Temporospatial tissue interactions regulating the regeneration of the enamel knot in the developing mouse tooth

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This certifies that the Doctoral Dissertation of Sung-Won Cho is approved.

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Now I am standing on the peak of a mountain through the climbing for five years. If I look back on these years, it was a very long and hard time for me. From the year 2001, when I stopped caring dental patient as a dentist and began to study the basic dental science, there have been professors giving invaluable advice to guide my way and colleagues sharing every moment in laboratory.

I would to like to give my thanks to professor Han-Sung Jung for giving a chance to study on tooth development and invaluable advice. I really appreciate Prof. Syng-Ill Lee, Prof. Kyoung-Nam Kim, Prof. Kyung Ah Park, Prof. Kyoo-Sung Cho, Prof. Hee-Jin Kim, Prof. Hitoshi Yamamoto, Prof. Kyung-Seok Hu, Dr. Jae-Young Kim and all members in Prof. Jung's laboratory.

My family support and trust me all through my life. I can be here because of their help and sacrifice. I would like to express special thanks to my family.

I am going to climb another mountain, which may be more rugged and higher than the mountains that I have climbed before. However, as long as I share time with my colleagues, friends and my family, it will be a great time.

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

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(*D*irected b*y* Professor Han-Sung Jung)

The enamel knot (EK), a transient signaling center in the tooth germ, has been known to regulate both the differential growth of dental epithelium and the tooth shape. Our studies evaluated the regeneration of the EK. The EK regions were removed in the E12, E14 and E16 dental epithelium, and remaining epithelia were recombined with its original dental mesenchymes. All these tooth germs coulddevelop into calcified teeth after three-week transplantation in kidney capsule, and regenerated one primary EK earlier and two or three secondary EKs laterin culture. The E16 tooth germ generated one primary EK firstly and many secondary EKs later, when it was simply recombined without removal of the EK. These results suggest that neither the dental epithelium nor the dental mesenchyme can dictate the pattern of the EKs formation directly. It is also suggested that the pattern of the EK in tooth germs restart from a primary EK and that the interaction betweenthe dental mesenchyme and dental epithelium is crucial to restart the patterning of the EKs.

Key words : enamel knot, tooth germ, regeneration, tissue interaction, fate map, molar

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I. INTRODUCTION

The mammalian tooth is one of the ectodermal organs, of which the development is controlled by reciprocal interactions between the epithelium and the mesenchyme. All ectodermal organs share similar signaling molecules during early morphogenesis, but each organ undergoes its own specific pattern formation later in development.[1,2]

The teeth develop from pharyngeal epithelium and the underlying neural crest-derived mesenchymal cells.[3] These neural crest cells derive from the midbrain region, and their final position in the maxillary and mandibular processes is associated with the original position of the cells in the neural crest as well as with the time when the cells leave the crest.[4,5]

Tooth formation is a continuous process that may be characterized by a series of distinguishable stages. The stages are classified according to the shape of epithelium of the tooth germ. Four different stages are recognized, for example the dental lamina stage, bud stage, cap stage or bell stage. The dental lamina stage is characterized by a thickening of the oral epithelium. The bud stage designates a rounded, localized growth of the epithelial cells of the dental lamina. Proliferating mesenchymal cells surround the bud and form an ectomesenchymal condensation. Gradually, the epithelial bud gains a concave surface to be considered as a cap-shaped enamel organ. The

enamel organ, dental papilla and dental follicle constitue the cap stage tooth germ. After the enamel organ and adjacent dental papilla increase further in size, the tooth germ proceeds from the cap stage to the bell stage, at which tooth germ establishes the definitive tooth shape showing epithelial tips of developing cusps.

In mice, tooth morphogenesis is initiated at E11, when the oral epithelium begins to thicken in the incisor and molar regions. The dental lamina in mice is very thin, unlike in other mammals. At late bud stage, the enamel knot begins to develop.[6]

The enamel knot (EK) is considered to be the most important structure in the determination of the number of cusps, as well as the shape of an individual tooth. In mouse molar tooth development, there are three signaling centers: the early signaling center, the primary enamel knot (EK), and the secondary EK.[7] Both the primary EK and the secondary EK share the expression patterns of the same genes. Signaling molecules such as *Fgf4, 9, Shh, Wnt10a, b,* and *Bmp2, 4, 7,* and transcription factors such as *Msx1, 2* and *Lef1* are expressed in the primary EK and the secondary EK.[8,9,10,11,12] Among these genes, *Fgf4* and *Slit1* can be used as EK markers, as these are two genes observable only in both the primary and the secondary EK.[13,14,15]

Many investigators have proposed that the primary EK acts as a signaling center, which may also regulate crown morphogenesis.[13,16] Signaling molecules expressed in EKs have been shown to stimulate cell division in both the dental epithelium and the dental papillae. However, no fibroblast growth factor (FGF) receptors appear to be present in EK cells, and these cells do not undergo mitosis when FGFs are added.[17] In addition, the cyclin-dependent kinase inhibitor, p21, which is involved in apoptosis, is also expressed in the primary EK.[8] Therefore, it has been suggested that both cell death in the primary EK and cell proliferation in the epithelium adjacent to the primary EK coordinate epithelial folding and the transition of the bud to the cap stage.[7,13]

This study examined the phenomena occurring after removing these epithelial signaling centers. At first, early signaling center, the primary EK and the secondary EKs were mechanically removed from the E12, E14 and E16 tooth germs respectively. The shape of calcified teeth from these tooth germs were analyzed and the regeneration of EKs were investigated in these tooth germs.

Furthermore, the precursor of regenerated EK and the EK-regeneration mechanism were both investigated by the various kinds of recombinations and cell labeling methods.

In general, to investigate the lineage and differentiation ability of certain specific cells, two strategies for intracellular labeling have been used, fluorescent dye markers and the transgene expression of the reporter genes.[18,19] Fluorescent dye markers are widely used for detecting cell proliferation and cell movement.[20] Recent extracellular cell labeling dyes are DiI and DiO, hydrophobic substances dissolving in the lipid membranes of the labelled cells and are well retained in the original cells and their progeny. They are intensively fluorescent, DiI producing red and DiO producing green emission. In tooth development, the lineage of the primary and secondary EKs has been investigated by the cell labeling using DiI.[21] Transgenic animals with the reporter genes, such as *lacZ* (β-galactosidase) and GFP (green fluorescent protein), are suitable to be investigated for the cell lineage, migration, proliferation, and differentiation during the development and the regeneration processes. Recently, *lacZ* system was found to be more popular than the GFP system, which has the nonspecific fluorescence, interferences of the background and immune responses within the host.[21,22] ROSA26 mice expressing the *lacZ* have been widely used not only to study the ability of stem cells to differentiate into various cell types including endothelial cells,[23,24,25] smooth muscle cells,[26,27] nerve cells[28], skeletal muscle cells[29] and cardiomyocytes[30] but also to investigate the targeted gene activity during embryonic development.

In this study, *lacZ* transgenic mice were used to distinguish the

epithelium from the mesenchyme and to identify the non-contamination of epithelial cells into the mesenchyme during separation. DiI was used for the investigation of the target cell lineage.

II. MATERIALS AND METHODS

1. Removal of the EK region from the tooth germ at different stages

Tooth germs at the early bud stage (E12), the cap stage (E14), and the early bell stage (E16) were carefully isolated from the mandibles of ICR mice embryos. The anterior and posterior portions of the first molar in the E12, E14 and E16 tooth germs were initially excised. The dental epithelium and dental mesenchyme of the first molars were separated from each other after 12 minutes of incubation in dispase II (Boehringer Mannheim). The apical half of the dental epithelium, including the signaling centers, was mechanically removed with fine tungsten needles. The remaining dental epithelium at E12 was referred to the remaining E12 epithelium. The remaining dental epithelium at E14 and E16 were referred to the remaining E14 epithelium and the remaining E16 epithelium respectively (Fig. 1).

