Tooth Formation
in Reaggregated Dental Mesenchyme

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Tooth Formation
in Reaggregated Dental Mesenchyme

Directed by Professor HAN-SUNG JUNG

The Master' s Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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2002년 12월

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(Directed by Professor HAN-SUNG JUNG)

Induction of tooth formation by dental epithelium and ectomesenchyme is of fundamental importance during tooth development. It has been known that dental epithelium has the potential to induce tooth formation before bud stage, whereas dental mesenchyme has the potential to induce tooth formation after bud stages.

In this study, different numbers of dissociated dental mesenchymal cells were recombined with a piece of the same sized oral epithelium. Subsequently, different types of teeth were observed after incubation in kidney capsule.

Using the reaggregated system, the reaggregated teeth were obtained. After 3 weeks in kidney capsule, 6 to7 cusps, which are mesially inclined crest, were

produced in control. This has 6 tall cusps and 1 small cusp. However, the cusps of the reaggregated tooth had various shapes. Three types of cuspal patterns of reaggregated tooth were identified, such as the M1-like (3.3 x 10^4 cells), the crater-like (1.4 x 10^4 cells), the slope-like (2.9 x 10^4 cells). The reaggregated tooth was examined, by H-E staining of paraffin sections after decalcification. The reaggregated tooth showed cusps, dentinal tubules running to the dentin surface, and mantle dentin in the dentin surface in the same way as in the control teeth. *Bmp-4* and *Shh* were expressed in epithelium of reaggregated tooth. When Di.I. was injected into the mesenchymal cells just below the enamel knot, the Di.I. labeled cells spread over all the mesenchyme of reaggregated tooth, which showed no predetermination of dental mesenchymal cells. The reaggregates made from the oral epithelium and the reaggregated dental mesenchymal cells from E11.5 gave rise to the M1-like tooth. The M1-like reaggregated teeth (5.9 x 10^4 cells) were produced when the incisor epithelium was recombined with the reaggregated mesenchyme of the first molar. The reaggregated tooth, which recombined the epithelium at E11.5 with the reaggregated mesenchymal cells at E13.5, was slope-like tooth (6.8 x 10^4 cells).

To sum it up, the cuspal pattern of the reaggregated tooth is possibly associated with the mesenchymal cell numbers. Moreover, it is predicted that the numbers of dental mesenchyme cells could determine the diversity of tooth patterns.

Key words: bioengineering tooth, embryonic tooth development, epithelial-mesenchymal interaction, cell number, reaggregation

Tooth Formation in Reaggregated Dental Mesenchyme

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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.[1,2] Epithelial-mesenchymal recombination experiments showed the potential to form a skin appendages from mesenchymal cells in the dermis.[3]

The tooth is an excellent model to study reciprocal tissue interactions 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.[4] The teeth develop from the epithelium lining the oral region and from the ectomesenchyme derived from the caudal mesenphalic and rostal rhombencephalic neural crest.[5]

At Embryonic day 11 (E11), oral epithelium thickening is the first indication of tooth morphogenesis in mice. Subsequently, this thickening proliferates (Fig. 1-A) 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 (Fig. 1-B). An enamel knot, which is a putative signaling center, appears at the cap stage (E13.5) (Fig. 1-C and 1-D). 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 .[6,7]

A characteristic feature of molar tooth morphogenesis is the primary and secondary enamel knots, which play various roles regulating the mammalian molar tooth shape.[7,8] The primary enamel knot appears during early tooth morphogenesis in the center of the tooth bud epithelium, which occurs above the forming dental papilla at the transition from bud stage to cap stage. At cap stage (E13.5), the primary enamel knot cells express several signaling molecules including Sonic hedgehog (*Shh*), Bone morphogenetic proteins (*Bmp-2, Bmp-4* and *Bmp-7*), as well as Fibroblast growth factor-4 (*Fgf-4*).[7]

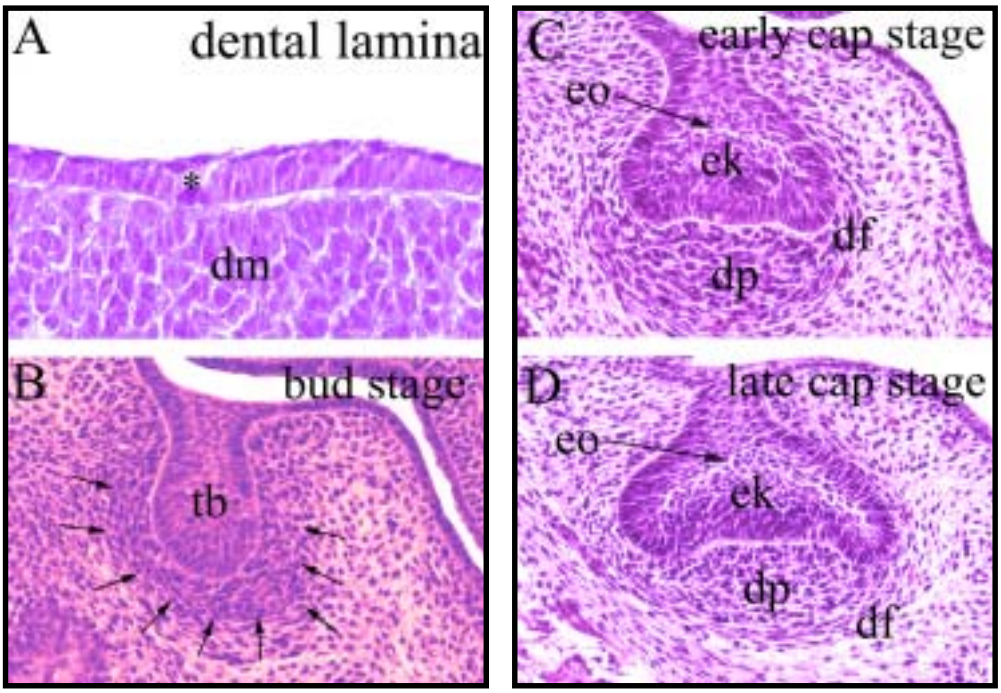


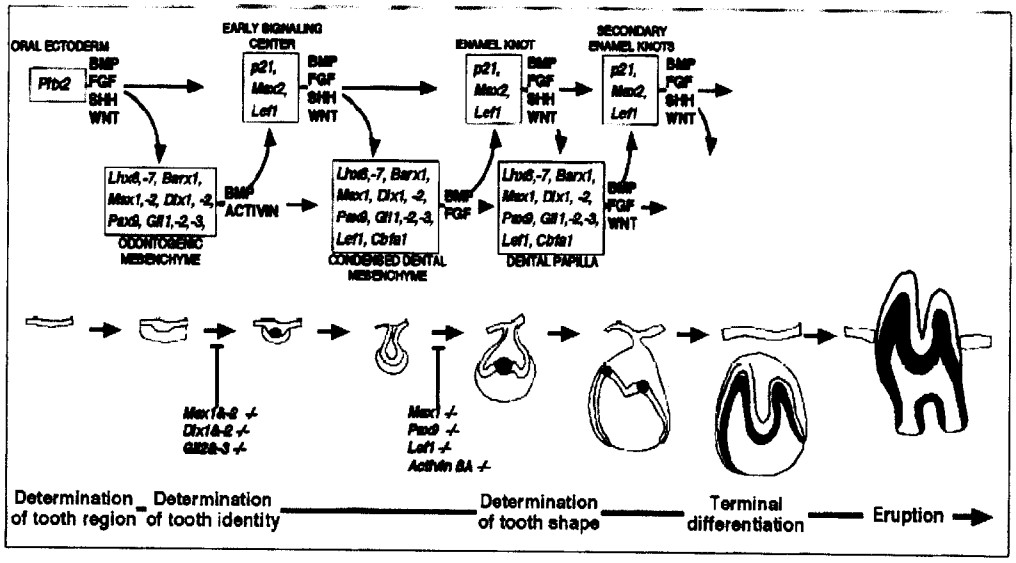
Figure 1. The Embryonic Tooth Development:

(A) The oral epithelium thickened to form the dental lamina. (B) The oral epithelium invaginates to the dental mesenchyme and forms a tooth bud. (C), (D) The enamel knot appears at the cap stage, which is characterized by epithelial folding and rapid cell proliferation leading to the establishment of the shape of a tooth crown. (*: dental lamina, dm: dental mesenchyme, tb: tooth bud, eo: enamel organ, dp: dental papilla, df: dental follicle, ek: enamel knot)

At bell stage (E14.5), it is believed that the secondary enamel knots determine the cusp sites and promote their growth.[9] At each stage, the oral epithelium and the ectomesenchyme signals to one another in order to coordinate their development (Fig. 2).

