Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 304 (2007) 499–507
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Patterning the size and number of tooth and its cusps

Jinglei Cai [1], Sung-Won Cho [1], Jae-Young Kim, Min-Jung Lee, Yoon-Geun Cha, Han-Sung Jung *

Division in Anatomy and Developmental Biology, Department of Oral Biology, Research Center for Orofacial Hard Tissue Regeneration, Brain Korea 21 project, Oral Science Research Center, College of Dentistry, Yonsei Center of Biotechnology, Yonsei University, 134 Shinchon-Dong, Seodaemoon-Gu, Seoul 120-752, South Korea

Received for publication 21 July 2006; revised 21 November 2006; accepted 3 January 2007
Available online 9 January 2007

Abstract

Mice and rats, two species of rodents, show some dental similarities such as tooth number and cusp number, and differences such as tooth size and cusp size. In this study, the tooth size, tooth number, cusp size and cusp number, which are four major factors of the tooth patterning, were investigated by the heterospecific recombinations of tissues from the molar tooth germs of mice and rats. Our results suggest that the dental epithelium and mesenchyme determine the cusp size and tooth size respectively and the cusp number is co-regulated by the tooth size and cusp size. It is also suggested that the mesenchymal cell number regulates not the tooth size but the tooth number. The relationships among these factors in tooth patterning including micropatterning (cusp size and cusp number) and macropatterning (tooth size and tooth number) were analyzed in a reaction diffusion mechanism. Key molecules determining the patterning of teeth remains to be elucidated for controlling the tooth size and cusp size of bioengineered tooth.

Keywords: Tooth patterning; Tooth size; Cusp size; Cusp number; Tooth number; Reaction diffusion mechanism; Rodent

Introduction

Ectodermal organs such as tooth, hair, mammary gland and feather share common morphological features, which develop from epithelial–mesenchymal interactions during the early stages of morphogenesis (Pispa and Thesleff, 2003). The epithelial–mesenchymal interaction in tooth development has been widely studied with the recombination and reaggregation methods (Mina and Kollar, 1987; Baba et al., 1996; Yamamoto et al., 2005). Furthermore, the heterospecific recombination of dental tissues has been used to examine tooth formation between mammals and birds (Kollar and Fisher, 1980; Wang et al., 1998; Chen et al., 2000). These previous recombination studies confirmed that the inductive potential of tooth formation shifts from the first pharyngeal arch epithelium to mesenchyme after the bud stage (Mina and Kollar, 1987), and those mesenchymal signals are necessary for the epithelial patterning and for the formation and maintenance of the epithelial compartments (Mustonen et al., 2002). However, the roles of the epithelium and mesenchyme in the patterning of teeth have not been studied sufficiently. In this study, we applied heterospecific recombination of embryonic dental tissues between mice and rats to investigate characteristics of the tooth patterning.

It is well known that the process of tooth development is almost the same in mice and rats, but is slower in rats. In the tooth patterning, the most discriminable differences in molars between mice and rats are the tooth size and cusp size, while the tooth number and cusp number are same between mice and rats. These tooth size, tooth number, cusp size and cusp number are four major factors of the tooth patterning. Our study compared these four main factors in various teeth which were formed by the heterospecific recombination and heterospecific reaggregation between the mouse and rat dental tissues from the presumptive mandibular first molars at both dental lamina and cap stages. Especially, for the cusp patterning, *Fgf4* expression was examined in the heterospecific recombinant tooth germs from the cap stage.

* Corresponding author. Fax: +82 2 312 8012.
E-mail address: hsjung@yumc.yonsei.ac.kr (H.-S. Jung).
[1] These two authors made equal contributions.

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.01.002

The patterning of an organ could be divided into macro- and micropatterning. In dermis formation, it has been suggested that the developing skin establishes several skin regions in the macropatterning process, and then the epithelium and mesenchyme become competent to execute micropatterning, the induction of individual feather primordia (Sengel, 1976). In our study, we divided the tooth patterning into the macropatterning and micropatterning. The macropatterning includes the tooth size and tooth number in maxilla or mandible, while the micropatterning of teeth includes the cusp size and cusp number in an individual tooth. Furthermore, the patterning of various recombinant teeth was discussed in terms of a reaction diffusion mechanism as previous studies on tooth patterning (Jernvall et al., 2000; Salazar-Ciudad et al., 2003).

Materials and methods

Preparation of tissues

The molar tooth germs of embryonic day 11 (E11) ICR mice and E13 Sprague–Dawley (S-D) rats show the similar developmental stage (dental lamina stage). The molar tooth germs of E14 ICR mice and E16 S-D rats also show the similar developmental stage (cap stage). The size of the mouse tooth germ is smaller than that of the rat tooth germs both at the dental lamina and cap stages. These four kinds of tooth germs were dissected and incubated in Dispase II (Roche, Germany) at 1.2 U/ml in PBS for 10 min, 13 min, 20 min and 30 min respectively. The tooth germs were washed in DMEM with 10% FBS, and the dental epithelium was separated from the dental mesenchyme. The mouse dental epithelium is smaller in size than the rat dental epithelium, and the size of the mouse dental mesenchyme is smaller than that of rat dental mesenchyme.

Tissue recombinations

Four types of recombinations were carried out as follows: 1) Homospecific recombination in mice at E14 (E14M-epi/E14M-mes: E14 mouse dental epithelium overlaid on E14 mouse dental mesenchyme) and rats at E16 (E16R-epi/E16R-mes) respectively; 2) Heterospecific recombination between dental tissues from mice and rats (M-epi/R-mes: E14M-epi/E16R-mes, E11M-epi/E13R-mes; R-epi/M-mes: E16R-epi/E14M-mes, E13R-epi/E11M-mes) (see in Figs. 2H′, L′, O′ and R′); 3) Heterospecific reaggregation between dental tissues from E14 mice and E16 rats (E14M-epi/E16R-*r-mes*: mouse dental epithelium overlaid on the rat *r*eaggregated dental *mes*enchyme, E16R-epi/E14M-r-mes): the reaggregated mesenchyme was prepared as previously described (Yamamoto et al., 2003), the number of cells in the E14 M-r-mes and E16 R-r-mes was adjusted as 6.0×10^4, which corresponds to the average number of mesenchymal cells in one molar of E16 rats; 4) Reaggregation in mice at E14 (E14M-epi/E14M-r-mes): the number of cells in the reaggregated mesenchyme was adjusted into 2.0×10^5, which corresponds to 10 times the average number of mesenchymal cells in one molar of E14 mice. The mouse reaggregated mesenchyme containing 2.0×10^5 cells were much larger than that the mouse dental epithelium in size. The epithelium, which was smaller in size than that of the mesenchyme in the case of the E11M-epi/E13R-mes, E14M-epi/E16R-mes, E14M-epi/E16R-r-mes, and E14M-epi/E14M-r-mes, was overlaid on the center of the mesenchyme. These recombinant tooth germs were cultured with Trowell-type organ culture method using DMEM with 10% FBS for 1, 2, 4 and 6 days.