2. Recombination

A. Recombination between remaining epithelium and dental mesenchyme

The RTG, which was made up of the remaining E12 epithelium and the E12 dental mesenchyme, was simplified as the E12 RTG. The remaining E14 epithelium and the remaining E16 epithelium were recombined with each original dental mesenchyme to form the E14 RTG and the E16 RTG respectively (RECOMBINATION #1 in Fig. 1).

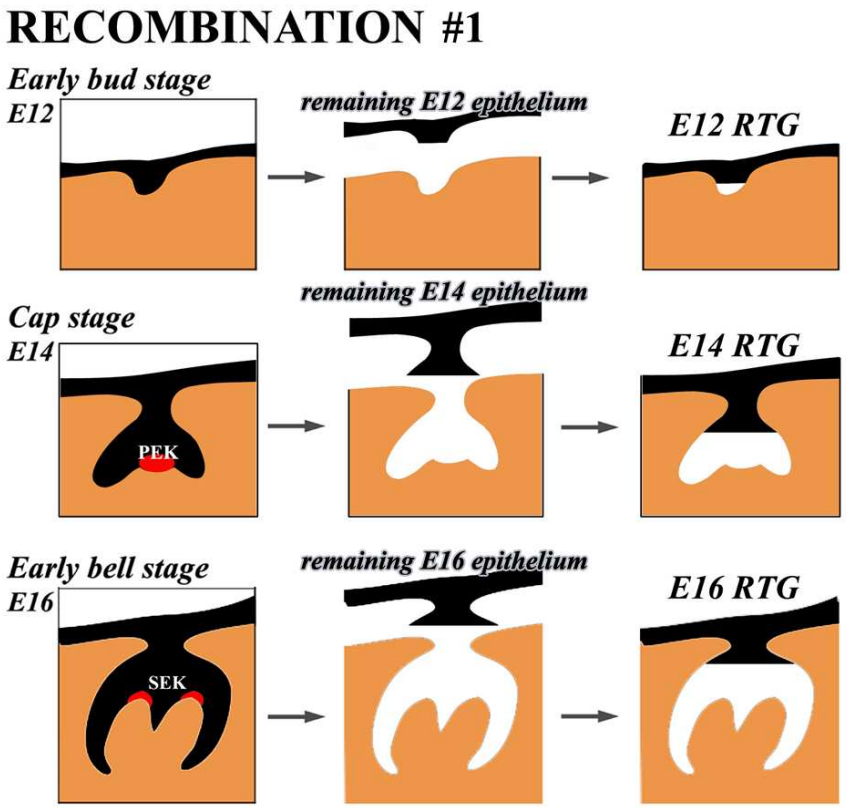

Fig. 1. Recombination method and various kinds of recombinant tooth germs (RTGs)

After being separated from the dental mesechyme, remaining epithelium without the primary EK(PEK) and secondary EK(SEK) were recombined with the original mesenchyme. The RTG between the remaining E12 epithelium and the E12 dental mesenchyme was simplified as the E12 RTG. The remaining E14 and E16 epithelia were recombined with each original dental mesenchyme to form the E14 RTG and the E16 RTG, respectively.

B. Recombination with cell labeling

After the dental papilla was labeled with lipophilic dye, the remaining E14 epithelium and the E14 dental mesenchyme were recombined (see RECOMBINATION #2 in Fig. 2).

C. Recombination without removal of secondary EKs

After being separated from the E16 dental mesenchyme, the E16 dental epithelium was recombined with the E16 dental mesenchyme without any mechanical removal of secondary EKs to form the E16 recombined tooth germs (rtgs) (see RECOMBINATION #3 in Fig. 2).

D. Recombination between apical half epithelium and dental mesenchyme

The apical half of the E14 dental epithelium could be considered as the enamel organ, therefore the RTG, which was composed of the apical half of the E14 dental epithelium and the E14 dental mesenchyme, was simplified as the E14 enamel organ RTG (see RECOMBINATION #4 in Fig. 2)

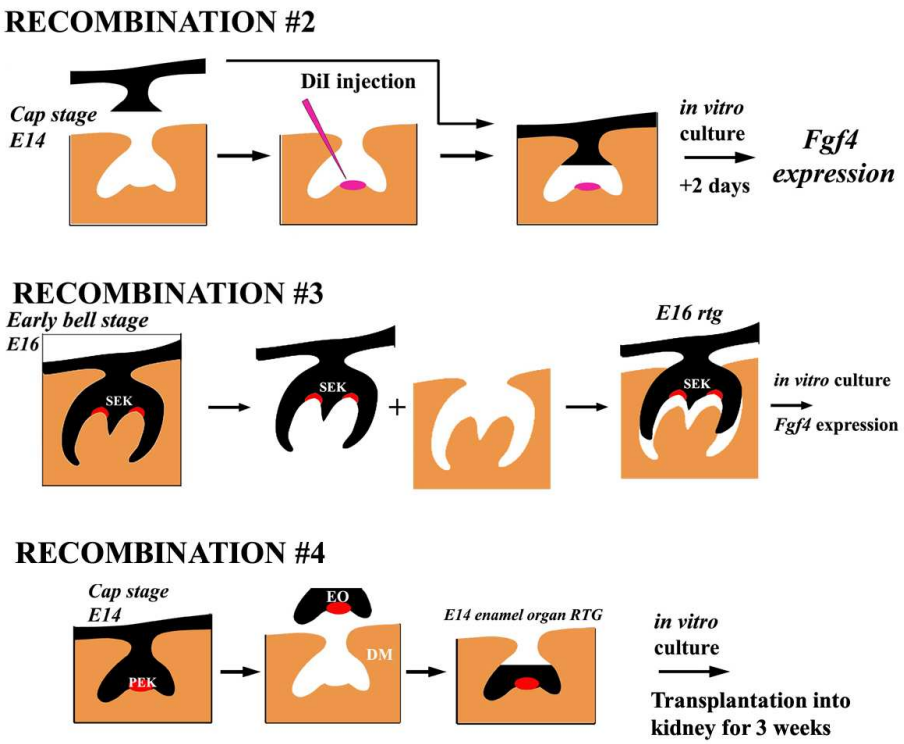

Fig. 2. Various kinds of recombination methods and tooth germs

(RECOMBINATION #2) After dissociation of the dental mesenchyme from the dental epithelium in E14 tooth germ, the dental papillae were labeled with DiI, and then recombined with the remaining E14 epithelium which had lost primary EK. Two days later, the E14 RTGs were in situ hybridized. (RECOMBINATION #3) The E16 dental epithelium with four secondary EKs (SEK) and the E16 dental mesenchyme with four dental papillae were recombined to form the E16 recombined tooth germ (E16 rtg). (RECOMBINATION #4) The apical half of the E14 dental epithelium, which can be regarded as the enamel organ (EO), was recombined with the E14 dental mesenchyme (DM) to form a RTG (E14 enamel organ RTG).

3. Cross-recombination between the E14 and E16 tooth germs

After removal of primary EK and secondary EKs from the E14 and E16 dental epithelium, the remaining E14 epithelium and the remaining E16 epithelium were cross-recombined with the E16 dental mesenchyme and the E14 dental mesenchyme respectively to make cross-recombinant tooth germs (cross-RTGs). The cross-RTGs of the remaining E14 dental epithelium and the E16 dental mesenchyme were called as the E14/E16 cross-RTGs. The E16/E14 cross-RTGs were composed of the remaining E16 dental epithelium and E14 dental mesenchyme (Fig. 3).

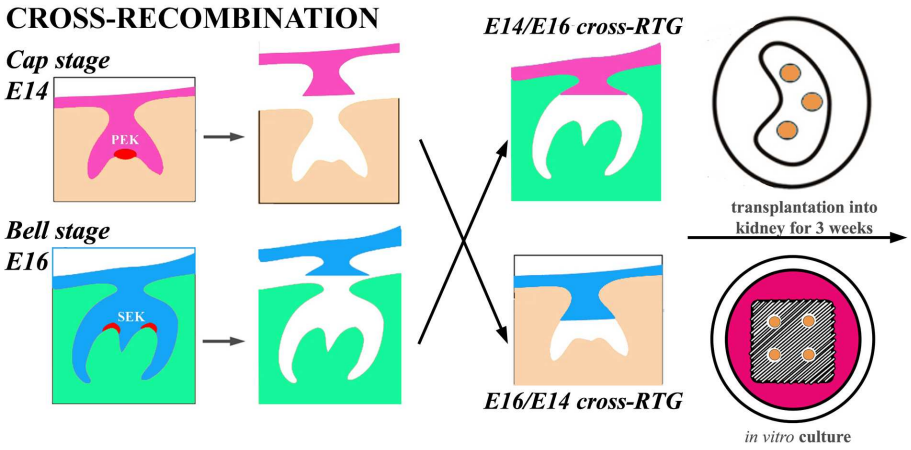

Fig. 3. Cross-recombination methods and various kinds of cross-RTGs.