Studies on the signaling molecules have revealed associations of the gene expression pattern with tooth morphogenesis. In recent years, the signaling pathways have been analyzed to determine the relationship between tooth evolution and the shapes of teeth.[12] Signaling molecules such as FGF8 and BMP specify the tooth area, and teeth always develop within the tooth row as with other organs.[10,11]

Shh, a vertebrate homologue of the *Drosophila* segment polarity gene hedgehog (*hh*), is expressed in the dental epithelium from the time of epithelial thickening to the completion of crown morphogenesis. The *Shh* plays a crucial role on tooth dentition and cell proliferation of the early dental epithelium. [12,13] The *Bmp-4*, the TGF-β super family member bone morphogenetic protein-4, has been shown in the dental lamina epithelium, which then shifts to the dental mesenchyme in the molar. This shift of *Bmp-4* expression pattern coincides with the shift potential to the dental mesenchyme.[14]



Mechanism of Developmet, 29 (2000), 19-29: Jukka J.and Thesleff I.

Figure 2. Schematic Representation of the Signals and Transcription Factors Mediating the Reciprocal Signaling between Epithelium and Mesenchyme during Tooth Development (yellow: tooth epithelium, red: enamel knot, blue: tooth mesenchyme, Mechanism of Development 29 (2000) 19-29: Jukka Jernvall and Irma Thesleff)

Recombination tissue experiments have demonstrated that the early epithelium might determine the tooth shape and this tooth forming potential shifts from the tooth epithelium to the mesenchyme after bud stage (Fig. 3-A and 3-B).[15,16] At E15-16, explants of the molars were divided into first molar (M1) and second molar (M2) district, and the parts were grafted separately, and cultured in the anterior chamber for 3 weeks. Grafts of isolated M1 tissue (at bell stage) produced a single M1 tooth, while grafts of M2 (at bud stage) developed both second and third molars (M3).[16] The other recombination experiments of the E16 and E13 tooth bud molar were transplanted into the anterior chambers of the eye for 20 days. The posterior tips of E13 tooth bud that were grafted to the E16 tooth bud explants produced 4 teeth of which the first was a normally shaped M1. The reciprocal, using the tissues from the same explants, developed a normal M1 and a normal M3.[16]

In mammals, the teeth are categorized as follows: incisors, canines, premolars, and molars. Their shapes identify these four different tooth families. Incisors have one cusp, canines are larger and sharper than incisors premolars have more cusps, and molars have diverse shape. The individual teeth are characterized as cusps. The cusps play roles in their part of the whole dentition. The mammalian upper molar teeth have two buccal cusps (paracone and metacone) and one lingual cusp (protocone). Most low molars have three lingual (paraconid, metaconid, entoconid) and two buccal cusps (protoconid, hypoconid) and one distal cusp. The major cusp in the disto-lingual corner of upper molar teeth is called the hypocone, which plays a role in crushing food.[17]

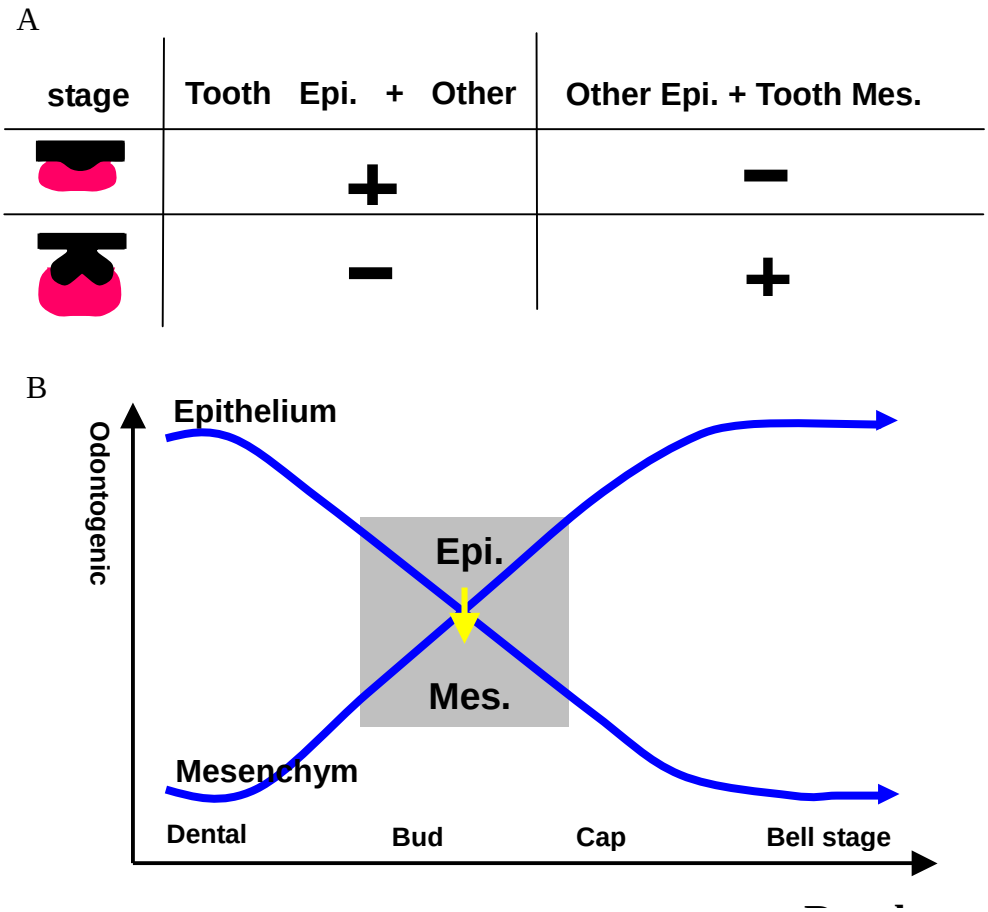


Figure 3. The Potential to Induce Tooth Formation:

(A) At dental lamina stage, when tooth epithelium was recombined with the other mesenchyme, tooth was produced. However, when other mesenchyme was recombined with the tooth epithelium, tooth formation did not induced. At cap stage, a tooth was produced with the same experiments as in the dental lamina stage only if other epithelium recombined with tooth mesenchyme. (B) Before the bud stage, the dental epithelium has the potential to induce tooth formation. After bud stage, dental ectomesenchyme has the information to induce tooth formation. (black: epithelium/ectoderm, pink: mesenchyme/mesoderm)

The reaggregated system has already been used in the case of a feather bud and a limb bud.[18,19] In the feather bud experiment, using the reconstitution assay, they traced back to the initial stage of the patterning process. The cells reset and self-organized into a periodic pattern without previous cues or sequential propagation.[18] In a reaggregated experiment of a limb bud, they attempted to study the patterning in the reaggregated system where the developmental axes were severely disrupted. Reaggregates from different regions of the leg mesenchyme developed into different digits according to *HoxD* genes.[19]

In this study, the reaggregated system was adopted for studying the tooth bud mesenchymal cells. In the reaggregated system the tooth mesenchyme at E13.5 is triturated into single cells before repelleting. It is then recombined with a piece of the same sized intact tooth epithelium and transplanted into a mouse kidney capsule for 3 weeks. Morphologically good tooth can be produced despite the fact the cells are completely disorganized.

