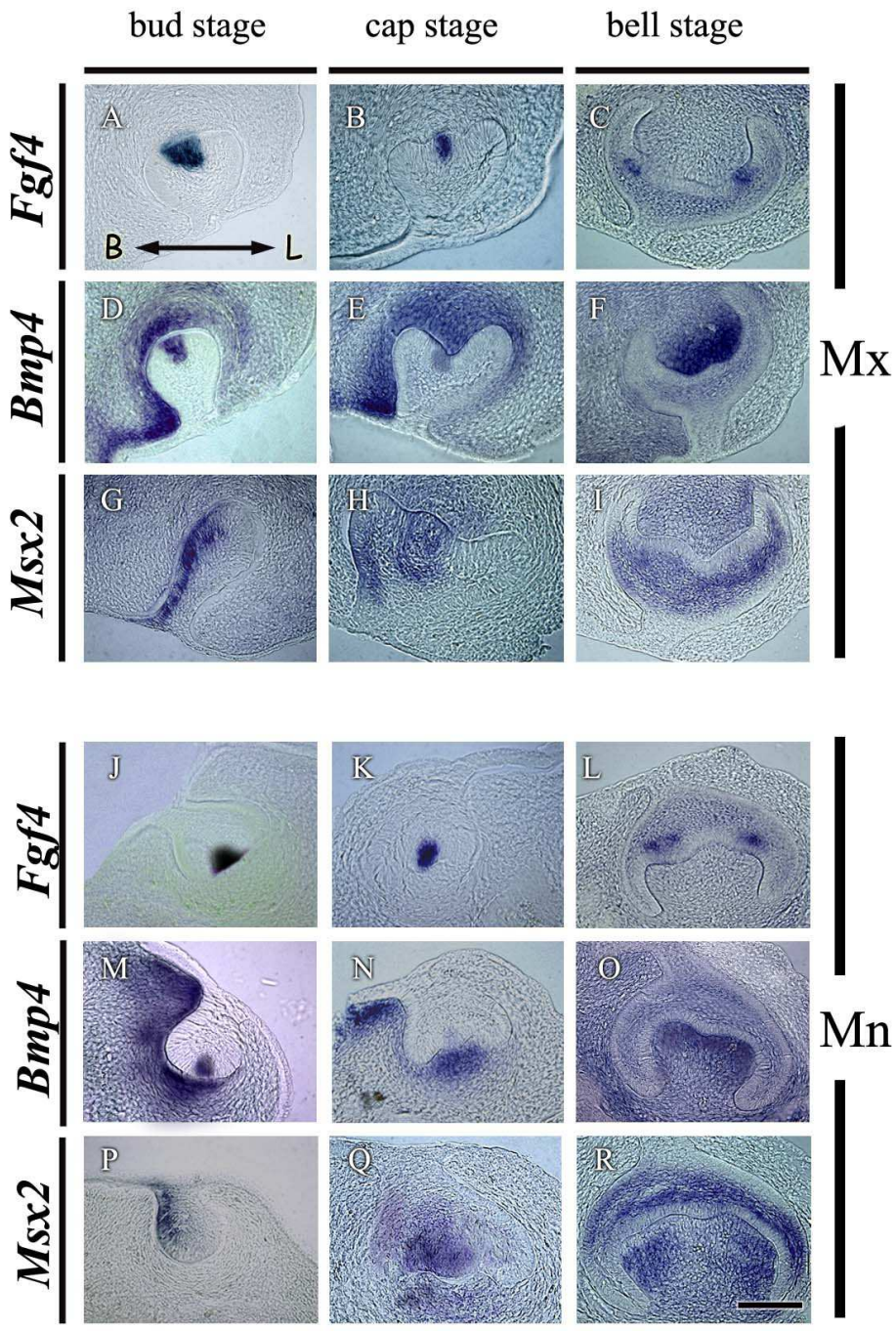

bud stage
cap stage
bell stage
Fgf4
Bmp4
Msx2
A
B
C
D
E
F
G
H
I
B
L
Mx
Fgf4
Bmp4
Msx2
J
K
L
M
N
O
P
Q
R
Mn

Figure 7. Expression patterns of *Fgf4, Bmp4* and *Msx2* in the tooth development.

(A and B) *Fgf4* is strongly expressed in the center region of the tooth germs corresponding to the primary EK at the bud and cap stages. (C) In the maxillary molar at the bell stage, intense *Fgf4* expressions are observed at the secondary EKs in both the buccal and lingual cusps. (J and K) *Fgf4* is strongly expressed in the center region of the tooth germs corresponding to the primary EK from the bud to cap stages. (L) In the mandibular molar at the bell stage, intense *Fgf4* expressions appears at the areas of the secondary EKs with some weak expression pattern in the bell stage. (D and M) At the bud stage, *Bmp4* expression, is detected at the primary EK and condensed dental mesenchyme around the epithelial bud with a somewhat stronger expression at the buccal side of the bud in the maxilla, the same *Bmp4* expression pattern is found in the mandible as well. (E and N) At the cap stage, intense *Bmp4* expression remains in the dental papilla mesenchyme, and weak expression of *Bmp4* is observed in the EK. (F and O) At the bell stage, strong *Bmp4* expression localize in the cuspal area of the dental papilla, and *Bmp4* expressions are observed with faint and broad expression pattern in dental lamina. (G and P) *Msx2* is expressed on the buccal side of the dental epithelium at the bud stage, but do not reach the

primary EK. (H and Q) *Msx2* is observed in the primary EK area and the buccal side of the dental epithelium at the cap stage. (I and R) At the bell stage, *Msx2* is expressed moderately in the dental epithelium and weakly in the dental papilla (scale bar; 100 μm).