The cross-recombinant tooth germs (cross-RTGs) of the remaining E14 dental epithelium and the E16 dental mesenchyme was called as the E14/E16 cross-RTG. The E16/E14 cross-RTG was composed of the remaining E16 epithelium and E14 dental mesenchyme. Cross-RTGs were also cultured for three weeks in kidney and two days *in vitro*

4. Transplantation of RTGs into the subcapsular layer of mouse kidney

The recombinant tooth germs, each over twenty, were transplanted into the subcapsular layer of an adult ICR mouse kidney, and cultured for three weeks. All surgery was performed after the administration of intra-peritoneal injections of anesthesia. No immuno-suppressive medication was used.

5. Whole mount *in situ* hybridization

In vitro cultured tooth germs were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). *In situ* hybridization was carried out by

treating the tooth germs with 20 g/ml proteinase K for four minutes at room temperature. Anti-sense RNA probes were labeled with digoxygenin (BMS, Korea). After *in situ* hybridization, the specimens were cryo-sectioned at a thickness of 9 ㎛.

11

6. Identification of the *lacZ* transgenic cells

Specimens were washed with 2mM MgCl2 in PBS for 5 min, rinsed three times with a rinse buffer (2mM MgCl2, 0.02% NP-40, 0.01% sodium deoxycholate in PBS) for 20 min at room temperature, stained with β-gal staining solution (1mg/ml of 5-bromo-4-chloro-3-indolyl-β -D-galactopyranoside (X-gal), 5mM potassium ferrocyanide, and 5mM potassium ferricyanide), and incubated at 37? for 1 hour. After the X-gal staining, the samples were washed again with PBS for 10 min, and mounted on slides.

7. Cell labeling with DiI

DiI (dioctadecyl tetramethyl indocarbocyanine perchlorate; Molecular Probes, Eugene, OR, USA) was used as a cell tracer for the observation of epithelial or mesenchymal cell migration during tooth development. A 0.3%solution w/v DiI in DMSO was microinjected, using 10 cm borosilicate capillary pipettes (BF120-94-10, Sutter Instruments, USA), pulled using a Flaming/Brown micropipette puller (Sutter Instruments, USA), and filled by capillary action. Lipophilic carbocyanine dye was then inserted into the membrane of the cells adjacent to the injection site with an electronic device. In order to determine the exact locations of the labeled cells, 9 ㎛ frozen sections were prepared. The location of the dye can then be determined using fluorescent microscopy.

III. RESULTS

1. RTGs can develop into calcified teeth

In order to investigate the necessity of signaling centers in tooth development, the early signaling center at E12, the primary EK at E14, and the secondary EK at E16 were surgically removed. Three weeks after being transplanted into a kidney, all RTGs at various stages were able to develop into calcified teeth.

Calcified teeth developing from various RTGs showed different shape each other. Teeth developing from the E12 RTG (Fig. 4A, a) and teeth from the E14 RTG (Fig. 4B, b) showed molar shape with cusps. On the other hand, the E16 recombinant teeth exhibited abnormal molar shape with very low cusp (Fig. 4C, c) or human incisor-like shape (Fig. 4D). These results indicate that although the E12 and the E14 RTGs had lost their signaling centers, they proved capable of forming calcified teeth with cusps. From these results, two possibilities can be considered for the necessity of signaling centers: (1) The signaling center is not necessary for the formation of tooth cusps; (2) The signaling center can be regenerated after being removed.

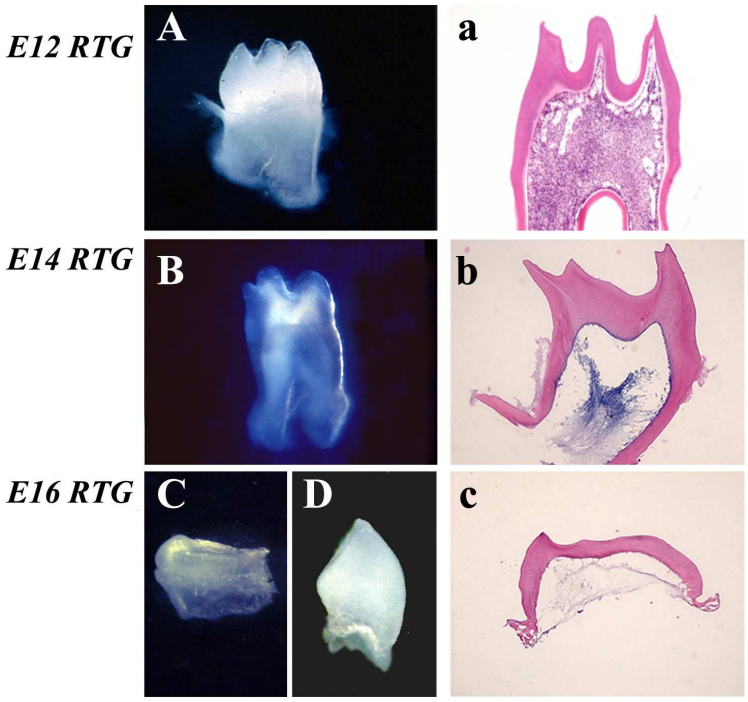

Fig. 4. Calcified teeth from RTGs.

Calcified teeth from various RTGs differed in shape from each other. (A) The E12 recombinant tooth showed molar shape with cusps. (a) The E12 recombinant tooth showed well forming tooth structure with cusps in the section. (B, b) The E14 recombinant tooth also exhibited a molar shape with cusps. (C, c) The E16 recombinant teeth showed abnormal molar shape with low cusp. (D) The E16 recombinant teeth also formed human incisor-like tooth.

2. *Shh* or *Fgf4*-expressing spots in RTGS

In order to prove the regeneration of the signaling center, RTGs were cultured in vitro and were in situ hybridized.

A. *Shh*-expressing spot in the E12 RTG

The existence of the EK in the E12 RTG was investigated by the *Shh* expression, because *Fgf4* is known not to express at the E12 tooth germ. In the E14 and E16 RTGs, the existence of the EK was investigated by the *Fgf4* expression.

After two days in culture, the E12 RTG showed only one dark blue spot expressing *Shh* in an upper view (Fig. 5A). After four days, the E12 RTG exhibited a large spot expressing *Shh* (Fig. 5B).

B. *Fgf4*-expressing spot in the E14 RTG

There was no *Fgf4*-expressing spot in the E14 RTG after 12 hours (Fig. 5C). This result proves that the EK region was totally excised during mechanical removal procedure. The E14 RTG showed one small *Fgf4*-expressing spot (arrow) after one day (Fig. 5D). After two days, the E14 RTG contained a round spot (arrow in Fig. 5E) or two spots (black and red arrows in Fig. 5F). Two spots (black and red arrows in Fig. 5F) were so distant from each other that might be considered as the one spot in each tooth germ (black arrow in gray dotted circle and red arrow in pink dotted circle in Fig. 5F). After four days, the E14 RTG showed two (arrowheads in Fig. 5G) or three spots (arrowheads and red arrow in Fig. 5H). The size of two spots (arrowheads in Fig. 5G) was same, and the distance was very short between them, therefore these two spots might be included in one tooth germ (Fig. 5G). In the E14 RTG showing three spots (Fig. 5H), two spots (arrowheads) was same in size, and the distance between them was very short, while one spot was isolated. These results indicate that there were two tooth germs, one of which showed two spots (gray dotted circle in Fig. 5H), and the other of which showed one spot (pink dotted circle in Fig. 5H). In summary, the E14 RTG regenerated one spot first, which was followed by two spots later.