It is interesting that either the dental mesenchyme or the dental epithelium determines the cusps number, size, shape and identification of the individual tooth. This study examined what kind of tooth shape was produced if different numbers of mesenchymal cells were recombined with a piece of the same sized epithelium.

Furthermore, the cuspal pattern of the reaggregated tooth was maybe associated with mesenchymal cell numbers. Moreover, the results suggest that the number of dental mesenchyme cells could be used to determine the diversity of the tooth patterns.

Ⅱ. Materials and Methods

1. Experimental Animals

The 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. Mice embryos were obtained from time-mated pregnant mice. The designated embryonic day 0 (E0) was determined on the day that the presence of a vaginal plug was confirmed. The lower first molar and incisor tooth germ from the embryos at the development stage E11.5 and E13.5 were used in this study.

2. Reaggregated System

The tooth buds using intact epithelium and dissociated mesenchyme were dissected from the lower jaw of E13.5 under a stereo-microscope in phosphate-buffered saline (PBS). They were then incubated at room temperature in 1.2U/ml dispase II (Roche, Germany, 295 825) for 12.5 minutes and washed in Dulbecco's minimum essential medium (D-MEM, Bio Whittaker, USA, 12-640F) containing 10% fetal bovine serum (FBS, GIBCO, USA, 16000-044). Under a dissection microscope, the epithelium and the mesenchyme were separated using a fine needle. The epithelia (Fig. 4-A) remained intact in the media. The mesenchyme (Fig. 4-B) was pooled and gently triturated into single cells by drawing them through pipettes with decreasing

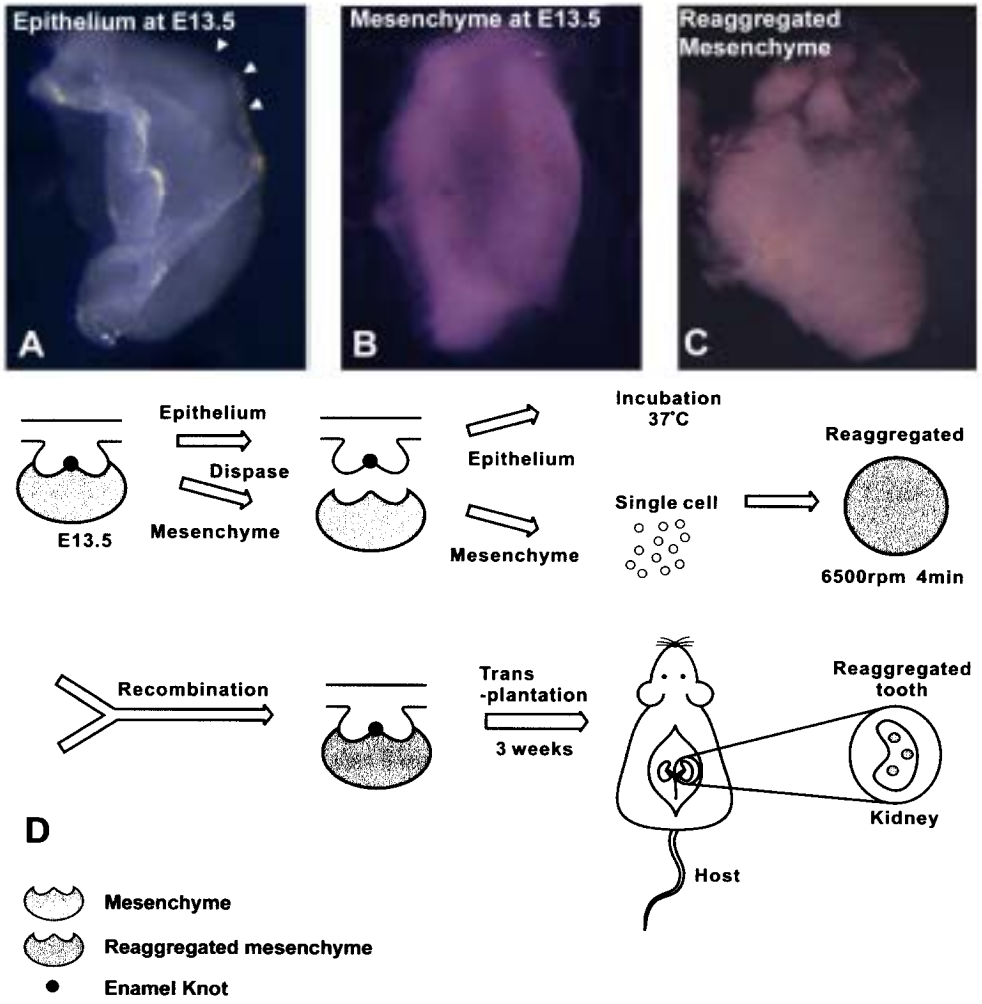


Figure 4. The Reaggregated System: intact epithelium and the repelleted mesenchymal cells make the reaggregated tooth

(A) The epithelium and (B) mesenchyme of the tooth bud that have been just separated at E13.5. (C) Repellet of freshly dissociated mesenchymal cells after triturating to single cells. (D) Schematic diagram of the reaggregated system (arrow heads: enamel knot region)

bores. The cells were filtered through nitex netting when necessary. The viability of the cells and the completion of dissociation were checked microscopically with Trypan blue inclusion. The dissociated cells were counted, repelleted by mild centrifugation (6,500 rpm for 4 minutes) (Fig. 4-C) and allowed to reaggregate for 1 hour at 37℃ on culture insert dishes (falcon). The epithelium was then placed on top of the repelleted mesenchyme and the reaggregated explants were cultured for 1 day at 37℃.

3. Kidney Transplantation

After 1 day incubation in 37℃ incubator, the reaggregated tooth was carefully separated from the filter. Using male adult mouse as a host, the reaggregated tooth was transplanted into the kidney capsule for 3 weeks. This *in-vivo* culture method can lead to the full-calcification of the tooth, which individual cusps can be recognized easily after 3 weeks incubation.

4. Histology

The reaggregated teeth were fixed with 4% PFA in PBS (pH 7.4) at 4°C overnight and decalcified by formic acid and sodium citrate. After dehydration, the reaggregated tooth was embedded in paraffin wax. Seven-micrometer thick sections using the standard techniques were examined histologically using Hematoxilin-Eosin staining.

5. Di.I. Microinjection

Di.I. (1,19-dioctadecyl-3,3,39,39-tetramethyl indocar-bocyanine perchlorate; Molecular probes, Eugene, OR) was used as a cell tracer to observe cell migration during tooth development. A 0.3% w/v Di.I in DMSO was used for the microinjection. The Di.I. injection was performed using 10 cm borosilicate capillary pipettes (Sutter Instruments, BF120-94-10), pulled using a Sutter Instrument Flaming Brown micropipette puller, and that were filled by capillary action. Using a device of electricity, the lipophilic carbocyanine dye inserts into the membrane of the cells adjacent to the injection site. The exact position of the dye can be determined using a fluorescent microscope.

6. *In situ* Hybridization

The reaggregated teeth were fixed in 4% PFA in PBS for 24 hours at 4°C . They were processed for cryo-sectioning. Sections were cut with ten-micrometer thickness and *in situ* hybridization of the tissue sections was carried out as described by Wiemers and Moser (1993).