5. Relationship between the enamel navel, enamel cord and enamel knot

Transverse semi-thin and ultra-thin sections of the tooth germs clearly demonstrated the presence of transient structures within the enamel organ. Here, the structures of EK, enamel cord and enamel navel in the enamel organ were investigated (Fig. 8). Although these structures were not recognizable until the late bud stage (data not shown), they were visible in the lower molar at cap stage. The enamel cord is a condensed cell cluster connected to the EK, which contains a strand of cells running from the enamel knot to the external dental epithelium, which appears to divide the dental organ in two (Figs. 8A, D and E)[30]. The enamel navel is a concave patch of cells in the outer enamel epithelium on the buccal side of the tooth germs (Figs. 8B and F). Even at the bell stage, the connection between the enamel navel, cord, and the EK was maintained on the buccal side (Fig. 8D), even though this connection was not always apparent depending on the position of the sections. The direction of the enamel cord at the bell stage was positioned along a vertical line relative to the oral epithelium (Fig. 8D), which is different from that at the cap stage (Fig. 8A). The enamel navel and cord were missing on the lingual side between the cap and bell stages (Figs. 8A, D and E).

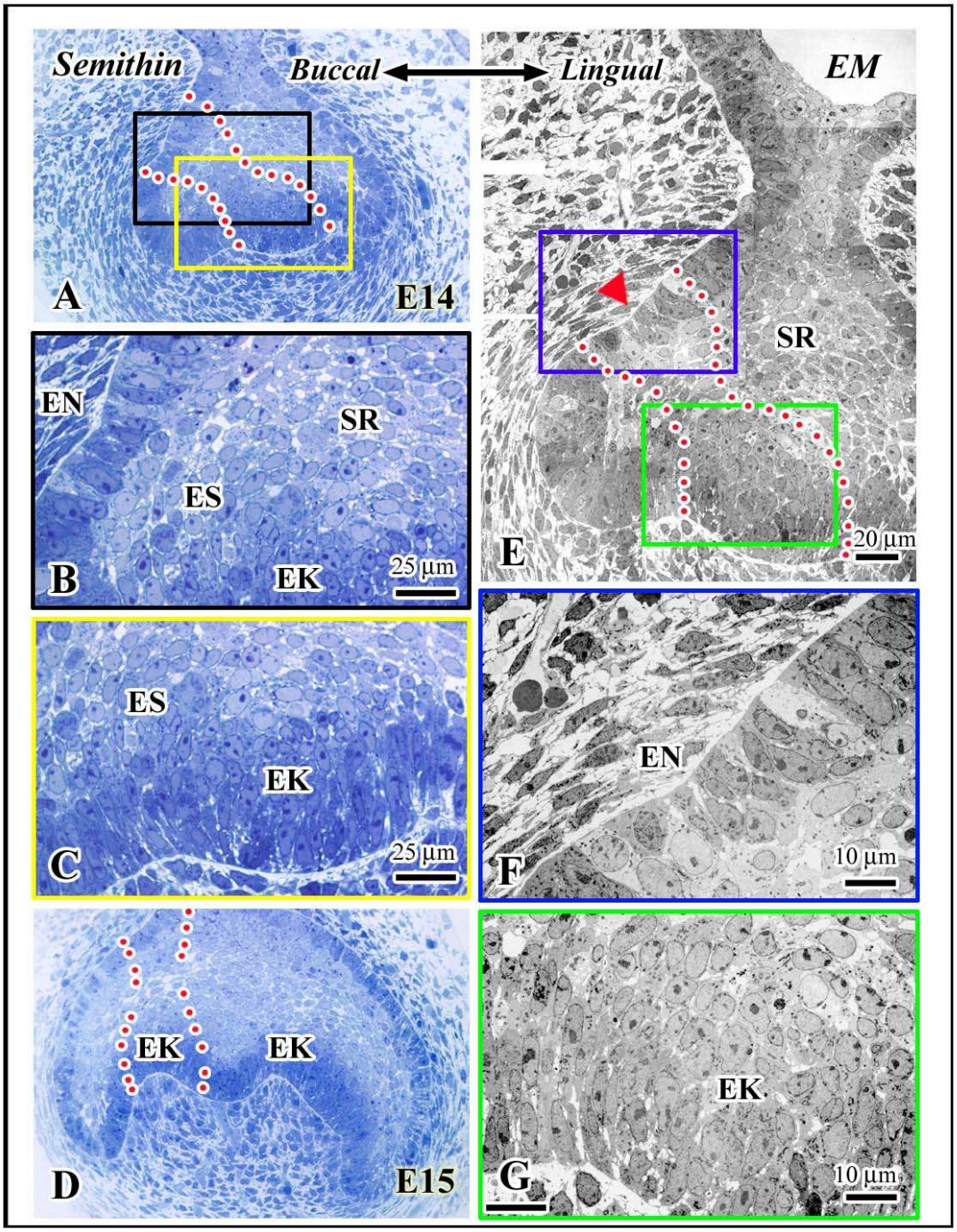

Semithin
Buccal
Lingual
EM
A
E14
SR
EN
SR
ES
B
EK
25 µm
E
20 µm
ES
EK
C
25 µm
EN
F
10 µm
EK
EK
D
E15
EK
G
10 µm

Figure 8. Spatial relationship among the enamel navel, enamel cord and EK.