C. *Fgf4*-expressing spot in the E16 RTG

The E16 RTG revealed one spot expressing *Fgf4* (arrow in Fig. 5I) after two days in culture and exhibited two or three spots in a row after four days (arrowheads in Fig. 5J, K).

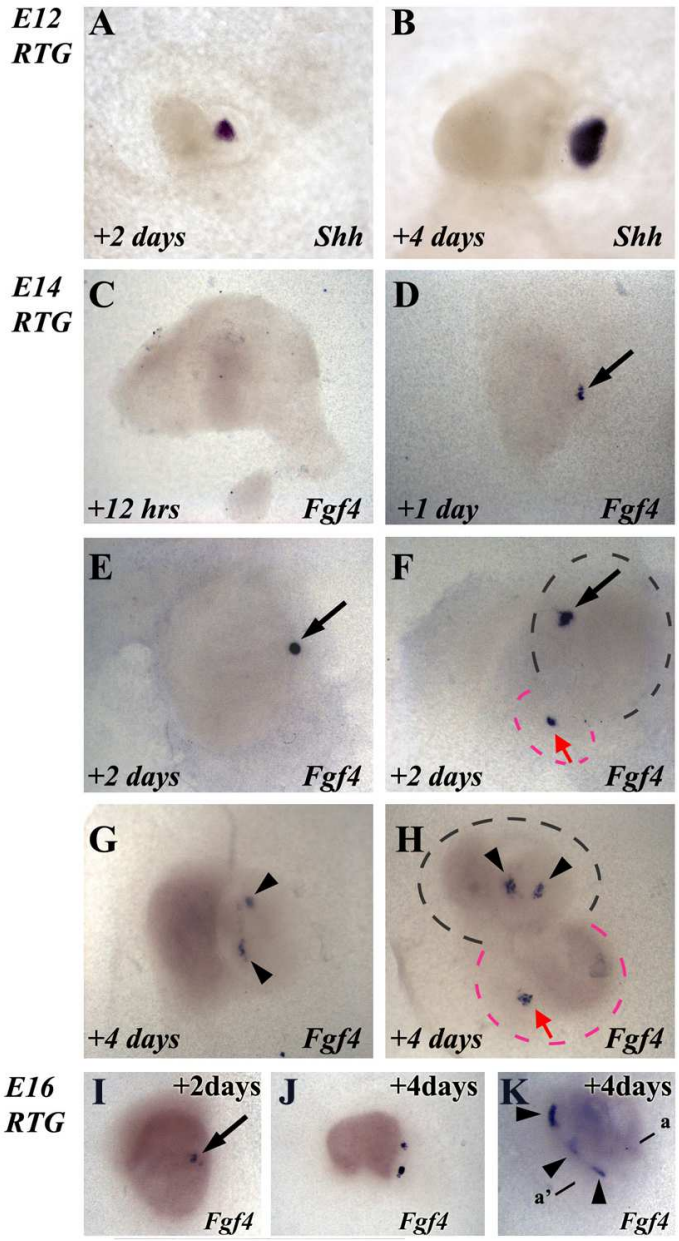

Fig. 5. *Shh* and *Fgf4*-expressing spots in the RTGs (upper view).

(A) After two days in culture, the E12 RTG showed only one dark blue spot expressing *Shh*. (B) After four days, the E12 RTG exhibited a large spot expressing *Shh* abundantly. (C) The E14 RTG did not show any spot expressing *Fgf4* after 12 hours. (D) The E14 RTG showed one small spot (arrow) expressing *Fgf4* following one day in culture. (E) At the end of two days, the E14 RTG had a big *Fgf4*-expressing spot (arrow). (F) The E14 RTG had two *Fgf4*-expressing spots (black and red arrows) after two days in culture. One spot (black arrow) was big and distant from the other small spot (red arrow), therefore the big spot might be included in one tooth germ (gray dotted circle), and the small spot might be included in the other tooth germ (pink dotted circle). (G) After four days, in the E14 RTG showing two spots (arrowheads), the size of both spots was the same, and the distance between the two spots was short. (H) Some of the E14 RTG showed three spots in the RTG after four days. Two spots (arrowheads) were close to each other, but one spot (red arrow) was isolated. Two spots might be included in one tooth germ (gray dotted circle), and the isolated spot might be included in the other tooth germ (pink dotted circle). (I) The E16 RTG showed only one spot (arrow) that expressed *Fgf4* after two days in culture. (J, K) After four days in culture, some the E16 RTG exhibited two (J) or three spots (three arrowheads in K). e, epithelium; m, mesenchyme

3. Localization of spots in the section of tooth germ

Fgf4 is known as a maker gene of the EK, therefore the EK can be defined as the *Fgf4*-expressing cluster in the dental epithelium facing the dental mesenchyme. In other words, the *Fgf4*-expressing spot, which was identified as the epithelial cell cluster facing the dental mesenchyme, can be considered as the EK. To identify the location of the spot in the tooth germ, all in situ hybridized RTGs were sectioned.

A. EK in the E12 RTG

After two days in culture, the E12 RTG appeared as an early cap stage tooth germ and exhibited *Shh*-expressing cell cluster located in the epithelium facing the mesenchyme (Fig. 6A). After four days, the E12 RTG appeared to be in the late cap stage, and it showed the wide *Shh* expression in the epithelium facing the mesenchyme (Fig. 6B). These results indicate that these *Shh* was expressed in the epithelial cluster facing the mesenchyme and that the first *Shh*-expressing spot in the E12 RTGs (Fig. 5A) was the EK.

B. EK in the E14 RTG

The E14 RTG was at the cap stage after two days in culture and showed a *Fgf4*-expression (Fig. 6C) and the limited *Shh* expression (Fig. 6D) in the epithelial cell cluster facing the dental mesenchyme. After four days in culture, the E14 RTG appeared to be at bell stage, and contained two cell clusters expressing *Fgf4* strongly in the inner enamel epithelium (Fig. 6E). These results indicate that all spots in the E14 RTGs (Fig. 2D, E, F, G, H) were the regenerated EKs.

C. EK in the E16 RTG

In the E16 RTGs after two days in culture, a few epithelial cells facing the mesenchyme showed the expression of *Fgf4* (Fig. 6F), while *Shh* was

expressed widely in the epithelium facing the mesenchyme (Fig. 6G). These localized *Fgf4* expression and wide *Shh* expression in the epithelium is coincident with that of secondary EK. In the section of the E16 RTG along the line (from a to a' in Fig. 5K), the E16 RTG showed one cell cluster expressing *Fgf4* in inner enamel epithelium after four days in culture (arrow in Fig. 5H). These results indicate that all spots in the E16 RTGs (Fig. 2I, J, K) were the regenerated EK.