Transplantation of recombinants into the renal subcapsular layer of nude mouse

To calcify the recombinant tooth germs, they were cultured for 2 days *in vitro* and transplanted into the renal subcapsular layer of adult nude mice (Orientbio, Korea). All surgical procedures were performed under anesthesia administered intra-peritoneally. No immunosuppressive medication was used. After 3 weeks, the host mice were sacrificed, and kidneys were dissected to obtain the calcified teeth.

In situ hybridization

In vitro cultured tooth germs were fixed overnight in 4% paraformaldehyde in PBS. *In situ* hybridization was carried out by treating the tooth germs with 20 μg/ml proteinase K for 3 min at room temperature. The anti-sense RNA probes were labeled with digoxygenin (BMS, Seoul, Korea). Whole-mount *in situ* hybridization was performed as our previous study (Kim et al., 2003).

X-gal staining

The E14M-epi/E16R-mes tooth germs composed of the *lacZ* transgenic Rosa26 mouse dental epithelium at E14 and S-D rat dental mesenchyme at E16 were harvested after 5-day incubation in the renal subcapsular layer and processed by X-gal staining as described elsewhere (Sasaki et al., 2005). The specimens were washed with 2 mM $MgCl_2$ in PBS for 5 min, rinsed three times with a rinse buffer (2 mM $MgCl_2$, 0.02% NP-40, 0.01% sodium deoxycholate in PBS) for 20 min at room temperature, stained with a X-gal staining solution [1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide] and incubated at 37 °C for 1 h. After X-gal staining, the samples were sectioned and washed again with PBS for 10 min and mounted on a slide.

Results

Tooth size of first mandibular molar in adult mice and rats

The tooth size of first mandibular molar (M1) of adult mice and rats is measured by the mesiodistal length and buccolingual length. The mesiodistal length (2.82±0.021 mm) and buccolingual length (1.38±0.015 mm) in the adult rat M1 ($N=5$) (Figs. 1D, E) were roughly double the mesiodistal length (1.44±0.011 mm) and buccolingual length (0.80±0.019 mm) in the adult mouse M1 ($N=5$) (Figs. 1A, B). The proportion of the M1 mesiodistal length (1.44±0.011 mm) to the mandible length (12.55 mm) in the mouse was 1:9, which also corresponds to the proportion of the M1 mesiodistal length (2.82±0.021 mm) to the mandible length (24.58 mm) in the rat (Figs. 1C, F). The cusp size was not measured in the M1 of the adult mice and rats because of the severe wear in cusps.

Tooth size and cusp size of transplanted M1 in mice and rats

Both E14 mouse tooth germs ($N=9/9$) and E16 rat tooth germs ($N=4/4$) formed the calcified teeth after being transplanted into kidneys for 3 weeks. The mesiodistal length (1.83±0.017 mm) and buccolingual length (1.23±0.008 mm) of the rat transplanted M1 (rat control, Figs. 1J–L) were almost double the mesiodistal length (0.94±0.014 mm) and buccolingual length (0.56±0.013 mm) of the mouse transplanted M1 (mouse control, Figs. 1G–I). The result that the tooth size of the rat transplanted M1 is almost double the size of the mouse transplanted M1 as seen in adults mean that transplantation of tooth germs into the renal subcapsular layer can be used as a culture system for the investigation of the tooth size.

The cusps of the transplanted M1 have no abrasion compared with those in adult. The cusp size was investigated by measuring the intercuspal distance (between the neighboring