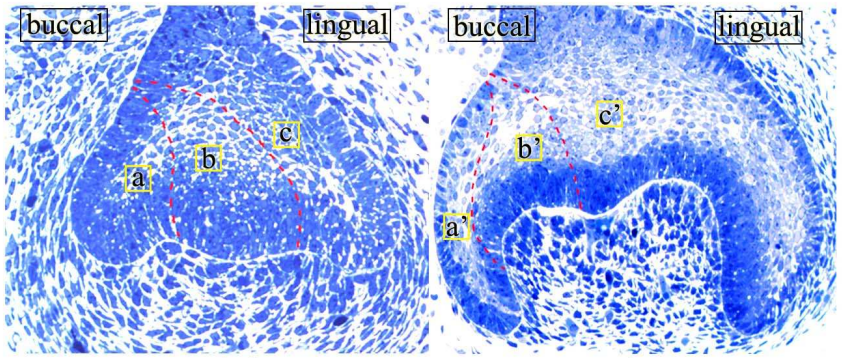
(A) At the cap stage (E14), the tooth germs possess a cap-shaped conformation including the inner and outer enamel epithelium, stellate reticulum (SR). The dotted lines indicate the boundary of three structure including the enamel navel (EN), enamel cord (EC or enamel septum), and EK. (B and C) Boxed areas denote a higher magnification of (A). (D) At the bell stage (E15), the size of tooth germs increase and result in the establishment of tooth shape. The relation among the enamel navel, cord, and EK is maintained at this stage (dotted lines). (E) The cell cluster of the enamel navel (arrow head), cord, and EK can be distinguished from the other components of dental epithelium (dotted lines). (F) The boxed areas denote a higher magnification of (E), the enamel navel, shows the concave features opposed to the dental follicle. (G) The enamel knot, shows the condensed cell cluster in the center of dental epithelium opposed to the dental papilla. EN; enamel navel, ES; enamel septum, SR; stellate reticulum, EK; enamel knot.

6. Cell density in enamel cord and other area of the dental epithelium

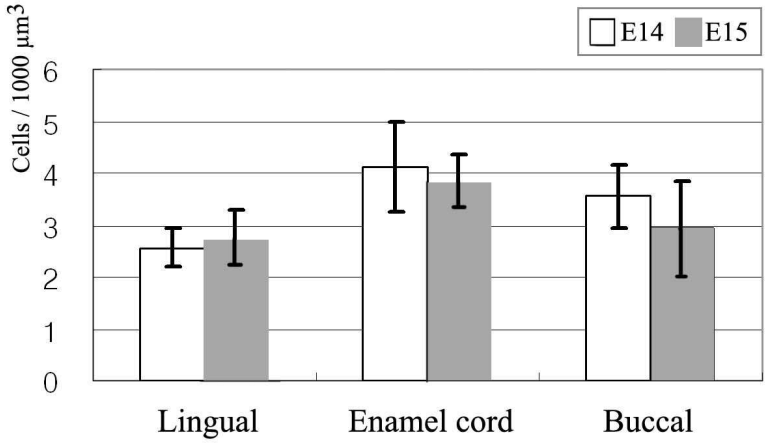
Surprisingly, it looks different cell condenses in the different areas of the enamel organ. Therefore, the number of cells were counted in the different areas of the stellate reticulum such as the buccal (a and a'), enamel cord (b and b') and lingual (c and c') area (Fig. 9). The cell population densities were measured in terms of cell counts per unit square (30 x 30 μm) (Fig. 9A) and were converted to cells per unit volume in 1 μm-thick section using the Abercrombie method.[31]

In the E14 dental epithelium, there were 3.6 cells/1000 μm^3 in the buccal area (N=13), 4.1 cells/1000 μm^3 in enamel cord area (N=15), and 2.6 cells/1000 μm^3 in lingual area (N=9). At the E15 dental epithelium, there were 3.0 cells/1000 μm^3 in buccal area (N=9), 3.8 cells/1000 μm^3 in enamel cord area (N=11) and 2.7 cells/1000 μm^3 in lingual area (N=8). Interestingly, the cell density was highest in the enamel cord, and higher in the buccal area than in the lingual part (Fig. 9C). This suggests that the enamel cord is a cell-dense structure in the stellate reticulum of the dental epithelium.

A



B



C

		Lingual	Enamel cord	Buccal
E14	average of cell number	2.6	4.1	3.6
	S.D.(±)	0.4	0.9	0.6
E15	average of cell number	2.7	3.8	3.0
	S.D.(±)	0.6	0.5	0.9

Figure 9. Cell density comparison in enamel cord, buccal, and lingual area.

(A) Tooth germ would be divided into buccal (a and a'), enamel cord (b and b') and lingual (c and c') parts and the number of cells in the quadrangle (yellow square; 30 x 30 μm) at each parts. (A-a; red dotted) Enamel cord structure is showed at E14. (A-b; red dotted) Enamel cord structure is observed at E15. The position of the enamel cord structure is similar during tooth development from E14 to E15. (B) The cell population densities measured in cell counts per unit area, using the method reported by Abercrombie (1946), assuming that the cells are 6 μm long in the plane normal to the section. (C) In the E14 dental epithelium, there are 3.6 cells/1000 μm^3 in the buccal area (N=13), 4.1 cells/1000 μm^3 in enamel cord area (N=15), and 2.6 cells/1000 μm^3 in lingual area (N=9). At the E15 dental epithelium, there are 3.0 cells/1000 μm^3 in buccal area (N=9), 3.8 cells/1000 μm^3 in enamel cord area (N=11) and 2.7 cells/1000 μm^3 in lingual area (N=8). S.D.; standard deviation

IV. DISCUSSION

1. The primary EK might be incorporated in the buccal secondary EK during tooth development

The primary EK appears during the bud to cap stage of tooth germs, and the secondary EKs appear at the sites of epithelial folding, which mark the cusp initiation sites. Both the primary and secondary EK shares similar characteristics such as their non-proliferating cell activity, gene expression pattern, and histological appearances.[14] Therefore, primary EKs may have some cellular continuity with the secondary EKs through the division of surviving cells in the primary EK and their migration into the secondary EKs.[12, 32] However, a recent study has reported that primary EK cells do not migrate to form secondary EKs.[27] This study has investigated the relationship between the primary and secondary EKs not only in the mandibular molar but also in the maxillary molar from both developmental and evolutional points of view.