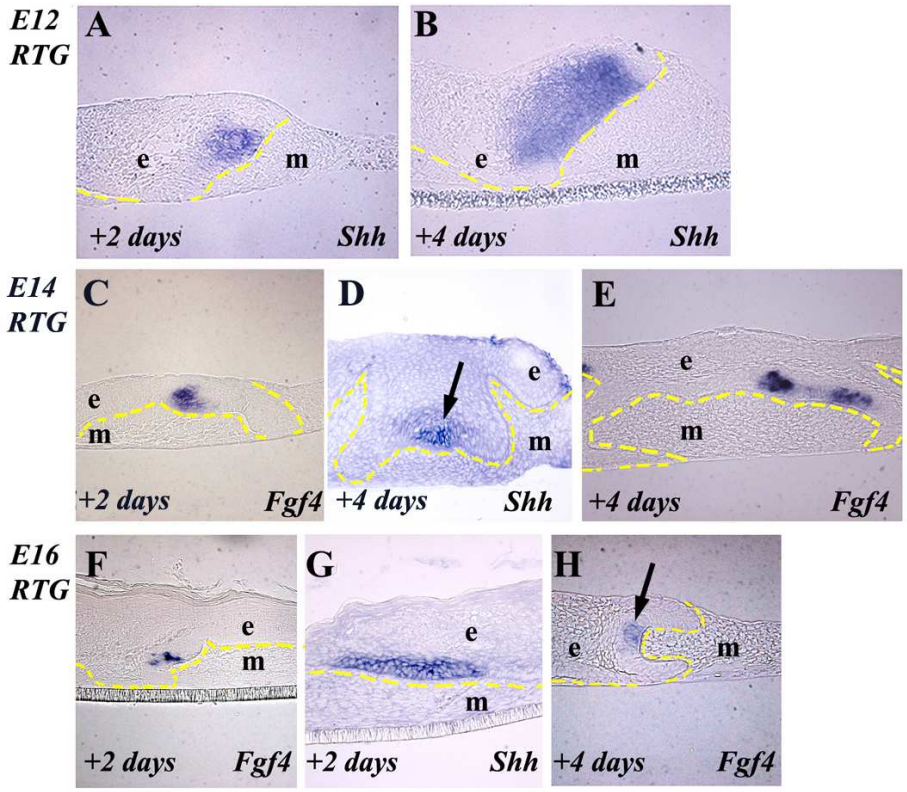

Fig. 6. *Shh* and *Fgf4* expression in the RTGs (section view).

(A) After two days in culture, the E12 RTG showed a cell cluster expressing *Shh*. (B) After four days, the E12 RTG appeared to be in the cap stage, and exhibited abundant *Shh* expression. (C) The E14 RTG entered cap stage after two days of culture, and developed cell cluster expressing *Fgf4* in the epithelium facing the mesenchyme. (D) The E14 RTG showed the limited *Shh* expression in the epithelial cell cluster facing the mesenchyme. (E) Following four days of culture, the E14 RTG showed two EK-like cell clusters expressing *Fgf4* strongly. (F) The E16 RTGs, after two days in culture, exhibited the expression of *Fgf4* in a few cells of the epithelium facing the mesenchyme. (G) *Shh* was expressed widely in the epithelium facing the mesenchyme. (H) In the section of the E16 RTG along the line (from a to a' in Fig. 2J), the E16 RTG showed one cell cluster expressing *Fgf4* in inner enamel epithelium after four days in culture (arrow). e, epithelium; m, mesenchyme.

4. Complete separation of epithelium from mesenchyme

The remaining epithelium and dental mesenchyme of the *lacZ* transgenic ROSA26 mice were recombined with the dental mesenchyme and remaining epithelium of the wild type ROSA26 mice in an attempt to clarify the complete separation of the dental epithelium from the dental mesenchyme and to exclude the possibility of cell contamination between the epithelium and mesenchyme. The tooth germs with the *lacZ* transgenic epithelium and the wild type mesenchyme ((+)epi/(-)mes in Fig. 7A, C) showed a dark blue stained epithelium after X-gal staining (Fig. 7A). This shows that the dental epithelia were clearly separated from the dental mesenchyme without mesenchymal cell contamination in the epithelia. On the other hand, the tooth germ with the wild type epithelium and *lacZ* transgenic mesenchyme ((-)epi/(+)mes in Fig. 7B, D) also showed a blue stained mesenchyme after

X-gal staining (Fig. 7B). This shows that the dental epithelia were clearly separated from the dental mesenchyme without epithelial cell contamination in the mesenchyme.

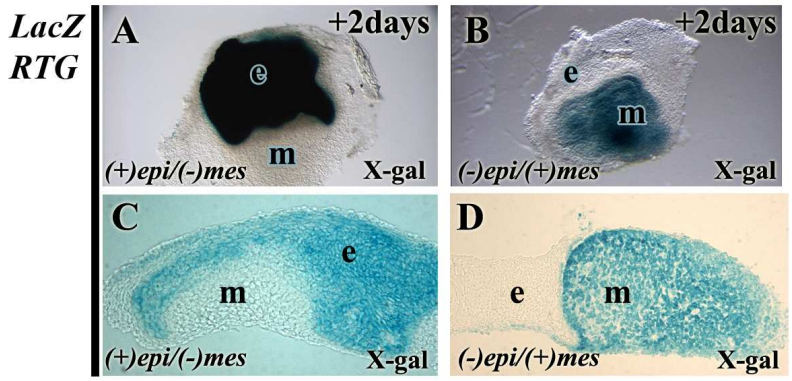

Fig. 7. Recombination between *lacZ* transgenic tooth germs and wild type tooth germs

(A) Tooth germs with *lacZ* transgenic epithelium and wild type mesenchyme ((+)epi/(-)mes) showed a dark blue stained epithelium after X-gal staining. (B) The tooth germ with the wild type epithelium and *lacZ* transgenic mesenchyme ((-)epi/(+)mes) showed blue stained mesenchyme. (C) Only the epithelial cells of the (+)epi/(-)mes tooth germs showed a blue color in the section. (D) Only the mesenchymal cells of the (-)epi/(+)mes tooth germs showed a blue color in the section.

5. Different characteristics in both epithelium and mesenchyme between the E14 and E16.

To investigate the difference between the remaining epithelia at E14 and E16 and between the mesenchymes at E14 and E16, the E16 remaining

dental epithelium was cross-recombinant with the E14 dental mesenchyme to form a recombinant tooth germ (E16/E14 cross-RTG) and the E16 dental mesenchyme was cross-recombinant with the E14 remaining dental epithelium to form another RTGS (E14/E16 cross-RTG) (see CROSS-RECOMBINATION in Fig. 3). After 2 days of in vitro culture, the E14/E16 cross-RTG developed into the early bell stage tooth germ. Two secondary EK-like cell clusters expressing *Shh* and *Fgf4* strongly, and the sharp epithelial edge, which is the trace of the primary EK, could be found (Fig. 8A, B). After being transplanted for 3 weeks into the subcapsular layer of kidney, all cross-RTGs formed calcified teeth, which were different from each other in size and shape. The E14/E16 cross-RTG formed a wide but short calcified crown with a few low cusps after 3 weeks transplantation into the kidney (Fig. 8C).

On the other hand, the E16/E14 cross-RTG, which was composed of the E16 remaining epithelium and the E14 dental mesenchyme, developed nto the cap stage tooth germ showing *Shh* and *Fgf4* expression in epithelium after two days of *in vitro* culture (Fig. 8D, E). The *Shh* expression (Fig. 8D) and *Fgf4* (Fig. 8E) expression in this cross-RTG was weak and restricted to a few cells of the inner dental epithelium. The E16/E14 cross-RTG also formed a calcified tooth after 3 weeks of transplantation (Fig. 8F). This result indicates that the developing stage in the E14/E16 cross-RTG was faster than that of the E16/E14 cross-RTG. This indication might be resulted from two assumptions. One is that the remaining E14 epithelium had less damage than the remaining E16 epithelium did. The other is that the E16 dental mesenchyme induced the EK generation faster than the E14 dental mesenchyme did.

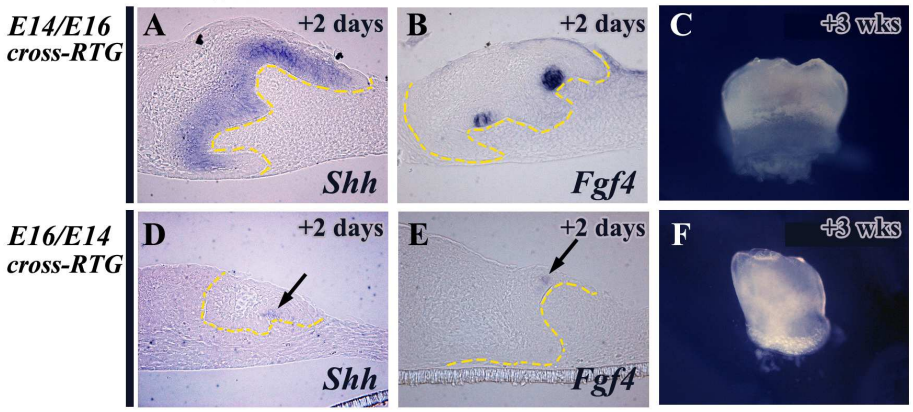

Fig. 8. Development of the cross-RTGs

(A, B) The E14/E16 cross-RTGs showed bell-staged tooth germs showing two secondary EK-like cell clusters expressing *Shh* (A) and *Fgf4* (B) strongly after two days *in vitro* culture. (C) After 3 weeks transplantation into the kidney, the E14/E16 cross-RTGs formed the calcified teeth. (D-E) The E16/E14 cross-RTGs at cap stage showed weak *Shh* expression (D) and weak *Fgf4* expression (E) in a few cells of the inner dental epithelium. (F) The E16/E14 cross-RTGs formed the calcified teeth.