Ⅲ. Results

1. The Reaggregated Tooth from the Reaggregated Mesenchymal Cells at E13.5

A. The Reaggregated Tooth at E13.5

The reaggregated system (Fig. 4-D) enables all mesenchymal cells to be reset to an equivalent state and have the same probability to become cusps or grooves. The control tooth, which was dissected from a presumptive tooth bud at E13.5, was transplanted into the kidney capsule for 3 weeks. Firstly, when the control teeth were taken from the kidney capsule, they are surrounded by alveolar bone, and soft bone like structures (Fig. 5-A). The reaggregated tooth with intact epithelium was recombined with the dissociated mesenchymal cells that were also surrounded by alveolar bone. It also possessed cusps and roots in the same way, as did the control tooth (Fig. 5-B). The control tooth had 6 to 7 cusps of mesially inclined crests. It had 6 tall cusps and 1 short (hypocone). To compare this with the control tooth, the reaggregated tooth is smaller and shorter. The cuspal patterns of the reaggregated tooth vary.

B. The Cuspal Pattern of the Reaggregated Tooth at E13.5

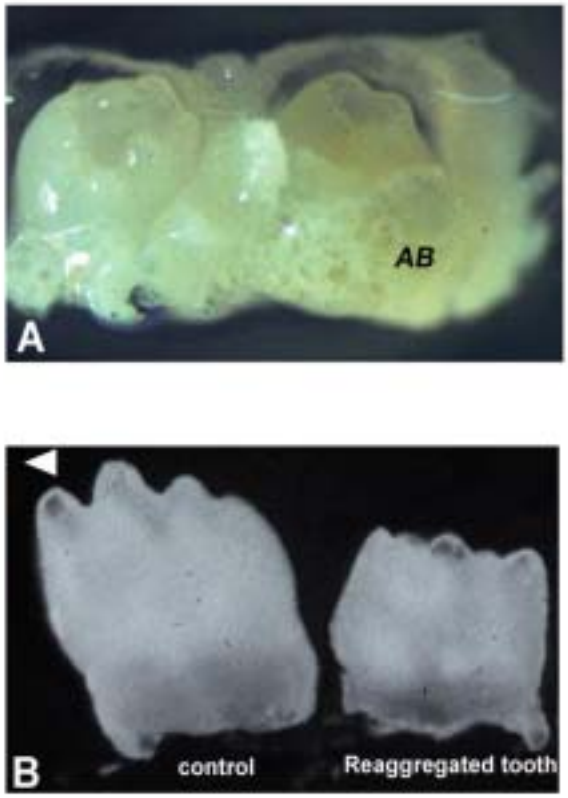


Figure 5. Comparison of the Control Tooth with the Reaggregated Tooth

(A) Two control teeth surrounded by alveolar bone were taken from the kidney capsule after 3 weeks. (B) The control (left) and the reaggregated tooth (right). (arrow head: mesial direction, AB: alveolar bone)

Three types with the cuspal pattern of the reaggregated tooth were identified. Type is the M1-like tooth (8 from 22), which is similar to the first molar (Fig. 6-A). The M1-like tooth has 6 to 7 cusps, which is similar to the control tooth. However, when comparing the control tooth, the cusps of the M1-like tooth are flattened, and the height and size of the cusps was the same (Fig. 6-B). The type Ⅱ tooth is the crater-like tooth (2 from 22). It has 5 cusps. Four of them has the same height, and is flattened. However, one of them, which is located on the center, is small (Fig. 6-C). The type Ⅲ tooth is the slope-like tooth (11 from 22, Fig. 6-D), which has various numbers of cusp from 1 to 5. This cusp shape has mesially inclined crest. The mesial cusp is the highest and the largest (Fig. 6-E).

In particular, it was found that the reaggregated teeth were associated with the cell numbers. First of all, the control tooth was found to contain $1.12x10^4$ cells. The M-1 like tooth had $3.3x10^4$ cells, the crater like tooth had 1.4×10^4 cells, and the slope-like tooth had 2.9×10^4 cells (Table 1).

C. Histological Approach of the Reaggregated Tooth at E13.5

Histological observations were performed to compare the reaggregated tooth with the control tooth. The cusps, predentin and dentin were detected (Fig. 7-A, and 7-B). There were dentinal tubules that approached to the dentin surface in the dentin and mantle dentin on the dentin surface (Fig. 7-C).

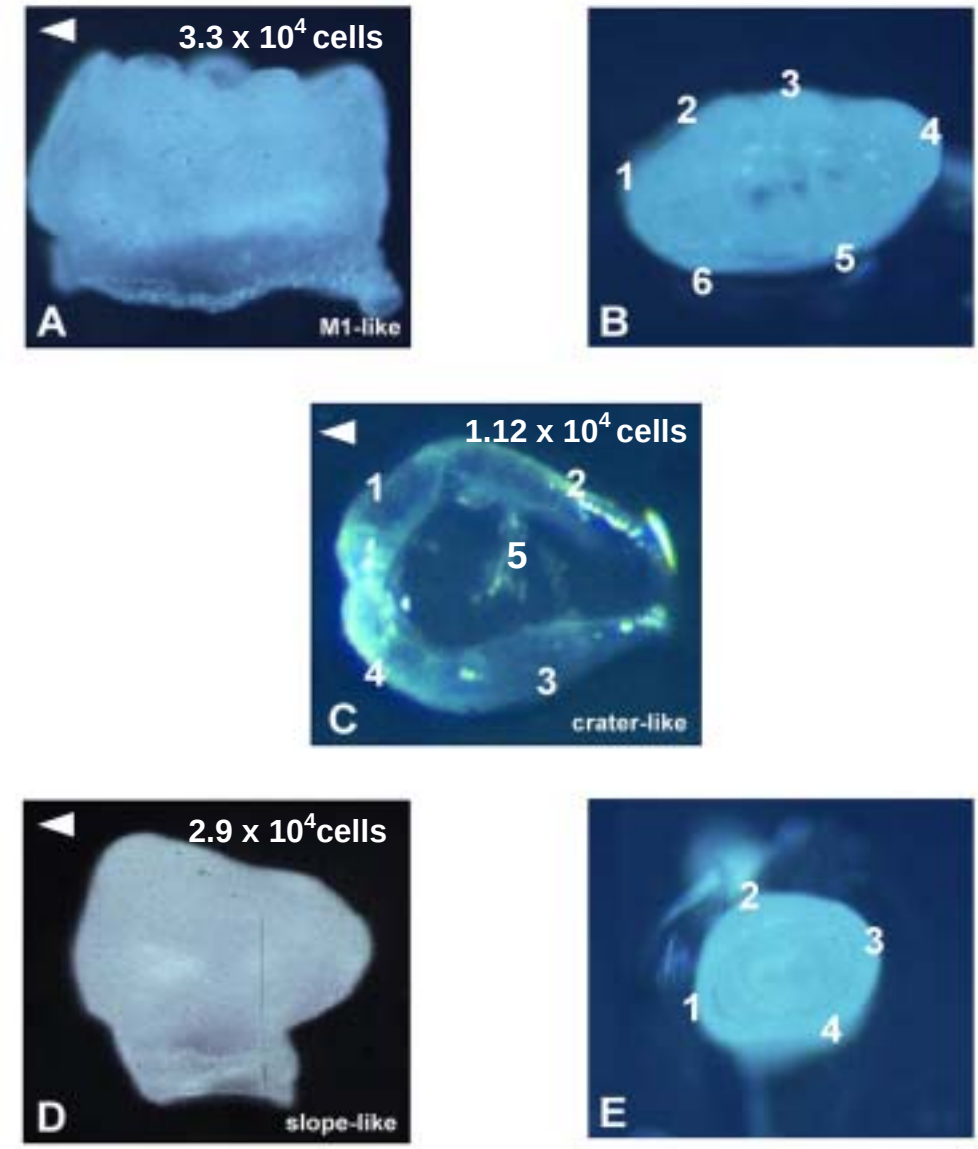


Figure 6. The Reaggregated Teeth: the reaggregated teeth obtained from the reaggregated mesenchymal cells at E13.5

(A) The M1-like tooth-side view, (B) upper view (C) The crater like tooth -upper view (D) The slope-like tooth -side view (E) upper view (number: cuspal number, arrow head: mesial direction)

Table 1. Cuspal Patterns of Reaggregated Tooth at E 13.5

Types	#	Height	Shape	Size	Cell Numbers
M1-like	6 to 7	Same	Flattened	Same	3.3×10^4
Crater-like	5	Same	Flattened	Same (center one is small)	1.4×10^4
Slope-like	1 to 5	Mesially highest	Mesially inclined crest	Mesially biggest	2.9×10^4

*** Control mesenchymal cell numbers : 1.12×10^4**

 # : cuspal numbers

Figure 7. Longitudinal Sections of Reaggregated Tooth at E13.5 after Decalcification: hematoxylin and eosin stained paraffin sections of the reaggregated tooth.