Initially, the locations of the primary EK and secondary EKs were investigated by chasing *Fgf4* expression patterns in the tooth germ (Fig. 4). It showed that the *Fgf4*-expressing domains are not the only domains of the EKs. It is known that the size of the *Fgf4*-expressing domains is smaller than that of

the primary EK domains. This could explain why the *Fgf4*-expressing spot is small in the E14.5 tooth germ. It is difficult to investigate the transition of the EKs in detail because the transition from the primary EK to secondary EKs occurs during one embryonic day (from E14 to E15). In order to examine the transitional events occurring in one embryonic day, the E14 molars were cultured *in vitro* for 8, 16 and 24 hours, and their EKs expressing *Fgf4* were investigated. Interestingly, as revealed in this experiment, EK marker, *Fgf4* expressions showed similar expression patterns in upper and lower molar. Morphology, locations of primary EK and buccal secondary EK were similar during tooth development. From these observations, I hypothesized that the primary EK maintain its original position and become incorporated into the buccal secondary EK, corresponding to the paracone in the maxilla and the protoconid in the mandible. In addition, the lingual secondary EK might be newly formed by interactions between the epithelium and the mesenchyme (Fig. 10). It is important to carefully exam the first expressed primary EK, which is located below the dental lamina in the tooth germ, and the other expressed *Fgf4*-domain, newly located near the lingual side of the first *Fgf4*-domain. After 24 hours of culture from E14, primary EK was still located below the dental lamina and the last expressed *Fgf4*-domain became more distant form the primary EK to the lingual side gradually. I have a question about the results

showed in Fig.4. Even though tooth germ development was in process, primary EK, the first *Fgf4*-domain expression did not move in any direction. If that was true, primary EK cells would become incorporated with secondary EK cells in the buccal main cusp during tooth morphogenesis. However, it was difficult to declare where one *Fgf4*-domain was divided in two, and the lingual *Fgf4*-domain moved gradually away from the buccal *Fgf4*-domain, or a newly formed *Fgf4*-domain on the lingual side moved gradually away from the buccal *Fgf4*-domain after the first *Fgf4*-domain appear. Based on *Fgf4* expression results, I hypothesized that primary EK would be remained buccal secondary EK. In order to define the precise relationship between primary EK and secondary EK. I examined the cellular event using DiI labelling.

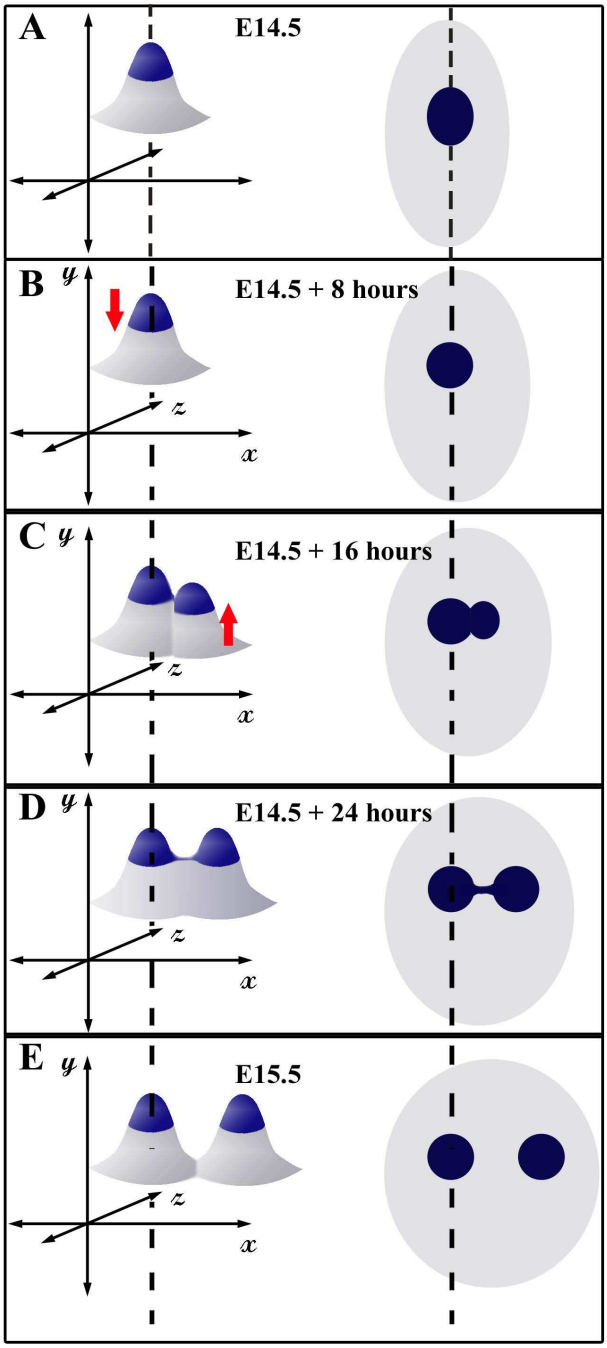

A
E14.5
B
y
E14.5 + 8 hours
z
x
C
y
E14.5 + 16 hours
z
x
D
y
E14.5 + 24 hours
z
x
E
y
E15.5
z
x

Figure 10. Schematic diagram showing the relationship between the primary EK and the secondary EK.

At the bud stage (E14), the primary EK with *Fgf4*-expressed domain appears in the center of tooth germ (A). After 8 hours culture, *Fgf4*-expressed domain is reduced slightly than that in the previous stage (B). After 16 hours, the primary EK becomes the secondary EK in the buccal cusp, and an additional new spot with *Fgf4* expression appears nearby resulting in the secondary EK in the lingual cusp (C). After 24 hours culture, the secondary EK in the buccal cusp remains in its original place and the secondary EK in the lingual cusp moves to the lingual side gradually and becomes a broad spot, resulting in two clear *Fgf4*-expressed domains (D). Two separated domains with *Fgf4* expression are clearly identified on day E15 (E). Thus, the primary EK does not move to any direction and is incorporated into the secondary EK corresponding to the first buccal main cusp (paracone and protoconid). (left : the frontal view, right : the occlusal view, X; buccal to lingual, Y; cusp formation potential, Z; anterior to posterior).