6. Origin of regenerated EK

In order to investigate the origin of regenerated EK, the movement of epithelial cells in the dental lamina was traced using a cell labeling technique with DiI at E11.5 (Fig. 9A, a). The dental lamina cells were labeled with DiI. After two days in culture, some of the DiI-labeled epithelial cells had moved towards the tip of the epithelial bud (Fig. 9B, b), but most of the labeled cells remained in their original positions. After four days, all DiI-labeled cells had moved towards the tip of the epithelial bud, resulting in the formation of the budding epithelium (Fig. 9C, c). This result presents the possibility of the cell migration from the dental lamina of the remaining epithelium to regenerate the enamel organ and the EK.

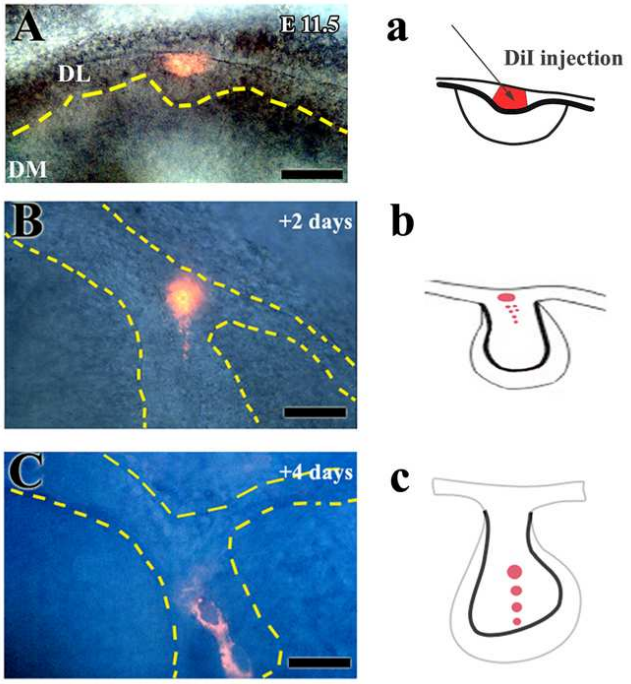

Fig. 9. Origin of regenerated EK.

(A, a) The epithelial cells in the dental lamina (DL) above the basement membrane (yellow dashed line) were traced by cell labeling with DiI (red) at E11.5 to investigate the precursor of the primary EK. (B, b) A few labeled cells in the dental lamina moved towards the tip of the epithelial bud after two days. (C, c) DiI-labeled cells moved in a row towards the primary EK after four days. DM: dental mesenchyme

7. The new position of the regenerated EK in RTGs

The fact that primary EK can be regeneratedin the E14 RTG leads us to identify the key tissue dictating the position of the EK. After dissociation of the dental mesenchyme from the dental epithelium in E14 tooth germ, the dental papillae were labeled with DiI, and then recombined with the remaining E14 epithelium (Fig. 6A). After two days in culture, the E14 RTG showed two *Fgf4*-expressing spots away from the DiI-labeled dental papillae in an upper view (black and red arrows in Fig. 6B). In the section of these specimens, DiI-labeled dental papillae were located between two tooth germs (gray dotted line and pink dotted line in Fig. 6C, D), each of which possessed the primary EK-like cluster expressing *Fgf4* (black and red arrows in Fig. 6C). This result implies that the position of EK is not determined by the dental papillae.

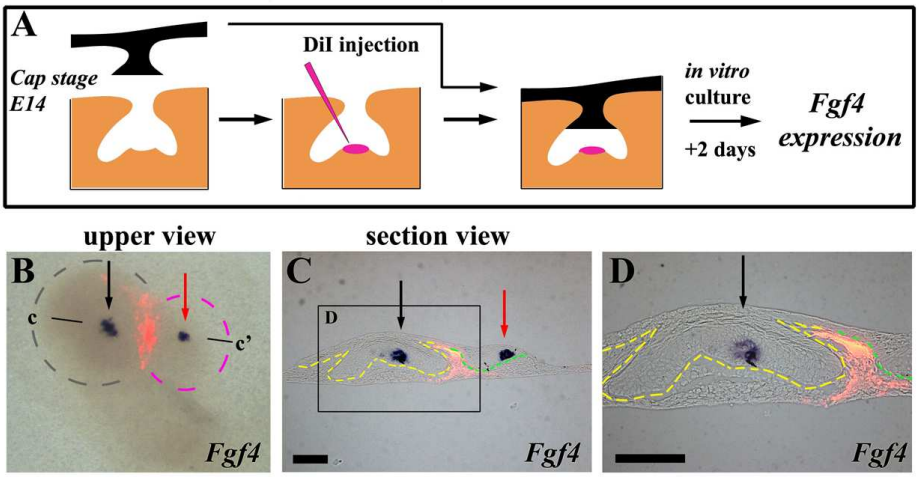

Fig. 10. The positional determination of the regenerated EK.

(A) After the dental mesenchyme had been dissociated from the dental epithelium in the E14 tooth germ, the dental papillae were labeled with DiI, and recombined with the remaining E14 epithelium, which had lost the primary EK. Two days later, the E14 RTGs were in situ hybridized. (B) After two days in culture, the E14 RTG contained one spot in each tooth germ (black arrow in the gray dotted circle and red arrows in the pink dotted circle) expressing *Fgf4* away from the labeled dental papilla in the upper view. (C) The DiI-labeled dental papilla was located between the two tooth germs, each of which showed EK expressing *Fgf4*. (D) The RTG developed into the cap stage and showed a primary EK-like cell cluster away from the labeled dental papilla.

8. Number of regenerated EKs in the E16 RTGs

The E16 RTGs, which consisted of the epithelium containing no EK and dental mesenchyme containing four dental papillae, regenerated one EK after two days in culture. This means that the dental mesenchyme alone cannot dictate the position and number of regenerated EKs. However, this does not mean that the position and number of regenerated EKs are dictated by a dental epithelium with EKs. Therefore, in order to clarify the role of the dental epithelium in dictating the position and number of regenerated EKs, EK formation in an E16 recombined tooth germ (E16 rtg) was investigated using an E16 dental epithelium possessing four EKs and an E16 dental mesenchyme possessing four dental papillae (RECOMBINATION #3 in Fig. 4D). The tooth germ showed four EKs expressing *Fgf4* at E16 (Fig. 4D). The E16 rtg showed one large EK after 12 hours (Fig. 4E), two EKs after 18 hrs (Fig. 4F) and four EKs after culturing for 24 hrs (Fig. 4G). Interestingly, all the E14 RTG, the E16 RTG and the E16 rtg showed one EK first. Although the E16 rtgs possessed four secondary EKs and four dental papillae, there was only one EK at the beginning in culture. This means that neither the dental epithelium nor the dental mesenchyme can dictate the pattern of EK formation directly.