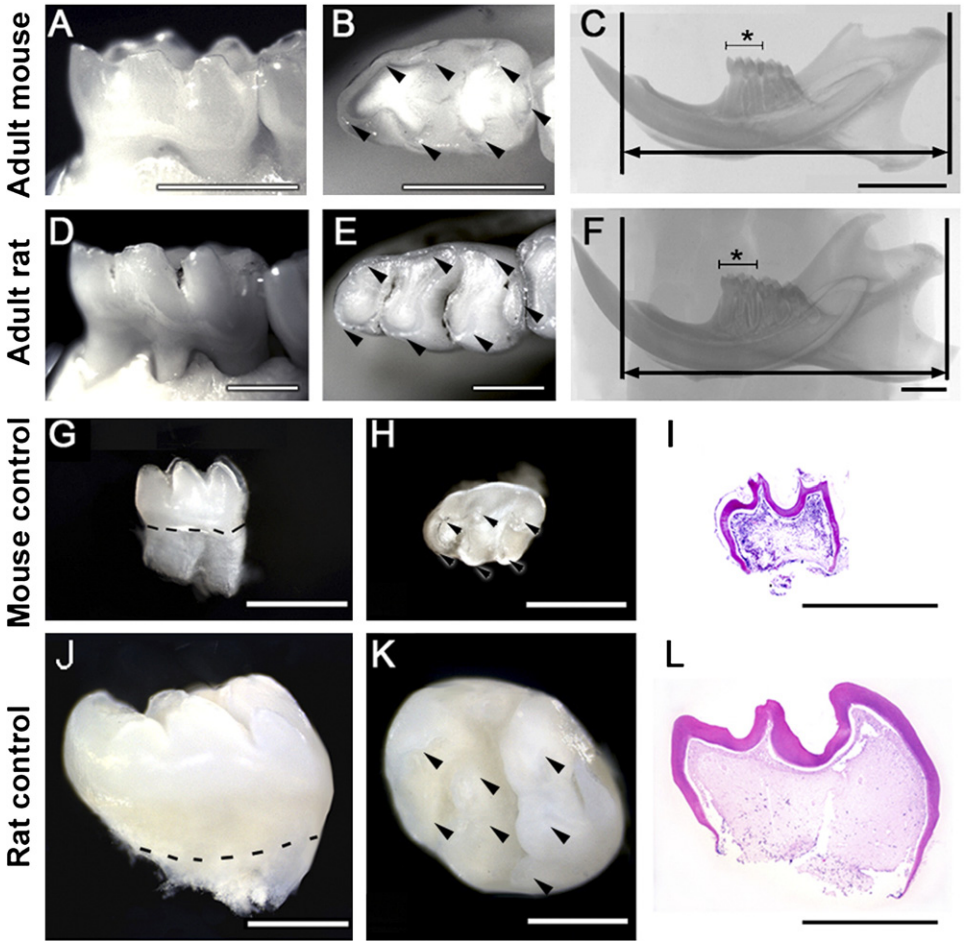


Fig. 1. Tooth size and cusp size of M1 in mice and rats. (A, B) The mandibular first molar (M1) of a 12-week-old mouse shows seven cusps in the lateral view (A) and occlusal view (B). (C) The ratio of the M1 length (asterisk) to the mandible length (two-way arrow between the most anterior end and the condyle head of the mandible) is about 1:9 in the lateral view of adult mice. (D, E) The M1 of a 12-week-old rat shows seven cusps in the lateral view (D) and occlusal view (E). (F) The ratio of the M1 length (asterisk) to the mandible length (two-way arrow between the most anterior end and the condyle head of the mandible) in adult rat is about 1:9, which is equal to that of adult mouse. (G–I) The mouse transplanted M1 developing from the E14 mouse tooth germs show six cusps. (J–L) The rat transplanted M1 developing from the E16 rat tooth germs, are larger in tooth size and cusp size than those of the mouse transplanted M1. Seven cusps are detected as well. The crown and root of teeth can be recognized (dotted lines in panels G, J). The cusp tips can be recognized (arrowheads in panels B, E, H and K). Scale bar: 1 mm in panels A, B, D, E, G–L; 2 mm in panels C, F.

cusp tips) of the calcified teeth. The intercuspal distance of the rat transplanted M1 (0.677±0.0047 mm) was about double that of the mouse transplanted M1 (0.325±0.0054 mm), indicating that the cusp size of the rat M1 was about double that of the mouse M1. This result suggests that transplantation into the kidney is a good system for studying cusp size of tooth.

Tooth and cusp size of homospecific recombinant M1 in mice and rats

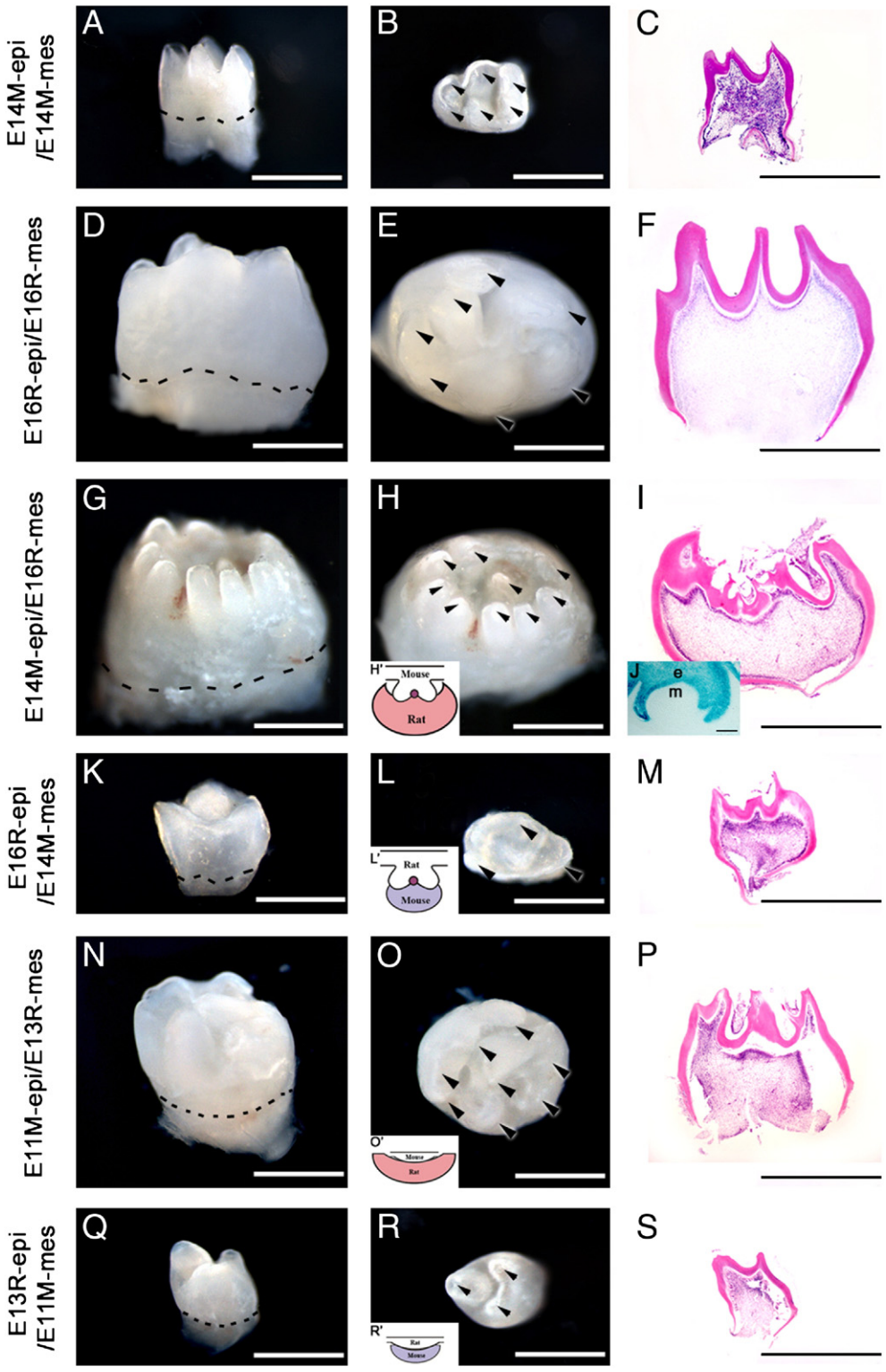
The calcified homospecific recombinant M1 of mice (N=9/9) (E14M-epi/E14M-mes, Figs. 2A–C) and rats (N=10/10) (E16R-epi/E16R-mes, Figs. 2D–F) had a similar tooth size and cusp size to their transplanted M1. The mesiodistal length (1.80±0.026 mm) and buccolingual length (1.14±0.078 mm) in the E16R-epi/E16R-mes (Figs. 2D, E) were about double the mesiodistal length (0.93±0.022 mm) and buccolingual length (0.53±0.027 mm) of the E14M-epi/E14M-mes (Figs. 2A, B). The intercuspal distance of the E16R-epi/E16R-mes (0.681±0.0073 mm) was about double that of the E14M-epi/E14M-mes (0.323±0.0052 mm). This result indicates that the recombination system can be used to study the tooth size and cusp size.