2. The cells of the primary EK may migrate into the buccal secondary EK, but not into the lingual secondary EK

The E14.5 tooth germ at cap stage is mesiodistally longer than the E13.5 tooth germ at early bud stage, and the primary EK at the E14.5 tooth germ is mesiodistally longer than that of the E13.5 primary EK.[33, 34] The long primary EK at the E14.5 tooth germ was removed apoptotically with the exception of the anterior portion in the area forming the first secondary enamel knot.[14, 35, 36] The primary EK in the E13.5 tooth germ corresponds to the anterior portion of the primary EK at the E14.5 tooth germ. In order to clarify the relationship between the primary EK and the buccal secondary EK, the primary EK cells were traced by the cell labeling method which has been suggested in the previous study.[27]

However, in contrast to the previous study, DiI was injected into the primary EK cells of the E13.5 sliced tooth germ. The present DiI-labeling experiment demonstrated that correctly DiI-labeled primary EK cells never migrated during the 48 hours of culture, and that the primary EK cells became incorporated with the secondary EK cells in the buccal main cusp during tooth development. These results support my hypothesis that the primary EK does not move but maintains its original position. Primary EK gives rise to the buccal secondary

EK, which determines the main cusp (the paracone in the maxilla and the protoconid in the mandible) (Fig. 10).

The recent study using a similar DiI-labeling method has reported that primary EK cells did not migrate to form a secondary EK, and concluded that the primary EK and secondary EK were not derived from the same cells.[27] The discrepancy between previous reports and my study might be due to differences in experimental design, such as the developmental stages of tooth germs and the DiI injection point. It is hard to define the precise morphological change, since tooth germ is too small and develops so fast. Although it would be shown various results, in my study, I was able to define the pattern of cell migration after summarizing the results from the experiment of injecting DiI in EK at E13.5. These results showed that the origin of buccal secondary EK would be primary EK. In contrast, in tooth buds, which were injected with DiI at the buccal side of the EK, the labelled cells were located not far away from the injection point. In tooth buds, which were injected with DiI at the lingual side of the EK, the labelled cells migrated to the whole lingual area.

3. The primary EK during development may be the primitive (main) cusp

According to the Cope-Osborn theory, the primitive form of the tooth in the maxilla and mandible is a simple cone (referred to as the protocone in the maxilla and the protoconid in the mandible), and is observed in many reptiles. Small mesial and distal cusps (the paracone and metacone in the maxilla and the paraconid and metaconid in the mandible) are added to the primitive cone.[22] In the subsequent stages, the relative position of these three cusps shifts to form a triangular cusp arrangement which the cusps are located opposite of each other; the primitive cusp is situated lingually in the maxilla (protocone) and buccally in the mandible (protoconid). Paleontological evidences and the study of the cusp development sequences of several mammals have shown that the first cusps to develop are the paracone in the maxilla and the protoconid in the mandibles. This study demonstrated that the primary EK was incorporated into the secondary EK in the paracone of the maxilla and in the protoconid of the mandible. The results from this study provide experimental evidence that the primary EK plays an important role in determining the buccal main cusp (the paracone and protoconid) during tooth development. This also suggests that the paracone and the protoconid might correspond to the primitive (main) cusps.

4. Enamel cord and enamel navel might be functionally significant in determining the position of the primary EK

The formation of the cap-shaped tooth germ involves a transient structure within the enamel organ, known as the enamel knot, enamel cord, and enamel navel.[37] In order to define the precise cellular mechanisms in lineage of enamel knot cells and structure of enamel organ in tooth development, semi-thin and ultra-thin section, *in situ* hybridization and cell count were examined. Semi-thin and ultra-thin sections of the cap stage enamel organ of molars showed morphological structures such as the primary EK, enamel cord (septum), and enamel navel. The connection between the enamel navel, cord, and EK was maintained in the buccal side even during bell stage. Interestingly, these structures are always observed in the buccal enamel organ. According to the result from the *in situ* hybridization showing that *Msx2* expression is overlapped with the primary EK, enamel cord, and enamel navel, it was suggested that these transitory structures are involved in the association between the tooth position and the tooth shape.[38] The fact that cell density of enamel cord as well as the primary EK was much higher than that of other area in the stellate reticulum at the cap and bell stage strongly suggests that enamel navel and the primary EK are connected by the enamel cord.

Overall, the transitory structures consisting of the primary EK, enamel cord, and enamel navel might be involved in specifying the tooth shape and in maintaining the location of the primary EK in the tooth germ. It is generally believed that the enamel navel and enamel cord in connection with the EK specify the position of the first buccal cusp (paracone and protoconid), which serves as a reference point for the later developing cusps.[38, 39]

5. Possible roles of *Bmp4* and *Msx2* involved in determination of tooth morphogenesis

The genes such as *Bmp4, Msx2,* and *Fgf4* showed distinct developmental regulated expression patterns during tooth organogenesis. Interestingly, the gene expression pattern in the upper molars was similar to that in the lower molars. During bud to cap stage, *Bmp4* was expressed mainly in the mesenchymal cells facing the buccal side of the dental epithelium in addition to the EK. Interestingly, after the cap stage, the maxillary and mandibular tooth germs expanded into the lingual area, which is the negative area for *Bmp4*. From these findings, it could be suggested that *Bmp4* might be an important factor inhibiting buccal growth of the dental epithelium. The fact, that *Msx2* is expressed on the buccal enamel cord at cap and early bell stage, suggests that the enamel cord might be closely related to the positioning of the primary EK by connecting the enamel navel and primary EK during tooth development. In addition, *Msx2* was detected on the outer enamel epithelium and stratum intermedia around the lingual and buccal secondary EK at late bell stage. At the same time, the position of the secondary EKs was determined. Moreover, it was reported that *Msx2* is the only transcription factor found in the EK[38], which is regulated by BMP4 during tooth development.[40] It is also involved in the