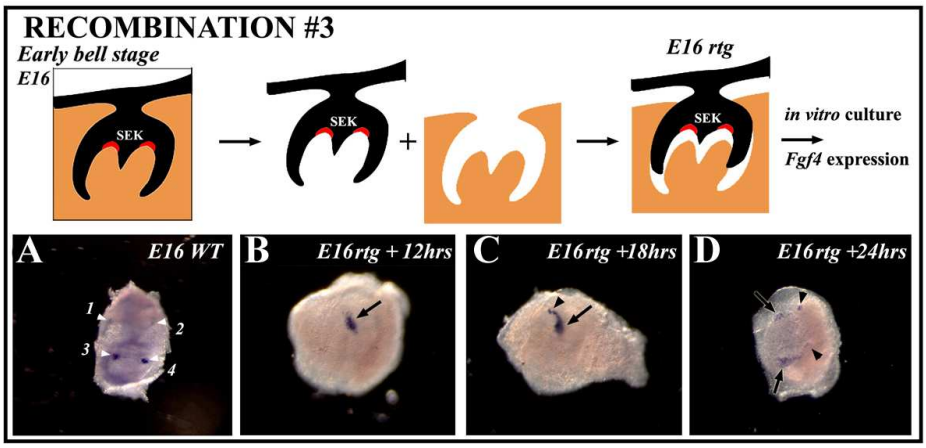

Fig. 11. Number of regenerated EKs in the E16 RTGs

(RECOMBINATION #3) The E16 dental epithelium with four EKs and the E16 dental mesenchyme with four dental papillae were recombined to form the E16 recombined tooth germ (E16 rtg). (A) The E16 wild type tooth germ (E16 WT) showed four EKs expressing *Fgf4*. (B-D) The E16 rtg showed one large EK after 12 hours (B), two EKs after 18hrs (C) and four EKs after 24hrs (D).

9. Apical half epithelium can develop into calcified tooth at E14

The apical half of the E14 dental epithelium, which can be regarded as the enamel organ, was recombined with the E14 dental mesenchyme to form another RTG (E14 enamel organ RTG in Fig. 12A). After three-week transplantation, this tooth germ showed calcified molar tooth with many evident cusps (Fig. 12B). Even though this RTG was composed of one enamel organ and one dental mesenchyme, this tooth germ developed into two tooth germs in vitro culture (Fig. 12C, D, E, F). This result is coincident with previous results that two tooth germs developed from the E14 RTG (Fig. 5F, H and Fig. 11B, C).

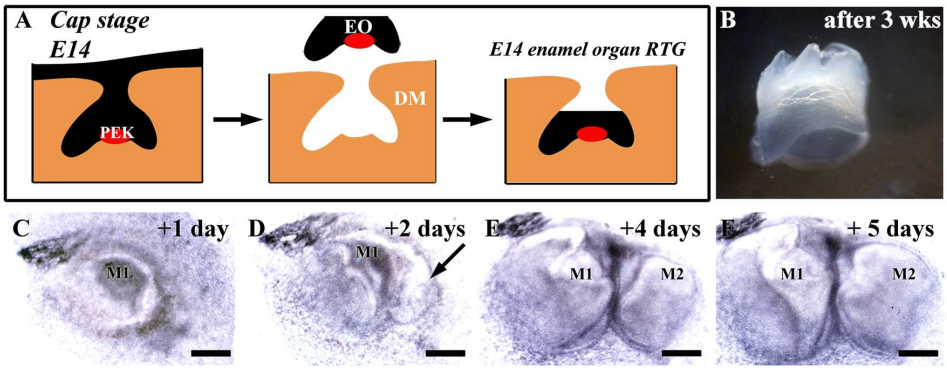

Fig. 12. Recombination of the apical half epithelium with dental mesenchyme at E14.

(A) The apical half of the E14 dental epithelium, which can be regarded as the enamel organ (EO), was recombined with the E14 dental mesenchyme (DM) to form a RTG (E14 enamel organ RTG). (B) After three week of transplantation, this tooth germ showed calcified molar tooth with many evident cusps. (C-F) Two tooth germs are developing into bell shape from one day to five days in vitro culture. (C) First molar tooth germ (M1) was developing after one day in culture. (D) After two days, second molar tooth germ (arrow) could be observed. (E) After four days, second molar tooth germ (M2) could be observed evidently next to the bell-staged M1. (F) Both of M1 and M2 appeared to be at bell stage. PEK: primary EK

IV. DISCUSSION

Tooth development begins with epithelial thickening on the future tooth position.[31,32,33] The primary EK appears at the center of the dental epithelium facing the dental mesenchyme from the late bud stage. The primary EK is considered as the most important structures in the determination of the number of cusps, as well as the shape, of an individual tooth.[13,16] Both the primary EK and secondary EK are the signaling centers in crown morphogenesis during tooth development,

If the EKs are removed from the developing tooth germ, what will happen? Will calcified teeth be formed with cusps? Will calcified teeth be formed without cusps? As a result, the E12, E14, and E16 RTGs developed calcified teeth after three weeks following transplantation into an adult mouse kidney. However, the shape and cusp height of the calcified teeth were different from each other according to the stage of RTGs. The fact that the E12 and E14 recombinant teeth exhibited cusps can be interpreted in two ways. One is that signaling centers may be not necessary for cusp formation. The other is that signaling centers can be regenerated to play important roles in crown morphogenesis. These two possibilities were evaluated by identifying the regenerated EK in the RTG.

Shh or *Fgf4* expression was used as a marker for regenerated EK. Firstly, *Shh* was expressed exclusively in the epithelial component of tooth germs from the dental lamina stage.[34] The *Shh* expression is confined to the primary EK during the cap stage[16,35] and this expression spreads laterally thereafter to the inner enamel epithelium, the stratum intermedium[36] and the stellate reticulum.[37] Because of these expression patterns, *Shh* was used as a marker for primary EK of the E12 RTG in this study. Secondly, *Fgf4* was used as the marker for the EK of the E14 and E16 RTG, as this is one of two genes observable in both the primary and secondary EK[13,14,15,38] but which also means that the primary EK can be distinguished from the secondary EK not only by the *Fgf4* expression but also other gene

expression such as *Shh*.

Accordingly, *Fgf4* was used as a marker for the EK of the E14 RTG, the E16 RTG and the E16 rtg. However, *Fgf4* cannot be used to distinguish between a primary EK from a secondary EK. However, the number of *Fgf4*-expressing spots in one tooth germ is helpful to distinguish the primary and secondary EK. During tooth development, two or more spots in one tooth germ can be regarded as the secondary EKs, and only one spot in one tooth germ can be considered as the primary EK when it will be followed by many secondary EKs.

1. EK can be regenerated after being removed mechanically

During the development of a mouse first molar, the primary EK appears as a line at E13, and as a spot at E14. There are two, four and six secondary EKs in an E15, E16 and E17 tooth germ, respectively (WT line in Fig. 13).

In this study, the E14 RTG showed one regenerated EK, which was followed by two EKs (E14 RTG line in Fig. 13). The pattern of regenerated EKs in the E14 RTG was similar to the pattern observed from E14 to E15 in normal development. This suggests that the first EK in the E14 RTGs is the primary EK, and the two EKs are the secondary EKs. On the other hand, the E16 RTG showed one regenerated EK first, followed by three EKs (E16 RTG line in Fig. 13). This pattern of regenerated EKs in the E16 RTGs was different to the pattern observed in normal tooth development. Therefore, it is difficult to name the first regenerated EK in the E16 RTG as the primary EK.

Interestingly, the E14 RTG, the E16 RTG and the E16 rtg showed one regenerated EK first. Even though the E16 rtgs contained four secondary EKs and four dental papillae, they showed only one EK after 12 hours, two after 18hrs and four after culturing for 24 hrs (E16 rtg line in Fig. 13). This pattern of the EKs in the E16 rtgs was similar to the pattern observed

from E14 to E16 in normal tooth development. This suggests that the first EK in the E16 rtgs is a primary EK. Furthermore, the EK pattern in tooth germs restarts from a primary EK. The fact that the E16 dental epithelium, which had many secondary EKs, generated a primary EK suggests that there are no significantly different characteristics between the primary EK and the secondary EK. Hence, the first EK in the E16 RTGs can be named a primary EK (E16 RTG line in Fig. 13). Additionally, the arrangement of three secondary EKs is similar to the arrangement of mamelons in human incisors. Therefore, three secondary EKs in a row in the E16 RTG might be related with the formation of human incisor-like teeth from the E16 RTG (Fig. 4D).