(A) Cusps, (B) predentin and dentin, and (C) mantle dentin in dentin surface and dentinal tubule running to the dentin surface were detected as same way as with the control tooth. (arrow head: cusps, arrow: dentinal tubules, PD: predentin, D: dentin, DT: dentinal tubule, MD: mantle dentin)

Therefore, reaggregated tooth appeared histologically similar to the control tooth.

D. Sectioned *in situ* Hybridization of *Bmp-4* and *Shh*

The *Shh* and *Bmp-4* expression in the reaggregated tooth at E13.5 were examined after 5 days incubation in the kidney capsule. In the case of the reaggregated tooth at E13.5 after 5 days in the kidney capsule, *Shh* was expressed only at the epithelium like control tooth (Fig. 8-A). The *Bmp-4* expression was comparable to the reaggregated tooth. *Bmp-4* expression was shown only at the epithelium of the reaggregated tooth (Fig. 8-B).

E. Di. I. Injected into the Mesenchymal Cells below the Enamel Knot

Di.I. was injected into the mesenchymal cells below the enamel knot to test whether the mesenchymal cells were predetermined or predisposed to becoming cusps.

If the cells becoming either dental papilla (just below the enamel knot) or dental follicles have a memory, the cells could move to the original positions after disassociation and reaggregation. Di.I. was injected into the dental papilla. The Di.I. labeled and un-labeled mesenchyme were triturated into single cells, and recombined with the un-labeled intact epithelium at E13.5.

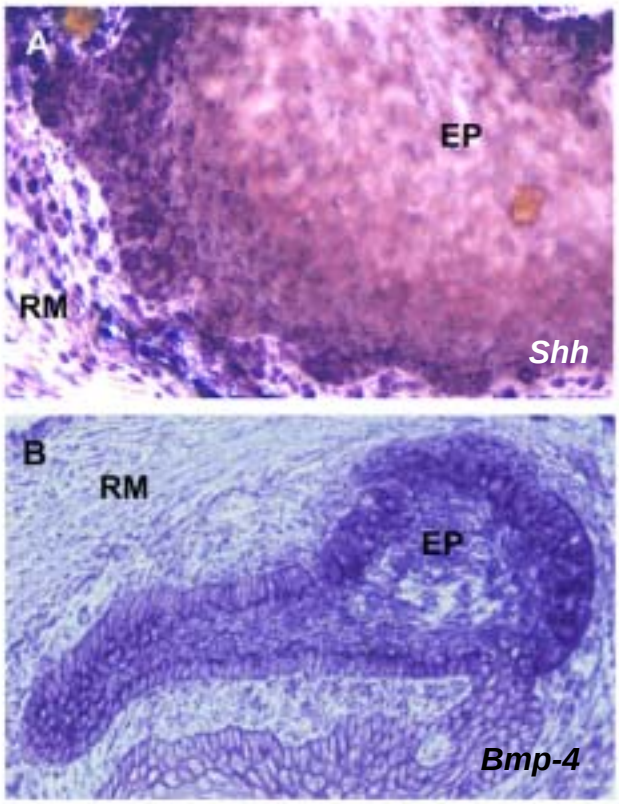


Figure 8. Reaggregated Tooth at E13.5 Processed for Section *in situ* Hybridization with *Shh* and *Bmp-4* RNA Probes: *Shh* and *Bmp-4* expressed only in epithelium after 5 days incubation in the kidney capsule.

(A) The *Shh* probe detected in the epithelium of the reaggregated tooth is similar to control. (B) *Bmp-4* expression is shown only at the epithelium of the reaggregated tooth. (EP: epithelium, RM: reaggregated mesenchyme)

After 5 days incubation in the kidney capsule, the labeled cells became randomly distributed in the cusps and grooves (Fig. 9). Therefore, all mesenchymal cells were reset to an equivalent state without any memory of their precious cues.

2. The Reaggregated Tooth from the Reaggregated Mesenchymal Cells at E11.5 and Diverse Reaggregated Teeth

A. The Reaggregated Tooth at E11.5

Using the reaggregated system, the intact epithelium was recombined with the repelleted mesenchymal cells at E11.5, and transplanted into the kidney capsule for 3 weeks using the same methods as done at E13.5. The reaggregated tooth was also produced. The reaggregated tooth at E11.5 was classified as the M1-like type (Fig. 10-A). The cusps number ranged from 3 to 6. The cusps were flattened. The height and size of the cusps were all the same (Fig. 10-B). Histological observation was used to identify this reaggregated tooth. As the reaggregated tooth at E13.5, cusps, root, predentin, and dentin were observed (Fig. 10-C and 10- D). The dentinal tubule and mantle dentin were also found in the reaggregated tooth at E11.5 (Fig. 10-E)

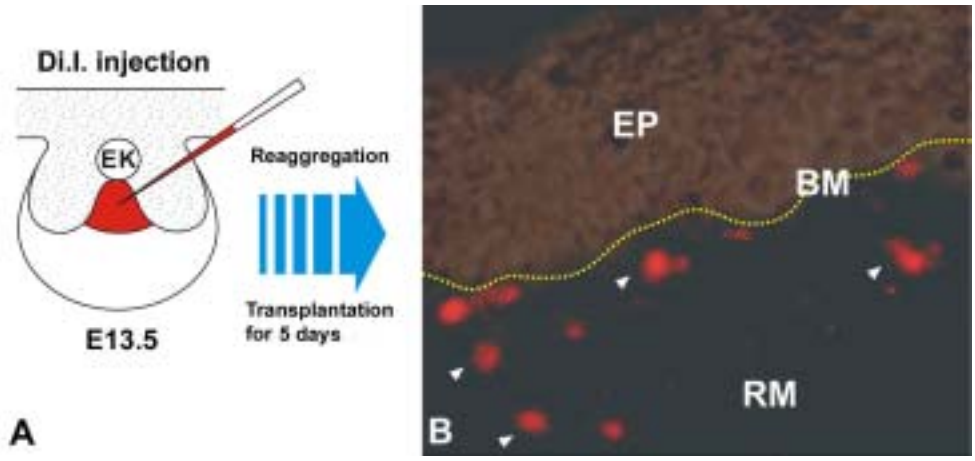


Figure 9. Di.I. Labeling to the Mesenchymal Cells below the Enamel Knot

(A) Prior to the separation of the epithelium and mesenchyme of the tooth bud, Di.I. was injected into the mesenchyme just below the enamel knot at E13.5. (B) When mesenchyme was triturated into single cells, recombined with the intact epithelium, and then transplanted in the mouse kidney capsule for 5 days, the labeled cells spread over the mesenchyme of the reaggregated tooth. (EP: epithelium, BM: basement membrane, RM: reaggregated mesenchyme, arrow heads: Di.I. labeled cells)