BMP4-dependent apoptosis pathway controlling silencing of the enamel knot and the interference with this function does not affect on early cusp patterning.[41] Consistent with this view, inhibition of apoptosis in the primary enamel knot with specific caspase inhibitors *in vitro* results in down-regulation of *Msx2* and *Bmp4* transcripts but normal cusp patterning.[12]

During the expression of these *Msx2* and *Bmp4* genes, the EK resembles the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA) in the developing limb bud, the notochord, and the floor plate of the spinal cord.[40] This suggests that the appearance of *Msx2* expression might be related to the fixations of the secondary EK on the lingual side at the late bell stage as well as on the buccal side during the cap to bell stages. These results show how the first buccal cusp and the second lingual cusp are evolved according to the advance of the tooth development (Fig. 11).

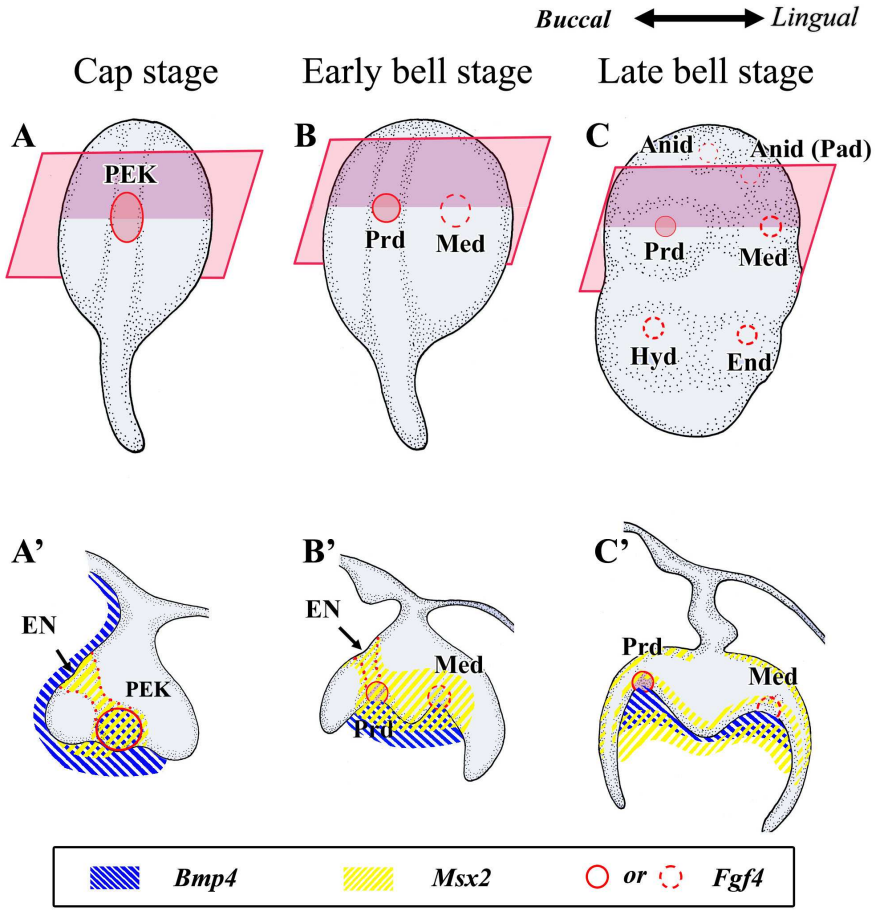

Buccal
Lingual
Cap stage
Early bell stage
Late bell stage
A
PEK
B
Prd
Med
C
Anid
Anid (Pad)
Prd
Med
Hyd
End
A'
EN
PEK
B'
EN
Med
Prd
C'
Prd
Med
Bmp4
Msx2
or
Fgf4

Figure 11. A scheme of the relationship between the primary and secondary EK from the cap to bell stages.

(A) In the upper view of the cap stage tooth germ, the primary EK (primary EK, red circle) is located in the center of tooth germ. (A') The section of the tooth germs demonstrates a cap-like appearance, and the primary EK with *Fgf4* expression is located in the center of the tooth germs (beneath the dental lamina). (B) In the upper view of the early bell stage tooth germ, the primary EK remains in its original place. (B') In the section of the early bell stage tooth germ, the primary EK remains in its original place (beneath the dental lamina) and gives rise to the buccal secondary EK corresponding to the future main cusp, e.g., the protoconid, whereas another secondary EK (red dotted circle) corresponding to the metaconid (med) appears in the lingual side of the buccal side. (C and C') In the late bell stage tooth germ, six secondary EKs corresponding to six cusps can be seen in the tooth germ in upper and section view. (A') *Bmp4* expression in the buccal mesenchyme of the tooth germ at cap stage may inhibit the buccal growth of the dental epithelium. (A' and B') *Msx2* is expressed on the buccal enamel cord connecting the enamel navel and the primary EK during tooth development. Anid; anteroconid, End; entoconid, Hyd; hypoconid, Med; metaconid, Pad; paraconid, Prd; protoconid.

V. CONCLUSION

The enamel knot (EK), located in the center of bud and cap stage tooth germs, is a transitory cluster of non-dividing epithelial cells. The morphological, cellular and molecular events leading to the relationship between the primary and secondary EKs have not been described clearly. In this study, the relationship between the primary and secondary EKs was investigated by analyzing gene expression pattern and tracing cell migration from the evolutional and developmental biology points of view.