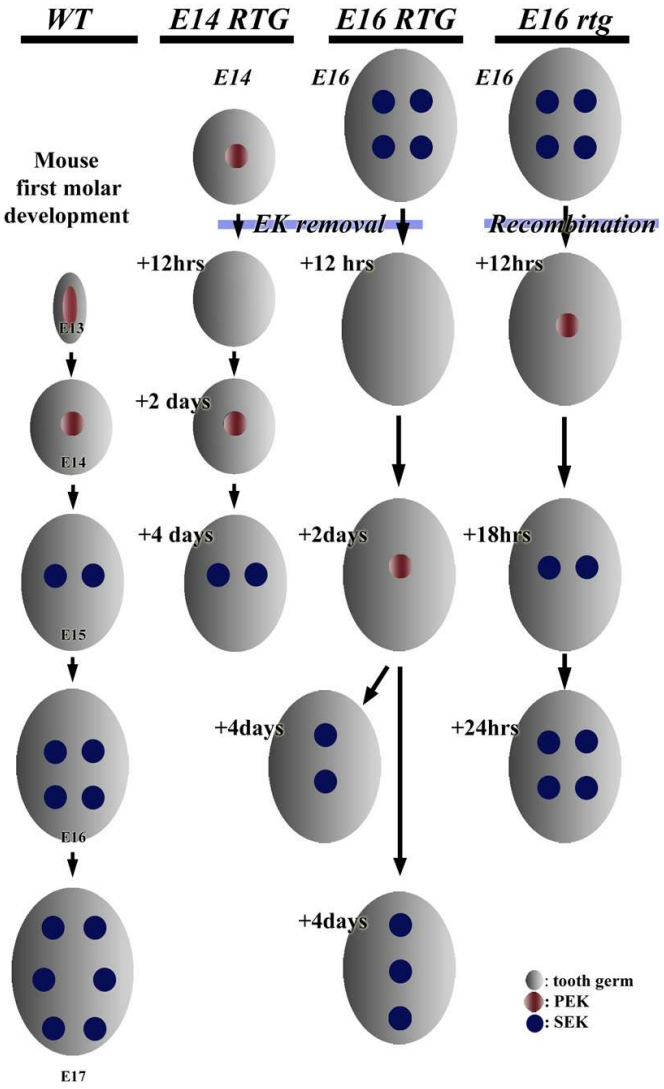

Fig. 13. Schematic diagram of the regenerating pattern of EKs in developing tooth germs

(*WT* line) During tooth development in a mouse first molar, the primary EK appears as a line (red line) at E13 and a spot (red spot) at E14. Two secondary EKs (blue spots) begin to form at E15, and four secondary EKs (blue spots) appear at E16. Six (or seven) secondary EKs were observed at the E17 mouse first molar. (E14 RTG line) The primary EK was removed

from the E14 RTG. The E14 RTG showed no EK after 12 hours, one primary EK (red spot) after two days, and two secondary EKs (blue spots) after four days in culture. (E16 RTG line) The E16 RTG lost four secondary EK as a result of the removal of the EK. The E16 RTG showed one primary EK (red spot) after two days, which was followed by two or three secondary EKs (blue spots) after culturing for four days. (E16 rtg line) The E16 rtg showed one primary EK first (red spot) after 12 hours, two secondary EKs after 18 hours (blue spots) and four secondary EKs after 24hours in culture (blue spots).

2. Interactions between the dental epithelium and the dental mesenchyme are important in the regeneration of the EK.

It is well known that during the early stages of tooth development, signals from the dental epithelium induce an odontogenic potential in mesenchymal cells as well as inductive potential shifts to the dental mesenchyme.[40,41,42,43] However, even though the dental mesenchyme has odontogenic potential, the EKs in the dental epithelium play important roles in cusp patterning via an interaction with the dental mesenchyme.[35]

In this study, the E16 RTGs and E16 rtgs possessing four dental papilla showed only one EK at the beginning. This means that the dental mesenchyme alone cannot dictate the position and number of regenerated EKs.

Furthermore, the role of the dental mesenchyme in determining the position of EK was investigated in E14 RTGs using a cell labeling method. The results showed that a new dental papilla had formed immediately below the regenerated primary EK, and DiI-labeled previous dental papilla was observed in the position of dental follicles of the E14 RTGs. This might come from the interaction between the remaining epithelium and the dental mesenchyme including the dental follicles and dental papillae. Before birth,

the dental follicles can interact with the dental epithelium to form a tooth.[44] However, this does not mean that the dental epithelium dictates the position of the regenerated EK. Although the E16 rtgs possessed four secondary EKs as well as four dental papillae, the E16 rtgs showed only one primary EK after culturing for 12 hours. This suggests that neither the dental epithelium nor the dental mesenchyme can dictate the pattern or number of EK formation. It is also suggested that the regeneration of the EK is regulated by an interaction between the dental epithelium and dental mesenchyme.

3. The E16 dental mesenchyme might induce the development of tooth germ faster than E14 did

To investigate the characteristics of the remaining epithelium and the mesenchyme respectively, the E14 remaining epithelium was cross-recombined with the E16 dental mesenchyme (E14/E16 cross-RTG) and vice versa (E16/E14 cross-RTG). While the E14/E16 cross-RTG developed into the early bell stage tooth germ after 2 days of *in vitro* culture, the E16/E14 cross-RTG developed into the cap stage tooth germ. The result that the developing stage of the E14/E16 cross-RTG was faster than that of the E16/E14 cross-RTG might be interpreted as two assumptions. One is that the remaining E14 epithelium had less damage than the remaining E16 epithelium did. The other is that the E16 dental mesenchyme induced the tooth development faster than the E14 dental mesenchyme did.

4. Regenerated EK can be originated from the remaining epithelium

In the investigation of the cell origin in the regenerated EK, labeled cells

in dental lamina at E11.5 moved in a row towards the tip of budding epithelium after four days in culture. This result suggests that cells in the remaining dental lamina epithelium of RTG might be the origin of the regenerated enamel organ and the regenerated EK.

5. Many teeth can be formed from one molar epithelium

In the E14 enamel organ RTG, developing tooth germs could be observed clearly under the microscope even after one day in culture, and the cusps of calcified tooth were evident. These results might be caused by the less damage on the apical half epithelium than on the remaining epithelium. The fact that two tooth germs were formed from the E14 enamel organ RTG as well as the E14 RTG indicates the great proliferating capacity of the dental epithelium.

V. CONCLUSION

Although a great deal of damage was inflicted on the dental epithelium during excising half of the epithelium, the regenerative and proliferative ability of the dental epithelium was powerful enough to circumvent this injury. After the half removal of the dental epithelium, the precursors of the regenerated EK, which remains in the dental lamina, relocated and regenerated the EKs. The interaction between the dental epithelium and the dental mesenchyme determines the characteristics and position of the regenerated EK.

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ABSTRACT(IN KOREAN)

치아 발생 중 사기질결절의 재생에 있어 상피와 간엽 간의 상호작용에 관한 연구

연세대학교 대학원 의과학과

조 성 원

<지도교수 정 한 성>

사기질결절은 치배의 치아상피 중심에서 일시적으로 존재하는 신호중심으로서, 치아상피의 성장을 조율하고, 치아의 교두를 형성하여 치아의 모양을 조절하는 기능을 가지고 있는 것으로 알려져 있다. 사기질결절의 중요성을 알아보기 위하여, 발생중인 흰쥐의 아래턱 치배에서 사기질결절을 제거한 다음, 남아있는 상피를 원래 치아간엽조직과 결합시켜 체외배양 및 체내배양을 실시하였다. 이렇게 치배형성의 신호중심을 제거했음에도 불구하고, 교두를 가지는 치아를 얻을 수 있었는데, 이러한 결과는 사기질결절이 재생되기 때문에 초래된다는 것을 알 수 있었다. 그러나 치배의 시기별로 사기질결절이 재생되는 양상은 차이가 있었다. 또한 이렇게 새로이 재생된 사기질결절의 위치를 결정하는데 있어서 치아상피와 치아간엽조직 간의 상호작용이 중요하다는 것을 알 수 있었다.

핵심되는 말 : 사기질결절, 치배, 재생, 조직간 상호작용, 운명지도, 어금니