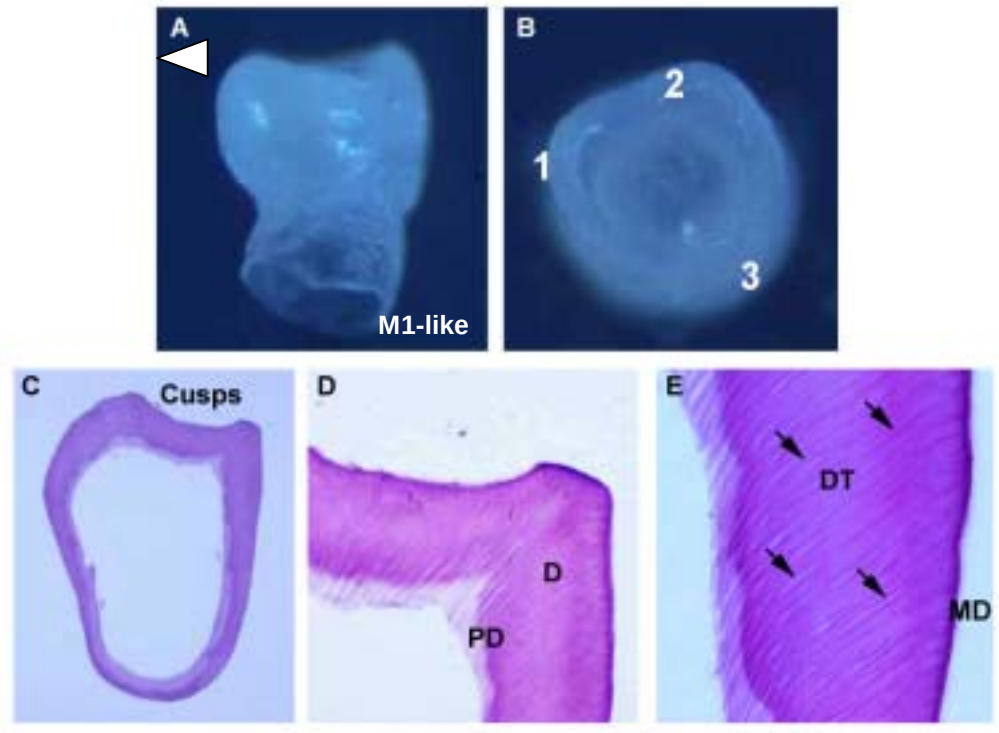


Figure 10. The Reaggregated Tooth Ⅱ: the reaggregated tooth made from the reaggregated mesenchymal from E11.5.

(A) M1-like tooth at E11.5 – side view (B) upper view (C) In order to observe the histology of the reaggregated tooth obtained from the E11.5 mesenchymal cells, H-E stainining was performed after longitudinal section. The cusps, predentin, dentin, dentinal tubules, and mantle dentin are observed like the reaggregated tooth from E13.5. (number: cuspal number, arrow head: mesial direction)

B. The Reaggregated Tooth Recombined Incisor with Molar at E13.5

The M1-like tooth type was made from the incisor epithelium that was recombined with the reaggregated mesenchymal cells of the molar at E13.5 (Fig.11-A and 11- B). In this case, the M1-like tooth had 3 to 6 cusps. The height and the size of this M1-like tooth were almost the same. The cusps were flattened. The number of cells in this M1-like tooth was 5.9×10^4 cells

C. The Reaggregated Tooth Recombined Epithelium at E11.5 with the Reaggregated Mesenchymal Cells at E13.5

The slope-like tooth was made from the epithelium at E11.5, which was recombined with the reaggregated mesenchymal cells at E13.5 (Fig. 11-C and 11-D). This slope-like tooth had 3 to 5 cusps and mesially inclined crest. The mesial cusp of this reaggreagated tooth was the largest and highest. This slope like tooth had 6.8×10^4 cells.

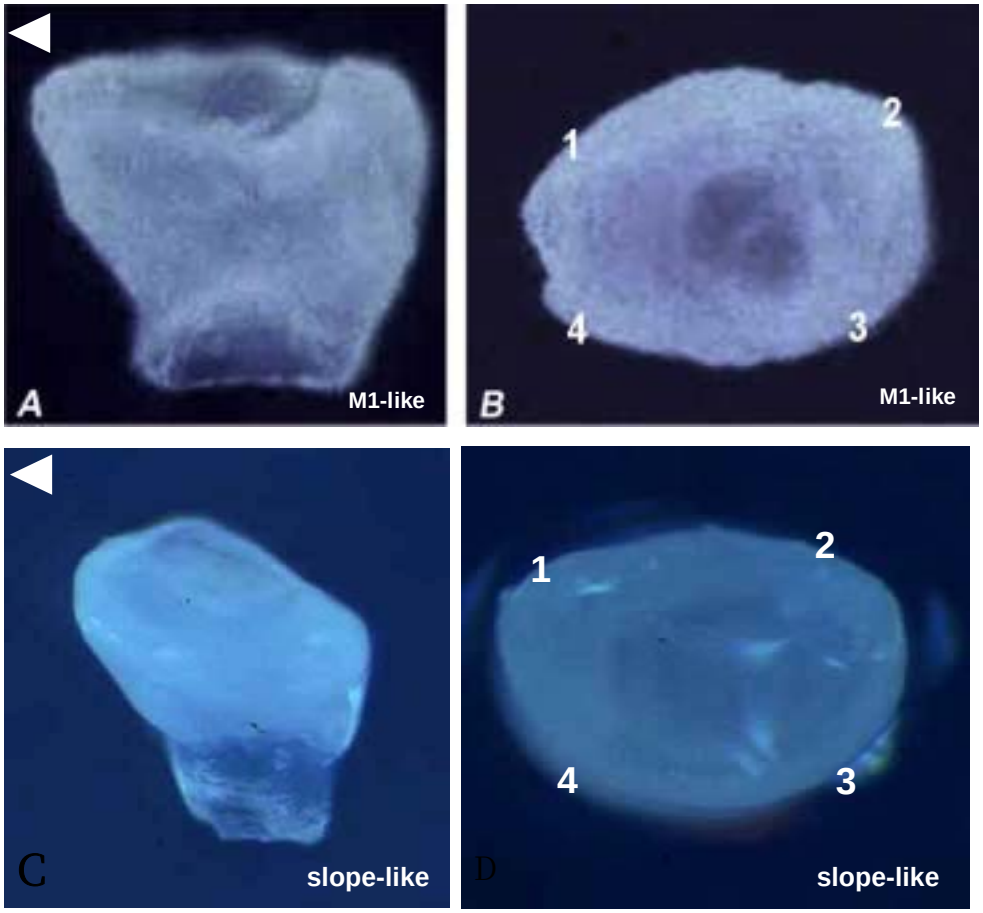


Figure 11. The Reaggregated Tooth III: the reaggregated tooth made from the reaggregated mesenchymal from E11.5.

(A) M1-like tooth at E11.5 –side view (B) upper view (C) In order to observe the histology of the reaggregated tooth obtained from the E11.5 mesenchymal cells, H-E stainining was performed after longitudinal section. The cusps, predentin, dentin, dentinal tubules, and mantle dentin are observed like the reaggregated tooth from E13.5. (number: cuspal number, arrow head: mesial direction)

Ⅳ. Discussion

Tooth morphogenesis is dependent on the developmental stages.

Previous recombination experiments have already shown that dental epithelium prior to E12.5 possess the potential to induce tooth development.[10] At E12.5, this potential shifts to the dental mesenchyme (Fig. 3). The mesenchymal cells of the dental papilla have been shown to regulate the tooth shape, i.e. an incisor tooth develops when the dental papilla of a cap or bell stage incisor tooth germ is combined with the molar epithelium.[20,21] To induce full calcification, Lumsden used the anterior chamber to supply the blood vessels.[22] To supply blood vessels for a recombined tooth, the recombined tooth was transplanted to the mouse kidney capsule for 3 weeks. After 3 weeks, the fully calcified reaggregated tooth was produced although the mesenchymal cells were reset to an equivalent state to become cusps or grooves.