Tooth and cusp size of heterospecific recombinant M1 between mice and rats

The heterospecific recombinant tooth germs from cap stage formed calcified teeth. The size of the heterospecific recombinant M1 (N=25/25) (E14M-epi/E16R-mes, Figs. 2G–I) were larger than that of another kind of the heterospecific recombinant M1 (N=20/25) (E16R-epi/E14M-mes, Figs. 2K–M). The mesiodistal length (1.82±0.037 mm) and buccolingual length (1.32±0.044 mm) in the E14M-epi/E16R-mes (Figs. 2G, H) were approximately double the mesiodistal length (0.92±0.034 mm) and buccolingual length (0.52±0.022 mm) of the E16R-epi/E14M-mes (Figs. 2K, L).

Interestingly, the 14M-epi/E16R-mes showed a similar size to the E16R-epi/E16R-mes, whereas the E16R-epi/E14M-mes showed a similar size to the E14M-epi/E14M-mes. On the other hand, the intercuspal distance of the E16R-epi/E14M-mes (0.654±0.0047 mm) was roughly double that of the E14M-epi/E16R-mes (0.335±0.0084 mm). The cusp size of the E14M-epi/E16R-mes and E16R-epi/E14M-mes was similar to that of the E14M-epi/E14M-mes and E16R-epi/E16R-mes respectively.

The calcified teeth were also obtained from the heterospecific recombinant tooth germs of both E11M-epi/E13R-mes (N=11/18) and E13R-epi/E11M-mes (N=10/25). Most of the calcified E11M-epi/E13R-mes (N=9/11) (Figs. 2N–P) were larger in size than the E14M-epi/E14M-mes. However, all



E13R-epi/E11M-mes (N=10/10) (Figs. 2Q–S) were a little smaller in size than the E14M-epi/E14M-mes. The mesiodistal length (1.51±0.019 mm) and buccolingual length (1.27±0.027 mm) in the E11M-epi/E13R-mes were approximately double the mesiodistal length (0.89±0.026 mm) and buccolingual length (0.58±0.037 mm) of the E13R-epi/E11M-mes (0.369±0.0096 mm). Thus, the cusp size of the E11M-epi/E13R-mes was larger than that of the E13R-epi/E11M-mes, and the tooth size of E11M-epi/E13R-mes was much larger than that of the E13R-epi/E11M-mes. Taken together, these results indicate that the M-epi/R-mes tooth germs develop into the larger teeth with smaller cusps, whereas, the R-epi/M-mes tooth germs develop into the smaller teeth with larger cusps.

In addition, the heterospecific recombinant tooth germs between the *lacZ* transgenic Rosa26 mouse dental epithelium at E14 and rat dental mesenchyme at E16 showed blue color only in the epithelium after X-gal staining (Fig. 2J). This result indicates no mouse epithelial cell contamination in the rat mesenchyme.

Cusp number of various teeth from mice and rats

All the transplanted M1 and homospecific recombinant M1 had six or seven cusps as seen in adult mouse M1 as well as adult rat M1 (Figs. 1G, H, J, K and 2A, B, D, E). Surprisingly, among the heterospecific recombinant M1, the E14M-epi/E16R-mes had eight to eleven cusps (Figs. 2G, H), and the E11M-epi/E13R-mes had six to nine cusps (Figs. 2N, O). However, both the E16R-epi/E14M-mes and the E13R-epi/E11M-mes had only one to three cusps (Figs. 2K, L, Q and R).

Tooth size of heterospecific reaggregated M1 between mice and rats

The heterospecific reaggregated M1 (N=7/11) (E14M-epi/E16R-r-mes, Figs. 3A–C) showed much larger in size than that of another kind of the heterospecific reaggregated M1 (N=8/12) (E16R-epi/E14M-r-mes, Figs. 3E–G). The mesiodistal length (1.52±0.024 mm) and buccolingual length (0.79±0.025 mm) in the E14M-epi/E16R-r-mes (Figs. 3A, B) were longer than the mesiodistal length (0.85±0.036 mm) and buccolingual length (0.57±0.041 mm) in the E16R-epi/E14M-r-mes (Figs. 3E, F).

Though the cell number of the R-r-mes was same as that of the M-r-mes at the moment of heterospecific reaggregation, the mesenchymal cell number of the E14M-epi/E16R-r-mes tooth germs (Fig. 3D) was much higher than that of the E16R-epi/E14M-r-mes tooth germs after 48 h in culture (Fig. 3H).

Tooth size and number of reaggregated teeth in mice

The mouse reaggregated tooth germs containing 2.4×10^5 cells in the reaggregated mesenchyme, formed the calcified teeth (N=10/10) (E14M-epi/E14M-r-mes, Figs. 3I–K). Interestingly, four or five teeth were developed from each E14M-epi/E14M-r-mes tooth germ (Figs. 3L, M) (N=7/10). The largest tooth among teeth from each E14M-epi/E14M-r-mes tooth germ showed a similar size to that of the mouse transplanted M1.

Heterospecific recombinant teeth and their cusp patterning

The cusp patterning in the heterospecific recombinant tooth germs at the cap stage (E14M-epi/E16R-mes, E16R-epi/E14M-mes) was investigated by examining enamel knots patterning with mouse *Fgf4* (m*Fgf4*), an enamel knot marker (Jernvall et al., 1994). Five m*Fgf4*-expressing spots were detected in the tooth germs of both E16 mice and E18 rats (Figs. 4A, F). This result indicates that m*Fgf4* can be used as the marker of the secondary enamel knot not only in the mouse epithelium but also in the rat dental epithelium. The E14M-epi/E16R-mes tooth germs showed one, three, five and eight m*Fgf4*-expressing spots after culturing for 1, 2, 4 and 6 days respectively (Figs. 4B–E). On the other hand, the E16R-epi/E14M-mes tooth germs showed one, one, two and three m*Fgf4*-expressing spots after 1, 2, 4 and 6 days respectively (Figs. 4G–J). These results indicates that the different patterning of the putative cusps in two kinds of heterospecific recombinant M1 (Figs. 2G, K).