The primary EK appears during the bud to cap stage of tooth germs, and the secondary EKs appear at the sites of epithelial folding, which mark cusp initiation sites. At first, I investigated the location of the primary EK and secondary EKs by chasing *Fgf4* expression patterns in tooth germ. The primary EK in the E13.5 tooth germ corresponds to the anterior portion of primary EK at the E14.5 tooth germ. To clarify the relationship between the primary EK and the buccal secondary EK, the primary EK cells were traced by the cell labeling method. The present DiI-labeling experiment demonstrated that correctly DiI-labeled primary EK cells would not migrate during the 48 hours of culture, and that the primary EK cells became incorporated with the secondary EK cells in the buccal main cusp during tooth development.

This study provide experimental evidence that the primary EK plays an important role in determining the buccal main cusp (the paracone and protoconid) during tooth development. Semithin and ultrathin sections of the cap stage enamel organ of molars demonstrated morphological structures such as the primary EK, enamel cord (septum), and enamel navel. It has been postulated that these transitory structures are always observed on the buccal enamel organ. It is suggested that the transitory structures consisting of the primary EK, enamel cord, and enamel navel might be involved in specification of the tooth shape and in maintenance of the primary EK location in the tooth germ. The prevalent presumption is that the enamel navel and enamel cord in connection with the EK specify the position of the first buccal cusp (paracone and protoconid), which serves as a reference point for the later developing cusps

1. Buccal secondary EK would be originated from primary EK.
2. Connections among enamel knot, enamel cord and enamel navel could be examined from cap to bell stage in tooth development.
3. Differential expressions of *Bmp4* and *Msx2* would involve in tooth cuspal formation with polarity.
4. Buccal secondary EK, originated from primary EK would play an important role in determination of main cusp.

REFERENCES

1. Sawyer RH. The role of epithelial-mesenchymal interactions in regulating gene expression during avian scale morphogenesis. Epithelial-mesenchymal interaction in development. In: Eds., R. H. Sawyer and J. F. Fallon. New york: Praeger 1993.P.115-146.

2. Hogan BL. Morphogenesis. Cell 1999;96(2):225-233.

3. Pispa J, Thesleff I. Mechanisms of ectodermal organogenesis. Dev Biol 2003;262(2):195-205.

4. Ruch JV. Determinisms of odontogenesis. Revis Biol Cellular 1987;14:1-99.

5. Osumi-Yamashita N, Ninomiya Y, Doi H, Eto K. Rhombomere formation and hindbrain crest cell migration from prorhombomeric origins in mouse embryos. Dev Growth Differ 1996;38:107-118.

6. Maas R, Bei M. The genetic control of early tooth development. Crit Rev Oral Biol 1997;8:4-39.

7. Dassule HR, Lewis P, Bei M, Maas R, McMahon AP. Sonic hedgehog regulates growth and morphogenesis of the tooth. Development 2000;127(22):4775-4785.

8. Jernvall J, Kettunen P, Karavanova I, Martin LB, Thesleff I. Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: non-dividing cells express growth stimulating *Fgf-4* gene. Int J Dev Biol 1994;38:463-469.

9. Butler PM. The ontogeny of molar pattern. Biol Rev 1956;31:30-70.

10. Mustonen T, Tummers M, Mikami T, Itoh N, Zhang N, Gridley T, et al. Lunatic fringe, FGF, and BMP regulate the Notch pathway during epithelial morphogenesis of teeth. Dev Biol 2002;248(2):281-293.

11. Jernvall J, Aberg T, Kettunen P, Keranen S, Thesleff I. The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot. Development 1998;125(2):161-169.

12. Coin R, Kiefer S, Lesot H, Vonesch JL, Ruch JV. Inhibition of apoptosis in

the primary enamel knot does not affect specific tooth crown morphogenesis in the mouse. Int J Dev Biol 2000;44:389-396.

13. Vaahtokari A, Aberg T, Jernvall J, KerWen S, Thesleff I. The enamel knot as a signaling center in the developing mouse tooth. Mech Dev 1996;54:39-43.

14. Jernvall J, Thesleff I. Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev 2000;92:19-29.

15. Kassai Y, Munne P, Hotta Y, Penttila E, Kavanagh K, Ohbayashi N, et al. Regulation of mammalian tooth cusp patterning by ectodin. Science 2005;309(5743):2067-2070.

16. Cobourne MT, Sharpe PT. Sonic hedgehog signaling and the developing tooth. Curr Top Dev Biol 2005;65:255.

17. Kondo S, Townsend GC. Associations between carabelli trait and cusp areas in human permanent maxillary first molars. Am J Phys Anthropology 2006;129(2):196-203.

18. Luukko K, Loes S, Furmanek T, Fjeld K, Kvinnsland IH, Kettunen P. Identification of a novel putative signaling center, the tertiary enamel knot in the postnatal mouse molar tooth. Mech Dev 2003;120(3): 270-276.

19. Nie XG, Luukko K, Kettunen P. FGF signalling in craniofacial development and developmental disorders. Oral Diseases 2006;12(2):102-111.

20. Pispa J, Jung HS, Jernvall J, Kettunen P, Mustonen T, Tabata MJ, et al. Cusp patterning defect in Tabby mouse teeth and its partial rescue by FGF. Dev Biol 1999;216(2):521-534.

21. Loes S, Luukko K, Kvinnsland IH, Kettunen P. Slit1 is specifically expressed in the primary and secondary enamel knots during molar tooth cusp formation. Mech Dev 2001;107(1-2):155-157.

22. Osborn JW. Dental Anatomy and Embryology. In: Eds., Rowe AHR, Johns RB, Blackwell Scientific Publications Vol.1, Book.2 1981. P. 250-255, 336-356, 340-375.

23. Jernvall J, Keranen SVE, Thesleff I. Evolutionary modification of

development in mammalian teeth: quantifying gene expression patterns and topography. Proc Natl Acad Sci USA 2000;97:14444-14448.