Interestingly, using the reaggregated system, when the molar epithelium at E13.5 was recombined with the molar reaggregated mesenchyme at E13.5, diverse shapes of reaggregated tooth was made as follows: the M1-like tooth, the crater-like tooth, the slope-like tooth (Fig. 6). The M1-like tooth was made from the molar epithelium recombined with the molar reaggregated mesenchyme at E11.5 (Fig. 10-A). The reaggregated tooth, which recombined incisor epithelium with the molar reaggregated mesenchyme at E13.5, was observed to be the M1-like tooth (Fig. 11-A). The reaggregated tooth, which recombined the molar epithelium at E11.5 with the

molar reaggregated mesenchyme at E13.5, was produced as the slope-like tooth (Fig. 11-B). Therefore, the dental mesenchyme has the potential to produce not only tooth development, but also tooth morphogenesis (shape in development) after bud stage (Table 2).

Reaggregated tooth development was investigated using *in situ* hybridization to compare with the control. *Shh* was detected in the dental epithelium (Fig. 8-A). *Shh* has been known to play a crucial role during tooth development; *Shh* is detected in dental epithelium from the epithelial thickening to the completion of crown morphogenesis. Mice with *Shh* null mutation develop craniofacial abnormalities as well as abnormal dentition that are similar to what is observed in mice lacking *Gli-2* or *Gli-2/Gli-3*, which are downstream transcription factors in the *Shh* pathway. This suggests that *Shh* plays a role in tooth development.[15,23] If *Shh* activity from the dental epithelium was removed genetically, which is the sole source of *Shh* during tooth development, tooth growth and cytological organization within both the dental epithelium and mesenchyme of the tooth were altered.[24]

Surprisingly, *Bmp-4* was expressed in the epithelial region in the reaggregated tooth (Fig. 8-B). *Bmp-4*, which is dynamically expressed in the tooth tissues throughout morphogenesis, was shown to regulate the mesenchymal expression of several transcription factors such as *Msx-1*, *Msx-2*, *Pax-9 in vitro*. In addition, *Bmp-4* was shown to induce its own mesenchymal expression, *Lef-1*.[17, 25-29]

Table 2. The Potential to Induce the Tooth Formation

Epi. / Mes.		E11.5		E13.5	
		I	M	**I**	M
E11.5	**I**				
	M		**M1-like**		X
E13.5	**I**			X	
	M		**Slope-like**	**M1-like**	**Diverse**

(Epi.: Dental Epithelium, Mes.: Reaggregated Mesenchyme, I: Incisor, M: Molar, X: could not make the reaggregated tooth)

In reaggregated tooth, *Bmp-4* was expressed in the epithelial expression, which suggests that dental mesenchymal cells altered their own cell fate to allow them to adjust with the recombined epithelium. This area requires more clarification in a further study.

The reaggregated system means the mesenchymal cells were reset to an equivalent state.

As an epithelial appendage, tooth bud and feather bud require a similar morphological potential to produce a developing organ. In the reaggregated system of feather buds, the dissociated mesenchymal cells (dermis) have the ability to self-organize into periodically arranged primordial feathers that grow into normal feather buds.[19] The authors showed that all the mesenchymal cells had the same probability of becoming feather primordia and interpromordia (tooth cusps, crests and groove). No predetermined differences and no molecular 'memory' following mesenchymal dissociation (*in situ* hybridization, Di.I. experiment) and reaggregation were found, and the size of the feather primordia remained constant according to the number of available mesenchymal cells (size of cusps, crown and roots). Unquestionably, the Di.I. labeling experiment was used to show that reagreagated tooth mesenchymal cells have the same ability to become cusps or grooves. In addition, their fate was not predetermined and they lost their cell memory after dissociation and reaggregation (Fig. 12).

However, the reaggregated mesenchymal cells in developing chick limb buds have the different story. The recombinants limbs made from the dissociated and reaggregated mesenchyme from the different regions of the leg could grow, pattern and form identifiable, different digits.[20] In the posterior third reaggregates, *HoxD* genes (*Hoxd-9*, *Hoxd-11*, *Hoxd-13*) were expressed in the sub-ridge mesenchyme. In the anterior third reaggregates, *Hoxd-9* but not *Hoxd-11* was expressed. They suggested a model for specifying the identity of the cartilaginous elements in general, and reported that the digit in particular, is based on the *HoxD* genes. The different origin of the epithelial appendage results in a different potential as early development (Fig. 12). Indeed, it might be suggested that a tooth bud is related to skin development (scale) as shown in a shark study. Furthermore, early non-changeable Hox genes in a limb bud make it difficult to reorganize their own fates to reform in embryonic development.

The cuspal patterns are related with the mesenchymal cell numbers.

The reaggregated tooth was identified with three types. The M1-like tooth ($3.3x10^4$ cells) was similar to the first molar of the lower jaw. When compared to the first molar, the cusps of the M1-like tooth are flattened, and the sizes are all the same. The cuspal pattern of the M1-like tooth can be also classified to the loph type in mice, as suggested by Jernvall.[18] The loph type has from 2 to many buccal and lingual cusps, and has a transverse or longitudinal loph.

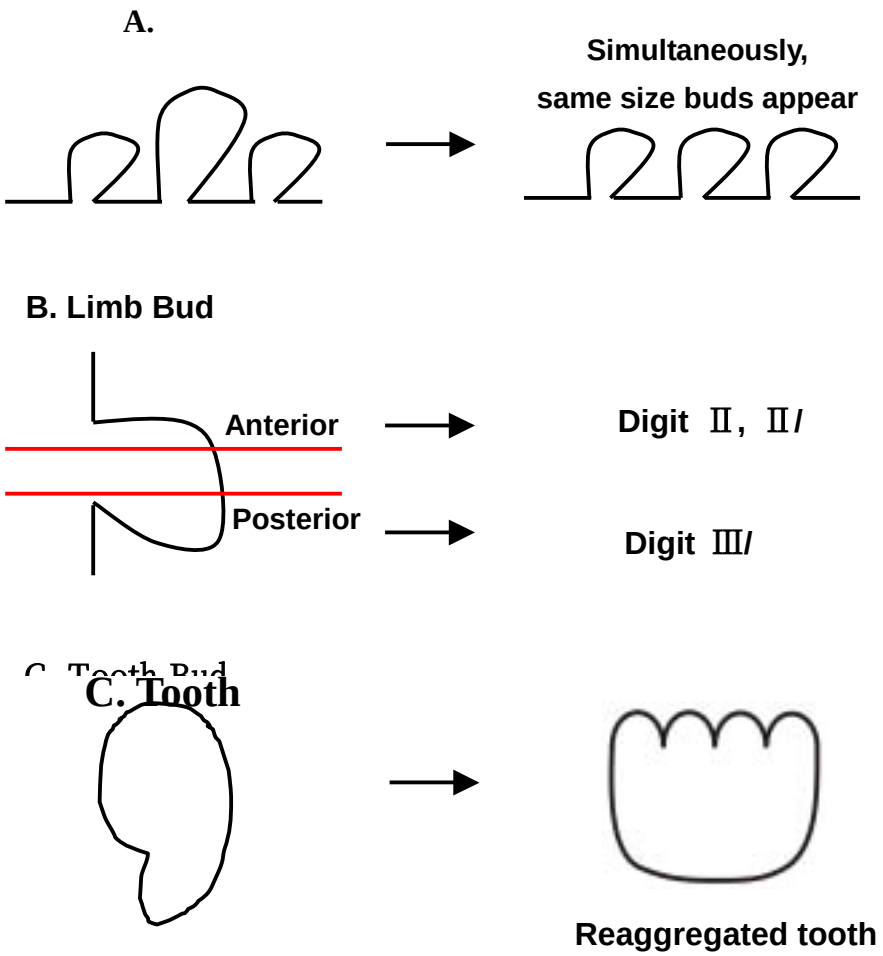


Figure 12. Comparing Feather Bud and Limb Bud with the Tooth Bud in Reaggregated System

(A) In reconstituted system of feather bud, all feather primordial appeared simultaneously after dissociation and reaggregation of the mesenchymal cells, and then recombining with the epithelium. (B) Of reaggreagates made from anterior leg bud mesoderm, digit Ⅱ, Ⅱ/Ⅲ were produced. Reaggregates made with the posterior leg bud mesoderm, gave rise to digit Ⅲ or Ⅳ. (C) The reaggregated tooth, which was made from epithelium with reaggregated mesenchyme, was produced.