Discussion

Tooth size and cusp size are determined by the mesenchyme and epithelium respectively

Previous tissue re-association studies in tooth development suggested that tooth crown morphogenesis is controlled by the epithelium before the bud stage and by the mesenchyme after the bud stage (Kollar and Baird, 1970; Schmitt et al., 1999). This previous suggestion, which came from the homospecific recombination between the incisor and molar tooth germs, is

Fig. 2. Tooth size and cusp size of M1 in various recombinant teeth. (A–C) The mouse homospecific recombinant M1 (E14M-epi/E14M-mes) obtained from the mouse epithelium recombined with the mouse mesenchyme, have a similar tooth size and cusp size to those of the mouse transplanted M1. Six cusps are also detected. (D–F) The rat homospecific recombinant M1 (E16R-epi/E16R-mes) show a larger tooth size and cusp size than those of the E14M-epi/E14M-mes, but show the same number of cusps as the rat transplanted M1. (G–I) The heterospecific recombinant teeth (E14M-epi/E16R-mes) show a tooth size as large as that of the E16R-epi/E16R-mes, while 10 cusps as small as the cusp size of the E14M-epi/E14M-mes. (J) The E14M-epi/E16R-mes tooth germs between the dental epithelium of the *lacZ* transgenic mouse and dental mesenchyme of S-D rat show blue color only in the epithelium after 5 days in the renal subcapsular layer. (K–M) The heterospecific recombinant M1 (E16R-epi/E14M-mes) show smaller size than that of the E14M-epi/E14M-mes crown, while only three cusps as large as the cusp size of the E16R-epi/E16R-mes. (N–P) The heterospecific recombinant M1 (E11M-epi/E13R-mes) are much larger in size than that of the E14M-epi/E14M-mes. (Q–S) The heterospecific recombinant M1 (E13R-epi/E11M-mes) shows a similar size to that of the E14M-epi/E14M-mes. (H′, L′, O′ and R′) The diagrams indicate the heterospecific recombinations between epithelium and mesenchyme, which showed different size respectively between mice and rats. The crown and root of teeth can be recognized (dotted lines in panels A, D, G, K, N and Q). The cusp tips can be recognized (arrowheads in panels B, E, H, L, O and R). “e” and “m” indicate epithelium and mesenchyme. Scale bar: 100 μm in panel J; 1 mm in panels A–I, K–S.

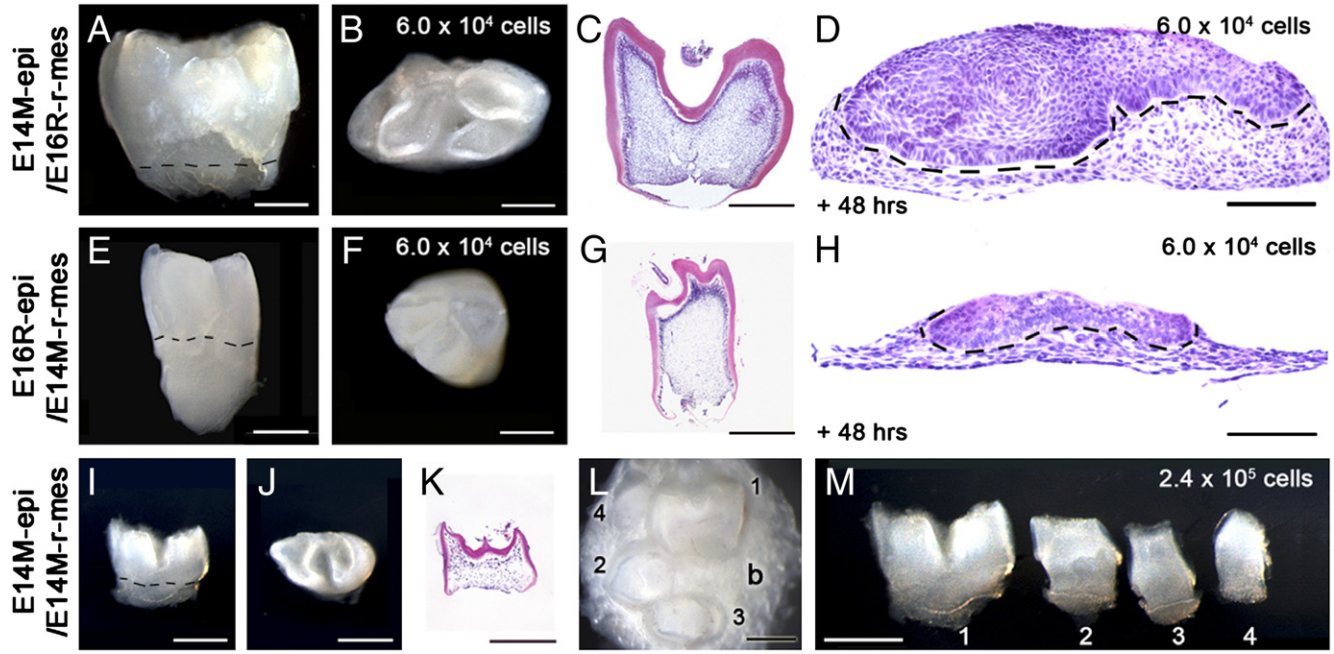


Fig. 3. The tooth size and the number of dental mesenchymal cells. (A–C) The heterospecific reaggregated M1 (M-epi/R-*r-mes*) are formed by overlapping the mouse dental epithelium on the rat *r*eaggregated *mes*enchyme containing 6.0×10^4 cells. (E–G) The heterospecific reaggregated M1 (R-epi/M-r-mes) are developed from the tooth germs between the rat epithelium and the mouse reaggregated mesenchyme containing 6.0×10^4 cells, showing a smaller crown than the M-epi/R-mes crown. (D, H) The M-epi/R-r-mes tooth germs (D) show larger mesenchyme (beneath the dotted line) after 48 h *in vitro* than the R-epi/M-r-mes tooth germs (H) did. (I–M) The mouse reaggregated teeth (M-epi/M-r-mes) are formed by overlapping one intact mouse epithelium on the mouse reaggregated mesenchyme containing 2.0×10^5 cells. The largest tooth shows a crown similar to that of the mouse transplanted M1. (L, M) Four teeth in the surrounding bone are detected from one M-epi/M-r-mes tooth germ with 2.4×10^5 cells in the reaggregated mesenchyme. The crown and root of teeth can be recognized (dotted lines in panels A, E and I). "b" indicates bone. Scale bar: 100 μm in panels D and H; 500 μm in panels A–C, E–G, and panels I–M.

concerning the tooth identity. However, our heterospecific recombination study at dental lamina and cap stages was carried out between the mouse and rat molar tooth germs. Our results are concerning not with the tooth identity but with the tooth size and the cusp size. The results show that the dental epithelium and mesenchyme play different roles during the tooth crown morphogenesis by revealing two main findings: 1) the mouse and rat dental mesenchyme memory has an intrinsic of their own final tooth size; 2) the mouse and rat dental epithelium has an intrinsic memory of their own final cusp size (Fig. 5).