24. Love A. Keywords & concepts in evolutionary developmental biology. Biol&Philos 2005;20(2-3):567-584.

25. Coin R, Lesot H, Vonesch JL, Haikel Y, Ruch JV. Aspects of cell proliferation kinetics of the inner dental epithelium during mouse molar and incisor morphogenesis: a reappraisal of the role of the enamel knot area. Int J Dev Biol 1999;43:261-267.

26.Keranen SV, Aberg T, Kettunen P, Thesleff I, Jernvall J. Association of developmental regulatory genes with the development of different molar tooth shapes in two species of rodents. Dev Genes Evol 1998;208:477-486.

27. Matalova E, Antonarakis GS, Sharpe PT, Tucker AS. Cell lineage of primary and secondary enamel knots. Dev Dyn 2005;233:754-759.

28. Honig MG, Hume RI. Dil and DiO: versatile fluorescent dyes for neuronal labelling and pathway tracing. Trends Neurosci 1989;12(9): 333-335, 340-341.

29. Jung HS, Francis-West PH, Widelitz RB, Jiang TX, Ting-Berreth S, Tickle C, et al. Local inhibitory action of BMPs and their relationships with activators in feather formation: implications for periodic patterning. Dev Biol 1998;196(1):11-23.

30. Ten-cate AR. Ten Cate's Oral Histology Development, Structure, and Function. In: Mosby Publications, fifth Edition, 1998. P.86-87.

31. Abercrombie M. Estimation of nuclear population from microtome sections. Anat Rec 1946;94:239-247.

32. Matalova E, Tucker AS, Sharpe PT. Death in the life of a tooth. J Dent Res 2004;83(1):11-16.

33. Shigemura N, Kiyoshima T, Kobayashi I, Matsuo K, Yamaza H, Akamine A, Sakai H. The distribution of BrdU- and TUNEL-positive cells during odontogenesis in mouse lower first molars. Histochem J 1999;31:367-377.

34. Shigemura N, Kiyoshima T, Sakai T, Matsuo K, Momoi T, Yamaza H, et al.

Localization of activated caspase-3-positive and apoptotic cells in the developing tooth germ of the mouse lower first molar. Histochem J 2001;33(5):253-258.

35. Matalova E, Witter K, Misecondary EK I. Apoptosis distribution in the first molar tooth germ of the field vole (Microtus agrestis) Tissue & Cell 2004;36(5):361-367.

36. Akhter M, Kobayashi I, Kiyoshima T, Matsuo K, Yamaza H, Wada H, et al. Possible functional involvement of thymosin beta 4 in developing tooth germ of mouse lower first molar. Histochem & Cell Biol 2005;124(3-4):207-213.

37. Zhao Z, Stock D, Buchanan A, Weiss K. Expression of Dlx during the development of the murine dentition. Dev Genes Evol 2000;210: 270-275.

38. MacKenzie A, Ferguson MW, Sharpe PT. Expression patterns of the homeobox gene, Hox-8, in the mouse embryo suggest a role in specifying tooth initiation and shape. Development 1992;115(2):403-420.

39. Ruch JV, Lesot H, Karcher-Djuricic V, Meyer JM, Olive M. Facts and

hypotheses concerning the control of odontoblast differentiation. Differentiation 1982;21(1):7-12.

40. Zhao Z, Weiss K, Stock D. Development and Evolution of dentition patterns and their genetic basis. In: Cambridge University Press, Cambridge P.152-172.

41. Marianna B, Stephanie S, Richard M. *Msx2* Controls Ameloblast Terminal Differentiation. Dev Dyn 2004;231:758-765.

ABSTRACT (IN KOREAN)

생쥐 첫째어금니에서 일차사기질결절과 교두 형성의 관계

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이 현 아

치배의 싹시기와 모자시기동안 그 중심부에 위치하는 사기질결절은 일시적으로 분화되지 않는 상피세포의 집단이다. 이는 치아형태형성을 위한 위치적 정보를 제공하는 역할과 이차사기질결절 유도를 통해 교두 성장을 조절하는 역할을 한다. 사기질결절은 발생과정동안 일차(싹시기~모자시기)와 이차(종시기)의 사기질결절 형태로 나뉘는데 두 구조물의 세포적, 분자적 관계에 대한 연구는 현재까지 미비한 수준이다. 사기질결절 발생과정의 명확한 이해를 위해서 사기질결절이 노출된 상태로 지속적인 관찰이 가능한 실험기법인 slice culture체외배양법으로 실험하였으며 친유성 형광염료인 DiI를 이용하여 사기질결절세포를 염색하여 그 운명을 추적하였다. DiI로 염색되어진 일차사기질결절의 세포들은 체외배양 48시간 후 구강 쪽 이차사기질결절로 이

동하였음을 알 수 있었다. 또한 사기질결절의 표지자로 알려진 *Fgf4*의 mRNA발현양상을 *in situ* hybridization 방법으로 확인해 본 결과 유전자 수준에서 일차사기질결절과 이차사기질결절의 연속성을 확인할 수 있었다. 그리고 semithin과 ultrathin section을 이용한 치배 구조관찰과 치배상피세포의 영역별(buccal, enamel cord, lingual) 세포의 밀도 계산을 통해 사기질끈(enamel cord)이라는 구조물이 일차사기질결절이 나타나는 모자시기부터 이차사기질결절이 나타나는 종시기에 이르기까지 사기질결절을 구조적으로 고정하고 있음을 알게 되었다. 따라서 사기질끈의 기능으로 인해 일차사기질결절은 구강 쪽의 이차사기질결절로 유지가 된다고 추측할 수 있었으며 구강 쪽 이차사기질결절은 발생과정을 거쳐 위턱의 paracone과 아래턱의 protoconid로 형성되어질 것으로 예상 할 수 있었다.

핵심 되는 말 : 치아발생, 사기질결절, 형광염료(DiI)추적, paracone, protoconid.