The crater-like teeth (1.4 x 10^4cells) are an unusual type. The crater-like tooth has 5 cusps. Four cusps have same size and height. One cusp is located on the center part of the crater-like tooth and is very small. If this center cusp is an incomplete tranverse loph, the crater-like tooth must be the mouse tooth. However, if this center cusp is not the transverse loph, the cuspal pattern of the crater-like tooth is similar to human teeth. The cuspal pattern of a human tooth has 4 round shapes. A human tooth has 2 buccal and 2 lingual cusps. The slope-like tooth (2.9 x 10^4 cells) has from 1 to 5 cusps. The slope-like tooth has mesially inclined crests. The mesial cusp is the largest and highest. This cusp type can be resembled of the otter's. This otter cuspal pattern contains 3 round and two buccal cusps.

This technique of reaggregated tooth from a mouse might be adapted to the bioengineering of human teeth. Using human embryonic dental mesenchymal cells (hEDMC) after bud stage, cells might be able to be cultured to sustain their own cell fates. When accessible mouse embryonic dental epithelium will be recombined with the reaggregated hEDMC, a tooth, which resembles a human tooth, could be best produced both biologically and_morphologically (Fig. 13). Recently, Young, et al. (2002) reset a tooth bud including epithelium and mesenchyme in a six-month old pig and tried to make a tooth using a scaffold.[30] In pigs, the third molar tooth bud was dissected, mechanically and enzymatically dissociated (reset). It was then seeded onto a tooth scaffold, and implanted in the omentum of athymic rats. After 30 weeks, they recognized a tooth structure. However, the tooth structure was very small and did not conform to the size and shape of the scaffold.

In teeth, the dental epithelium prior to E12.5 has the potential to induce tooth formation. The mesenchymal cells at E11.5 remain in the undifferentiated state. Undifferentiated cells can also be detected in the adult dental pulp. Stem cells are undifferentiated cells, which have self-renewal and multiple differentiation capacity. Many experiments suggested that stem cells exist in the dental pulp. The undifferentiated mesenchymal stem cells in the pulp are in essence dental pulp stem cells. If these stem cells can be obtained from dental pulp in an extracted third molar (wisdom tooth) and combined with an embryonic epithelium, a bioengineering tooth can be made.

Ⅴ. Conclusion

Using the reaggregated system that enables the tooth bud to be fully calcified, the reaggregated tooth has been produced. Although the size of the reaggregated tooth was smaller than that of control tooth, cusps, predentin and dentin and dentinal tubules running to the dentin surface in dentin and the mantle dentin in the dentin surface were histologically observed similar to control.

The reaggregated teeth at E13.5 were divided to three types: the M-1 like tooth (3.3×10^4 cells), the crater-like tooth (1.4×10^4 cells), and the slope-like tooth (2.9×10^4 cells). These results suggest that the cuspal pattern of the reaggregated tooth maybe associated with mesenchymal cell numbers. When Di.I. was injected into dental mesenchymal cells just below the enamel knot (the dental papilla), Di.I.-labeled cells became randomly distributed in the newly-formed cusps and grooves. All mesenchymal cells lost their own cell fates and non-prespecified dental mesenchymal cells develop into the reaggregated tooth.

The reaggregated tooth at E11.5 was classified as the M1-like tooth. The slope-like tooth was produced from the epithelium at E11.5, which was recombined with the reaggregated mesenchymal cells at E13.5. From these results, it is confirmed that the mesenchymal cells have potential to induce tooth morphogenesis after bud stage.

In use of odontogenic potential to form the tooth by epithelial-mesenchymal

interactions, it is high time that we could produce bioengineered human tooth to adjust individual favor. To obtain the human tooth, we prepare the human dental mesenchymal cells after bud stage, and then recombine with available potential epithelium, eg. oral epithelium from mouth, epidermis from skin etc. It should be note that all tissues must be from embryonic stages where embryonic potential remains within tissues. After required period of incubation in nude mice, we will be able to achieve fully calcified human teeth. It can be still problem to proceed above experiment in biology, sociology and theology. However, it will be not too long that we can have our own teeth to replace by bioengineered teeth.

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국문 요약

치아 간엽세포의 재합성과 치아 형성

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치아 상피 조직과 외배엽성 간엽 조직에 의해 치아 형성을 유도하는 것은 치아 발생동안 일어나는 가장 기본적이고 중요한 과정이다. 싹시기 이전에 치아의 형태형성을 유도하는 능력은 치아 상피에 있으며, 그 이후에는 그 능력이 치아 간엽 조직으로 옮겨진다는 것은 이미 알려진 사실이다.

본 연구에서는, 우선 간엽 조직을 단위세포로 만든 후, 그 수를 달리하여 재응집 시켜, 같은 크기의 상피 조직과 재조합 하였다. 이를 생쥐의 신장 막에 이식하여 3주후 관찰한 결과, 완전히 석회화 된 재합성 치아가 만들어졌다. 같은 방법 즉, 재응집 실험을 이용하여, 대조군 치아가 입술쪽으로 기울어진 6-7개의 교두가 생성되었다. 이중 6개는 크고, 1개는

작은 교두이다. 재합성 된 치아의 교두는 여러 가지 유형으로 나타났다. 재합성 치아의 교두는 3가지 유형으로 나누어질 수 있는데, M1-like, crater-like, slope-like로 나눌 수 있다. 재합성 치아와 대조군 치아를 조직학적으로 비교하기 위하여, 재합성 치아를 탈회한 후, 파라핀으로 포매 한 후 절편을 만들어, H-E 염색을 하였다. 그 결과, 대조군 치아와 마찬가지로 교두, 상아질, 외피상아질이 관찰되었다. 또한, 치아 발생동안 중요한 역할을 하는 유전자인 *Bmp-4*와 *Shh* 의 발현을 관찰한 결과, 둘 다 재합성 치아의 상피 부위에서 확인할 수 있었다. Di.I 를 법랑결절 바로 아래 간엽 세포에 주입시켰을 때, Di.I. 표지 된 세포들이 재응집 치아의 간엽 조직에 흩어져서 나타났다. 이는 치아의 간엽 세포들이 각각의 기억이 미리 정해져 있는 것이 아님을 의미한다.

E11.5 의 상피 조직과 재응집 된 간엽 세포를 재조합 하여 만든 재합성 치아는 M1-like 유형으로 나타났으며, E13.5 의 앞니의 상피와 어금니의 재응집 된 간엽 세포를 재조합 했을 시에는 M1-like 치아가 생성되어 졌다. 또한, E11.5의 상피 조직과 E13.5 의 재응집 된 간엽 세포를 재조합 하여 재합성된 치아를 만들었을 때에는, slope-like 치아가 만들어졌다.

이상의 결과를 토대로, 재합성된 치아의 교두 모양의 유형은 간엽 세포의 수와 관련이 있는 것으로 확인되었으며, 간엽 세포의 수를 조절하면 다양한 교두의 모양을 결정할 수 있을 것으로 생각되어진다.

핵심 되는 말: 생체 공학적 치아, 배아시기의 치아 발생, 상피-간엽 조직 간 상호작용, 간엽 세포의 수, 재합성