It has been suggested that the patterning of the individual feather primordia in the skin corresponds to the micropatterning of skin (Sengel, 1976), and that the size of feather bud is determined by the mesenchyme (Jung et al., 1998; Jiang et al., 1999). It is well known that the early development of tooth bud is similar to that of the feather bud. Therefore, it has been suggested that the patterning of feather buds is similar to the patterning of cusp (Jernvall and Thesleff, 2000). We think that the patterning of the feather buds (micropatterning of skin) is similar to the patterning of tooth buds (macropatterning of tooth), and that the patterning of the barbs in the individual feather is similar to the patterning of cusp in the individual tooth (micropatterning of tooth). Our recombination data support this by showing that the tooth size is regulated by the mesenchyme as the size of feather bud. Whereas, the cusp size, which is one factor of cusp patterning, was determined by the epithelium only. This indicates that both activators and inhibitors in controlling the cusp size are determined by the epithelium. Furthermore, it is possible to think two different mechanisms between activators and inhibitors. One is that the activator and inhibitor are in the epithelium. The other is that the activator in the epithelium determines and regulates the inhibitor in the mesenchyme. However, these are remained to be elucidated.

Cusp number is co-regulated by the tooth size and cusp size

A reaction diffusion mechanism has been applied to analyze and predict the patterning of structures such as digits in limb, feathers in skin and cusps in tooth (Kondo, 1992; Jung et al., 1998; Jernvall, 2000; Jernvall and Thesleff, 2000; Salazar-Ciudad and Jernvall, 2002; Jiang et al., 2004). It was suggested that the number of digits in the mouse limb is related both with the width of the mesoderm and with the length of the *Fgf4*-expressing apical ectodermal ridge (Litingtung et al., 2002; te Welscher et al., 2002). Furthermore, digit number of chick limb is also known to be related with the width of the bud and the length of the apical ectodermal ridge (Lee and Tickle, 1985; Brickell and Tickle, 1989). In our study, the cusp number was dependent on the tooth size and cusp size. The cusp and tooth size may correspond to the wave length and organ width respectively in a reaction diffusion mechanism. With the same organ width, the longer the wave length was, the less the waves were (e.g. R-cusps/M-crown compared with M-cusps/M-crown in Fig. 5). While, with the same wave length, the longer the organ width was, the more the waves were (e.g. M-cusps/M-crown compared with M-cusps/R-crown in Fig. 5). Therefore, it

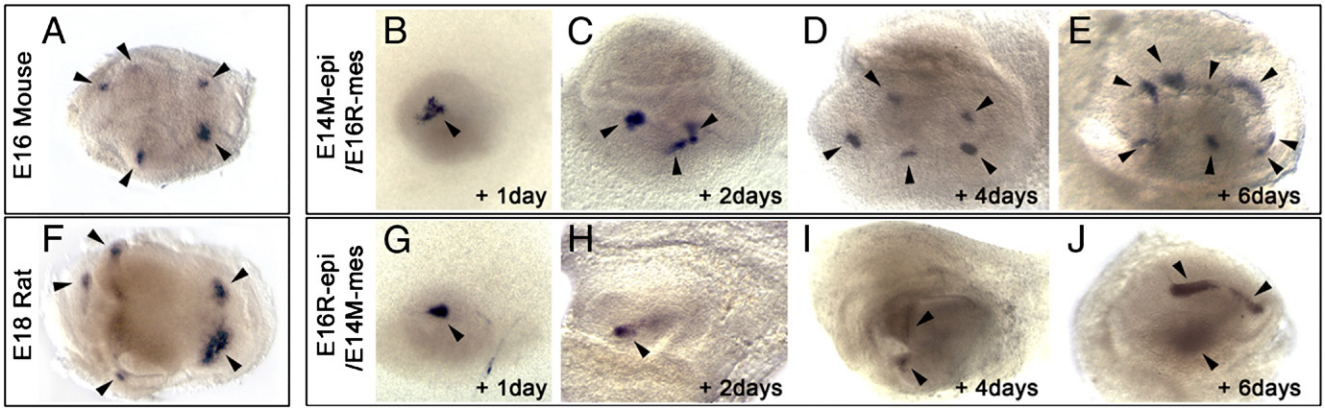


Fig. 4. *Fgf4* expression pattern in the heterospecific recombinant tooth germs. (A, F) Five spots of m*Fgf4* expression are detected in the E18 rat tooth germs (arrowheads in panel F), of which the m*Fgf4* expression pattern is similar to that of the E16 mouse tooth germs (arrowheads in panel A). (B–E) The E14M-epi/E16R-mes tooth germs show one, three, five and eight spots (arrowheads) of mouse *Fgf4* (m*Fgf4*) expression after culturing for 1, 2, 4 and 6 days *in vitro* respectively. (G–J) The E16R-epi/E14M-mes tooth germs show one, one, two and three spots (arrowheads) of m*Fgf4* expression after 1, 2, 4 and 6 days in culture respectively.

is suggested that the cusp size downregulates the cusp number, while the tooth size upregulates the number of cusp (Micro-patterning in Fig. 5). Multistructures, such as the digits, feathers, barbs, teeth, tooth cusps, show the periodic pattern in various region of body. This periodic pattern was represented as the wave-like pattern in the reaction diffusion mechanism. It has been suggested that the width of wave, where the concentration of the activator is higher than that of the inhibitor, symbolizes the size of multistructures and that the size of feather primordia could be changed by the artificial modification of the ratio of activators versus inhibitors (Jiang et al., 1999). In previous study on tooth, it was suggested that the secondary enamel knots was formed in the region where the activator wave is over the inhibitor wave (Jernvall and Jung, 2000). One interesting point in the reaction diffusion mechanism is that the periodic pattern may be composed of many wave units including the activator and inhibitor waves. The length of a wave unit may indicate the distance between multistructures. Therefore, we used the wave length as the indicator to analyze both tooth size and cusp size.

In feather, it was suggested that the ratio of the activator concentration versus the inhibitor concentration can be affected by the receptor number, intracellular signaling molecular concentration, extracellular ligand concentration, etc. (Jiang et al., 1999). Developmental regulations have been found in the periodic patterning in feather, in which it was suggested that the local activators are FGF4, SHH, and inhibitors are BMP2, BMP4 (Jung et al., 1998). Moreover, in tooth, it has also been suggested that the cusp patterning might be determined by the interaction of signaling molecules and that FGF4 might function as cusp activator, while BMPs and SHH could function as inhibitors regulating the distance between forming cusps (Jernvall and Thesleff, 2000). Furthermore, morphodynamic model based on the epithelia growth rate and an intrinsic rate of secreting activator was established. Candidate molecules for activators include BMPs and putative inhibitors include FGFs and SHH, antagonist of BMPs as well (Salazar-Ciudad and Jernvall, 2002). In this study, we tried to find out the roles of signaling molecules such as BMP4, FGF3, FGF10 and SHH in the developing tooth germs. However, no discernible differences could be found after exogenous treatment of BMP4, Noggin, FGF3, FGF10, SU5402, and 5E1 after 72 h *in vitro* (data not shown).

Tooth size is not affected by artificially changing dental mesenchymal cell number

It was reported that the size of an animal generally reflects cell number rather than cell size (Raff, 1996). Likewise, the size of an organ between different animals might reflect cell number rather than cell size. However, it was reported that artificially increasing the number of mesenchymal cells could not increase the size of feather primordia in skin (Jiang et al., 1999). Our results are consistent with these previous reports by showing 1) the bigger tooth contains more cells; 2) there is no effect on tooth size by increasing mesenchymal cell number. Therefore, it is suggested that tooth size may be regulated by the mesenchymal intrinsic factor. In addition, the same cell number of the R-r-mes and M-r-mes was adjusted artificially by reaggregation. However, the tooth size of the E14M-epi/E16R-r-mes was larger than that of the E16R-epi/E14M-r-mes. These results also indicate that the dental mesenchymal cells have a memory related with the final tooth size.

Number of teeth is regulated by mesenchymal cell number

Mice and rats have three molars and one incisor in each jaw quadrant. Moreover, it was reported that the mouse molar tooth germs produced M1 and M2, and three molars developed in some cases (Lumsden, 1979). This kind of tooth patterning in the mandible has been considered as the macropattening of teeth (Macropatterning in Fig. 5). In our study, artificially increasing dental mesenchymal cell number increased tooth number. This result is similar to a previous report on feathers, which suggested that an increase of the mesenchymal cell number increases the number of feather primordia (Jiang et al., 1999). Tooth number in macropatterning can be analyzed by a reaction

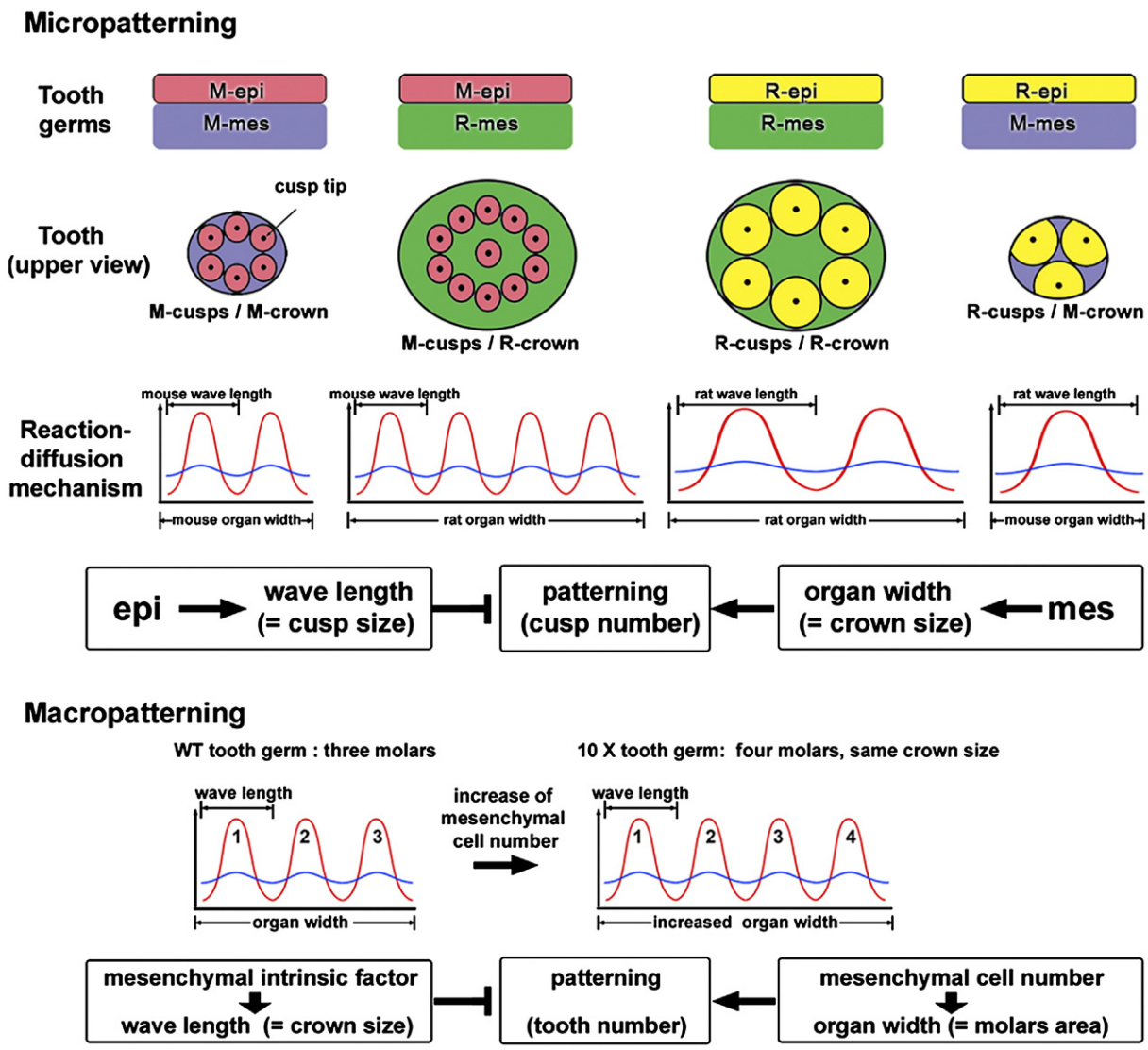


Fig. 5. Schemes of tooth patterning. *Micropatterning*: The mouse and rat dental epithelium forms the mouse cusp-sized cusp and the rat cusp-sized cusp respectively. The mouse and rat dental mesenchyme forms the mouse crown-sized crown and the rat crown-sized crown respectively. In terms of a reaction diffusion mechanism, the wave length, corresponding to cusp size, is shorter in mouse (mouse wave length) than in rat (rat wave length), and the organ width, corresponding to tooth size, is shorter in mouse (mouse organ width) than in rat (rat organ width). The wave length is same in M-epi/M-mes and M-epi/R-mes, while the organ width corresponding to the tooth size is larger in M-epi/R-mes (rat organ width) than M-epi/M-mes (mouse organ width). Under the same organ width, the longer the wave length is, the less the waves are (R-cusps/M-crown compared with M-cusps/M-crown). With the same wave length, the longer the organ width is, the more the waves are (M-cusps/M-crown compared with M-cusps/R-crown). Therefore, it is suggested that the cusp size downregulates the cusp number, while the tooth size upregulates the number of cusp. *Macropatterning*: While a wild type (WT) molar tooth germ at E14 forms three molars after 3 weeks in kidney capsule, the reaggregated tooth germ (10 × tooth germ), of which the mesenchymal cell number is 10 times as much as the cell number of a tooth germ, forms four molars without increase of tooth size after 3 weeks in kidney capsule. In macropatterning of teeth, the tooth size and molars area size correspond to the wave length and the organ width respectively. As the cell number increases, organ width increases but wave length does not increase. The number of waves corresponding to the tooth number is upregulated by the increased organ width. “M” and “R” indicate mouse and rat. “epi” and “mes” indicate epithelium and mesenchyme.

diffusion mechanism as well. The tooth size and dental mesenchyme size may correspond to the wave length and organ width in a reaction diffusion mechanism respectively. As the mesenchymal cell number was increased, organ width was increased, but wave length was maintained. Therefore, the number of waves corresponding to the tooth number is upregulated by the increased organ width.

It is well known that the cell proliferation is of fundamental importance in the organogenesis and that the cell proliferation increases the cell number, which determines the organ and body size. However, no discernable difference was detected in the PCNA (proliferating cell nuclear antigen) immunohistochemistry staining between two kinds of heterospecific recombinant tooth germs from cap stage (data not shown).

In conclusion, our results suggest that the tooth size and cusp size are determined by the dental mesenchyme and dental epithelium respectively and co-regulate cusp number by the epithelial–mesenchymal interactions. Tooth number as well as cusp number can be modeled as a reaction diffusion mechanism, in which the key molecules such as activators

and inhibitors may determine the micropatterning and macropatterning of teeth. It is necessary to elucidate these key molecules in order to adjust tooth size and cusp size in the future bioengineered teeth.

Acknowledgments

The authors acknowledge Prof. Cheryll Tickle for giving valuable advice and Prof. Yiping Chen for kindly providing the *Fgf4* probe. We also thank Dr. Sungwook Kwak for helpful discussions. This work was supported by grant No. R13-2003-13 from the Korea Science and Engineering Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.01.002.

References

Baba, T., Terashima, T., Oida, S., Sasaki, S., 1996. Determination of enamel protein synthesized by recombined mouse molar tooth germs in organ culture. Arch. Oral Biol. 41, 215–219.

Brickell, P.M., Tickle, C., 1989. Morphogens in chick limb development. BioEssays 11, 145–149.

Chen, Y., Zhang, Y., Jiang, T.X., Barlow, A.J., St. Amand, T.R., Yu, Y., Heaney, S., Francis-West, P., Chuong, C.M., Maas, R., 2000. Conservation of early odontogenic signaling pathways in Aves. Proc. Natl. Acad. Sci. U. S. A. 97, 10044–10049.

Jernvall, J., 2000. Linking development with generation of novelty in mammalian teeth. Proc. Natl. Acad. Sci. U. S. A. 97, 2641–2645.

Jernvall, J., Jung, H.S., 2000. Genotype, phenotype, and developmental biology of molar tooth characters. Am. J. Phys. Anthropol. 31, 171–190 [Suppl.].

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

Jernvall, J., Kettunen, P., Karavanova, I., Martin, L.B., Thesleff, I., 1994. 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. 38, 463–469.

Jernvall, J., Keranen, S.V., Thesleff, I., 2000. Evolutionary modification of development in mammalian teeth: quantifying gene expression patterns and topography. Proc. Natl. Acad. Sci. U. S. A. 97, 14444–14448.

Jiang, T.X., Jung, H.S., Widelitz, R.B., Chuong, C.M., 1999. Self-organization of periodic patterns by dissociated feather mesenchymal cells and the regulation of size, number and spacing of primordial. Development 126, 4997–5009.

Jiang, T.X., Widelitz, R.B., Shen, W.M., Will, P., Wu, D.Y., Lin, C.M., Jung, H.S., Chuong, C.M., 2004. Integument pattern formation involves genetic and epigenetic controls: feather arrays simulated by digital hormone models. Int. J. Dev. Biol. 48, 117–135.

Jung, H.S., Francis-West, P.H., Widelitz, R.B., Jiang, T.X., Ting-Berreth, S., Tickle, C., Wolpert, L., Chuong, C.M., 1998. Local inhibitory action of BMPs and their relationships with activators in feather formation: implications for periodic patterning. Dev. Biol. 196, 11–23.

Kim, J.Y., Mochizuki, T., Akita, K., Jung, H.S., 2003. Morphological evidence of the importance of epithelial tissue during mouse tongue development. Exp. Cell Res. 290, 217–226.

Kollar, E.J., Baird, G.R., 1970. Tissue interactions in embryonic mouse tooth germs: II. The inductive role of the dental papilla. J. Embryol. Exp. Morphol. 24, 173–186.

Kollar, E.J., Fisher, C., 1980. Tooth induction in chick epithelium: expression of quiescent genes for enamel synthesis. Science 207, 993–995.

Kondo, S., 1992. A mechanistic model for morphogenesis and regeneration of limbs and imaginal discs. Mech. Dev. 39, 161–170.

Lee, J., Tickle, C., 1985. Retinoic acid and pattern formation in the developing chick wing. SEM and quantitative studies of early effects on the apical ectodermal ridge and bud outgrowth. J. Embryol. Exp. Morphol. 90, 139–169.

Litingtung, Y., Dahn, R.D., Li, Y., Fallon, J.F., Chiang, C., 2002. Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. Nature 418, 979–983.

Lumsden, A.G., 1979. Pattern formation in the molar dentition of the mouse. J. Biol. Buccale 7, 77–103.

Mina, M., Kollar, E.J., 1987. The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. Arch. Oral Biol. 32, 123–127.

Mustonen, T., Tummers, M., Mikami, T., Itoh, N., Zhang, N., Gridley, T., Thesleff, I., 2002. Lunatic fringe, FGF, and BMP regulate the Notch pathway during epithelial morphogenesis of teeth. Dev. Biol. 248, 281–293.

Pispa, J., Thesleff, I., 2003. Mechanisms of ectodermal organogenesis. Dev. Biol. 262, 195–205.

Raff, M.C., 1996. Size control: the regulation of cell numbers in animal development. Cell 86, 173–175.

Salazar-Ciudad, I., Jernvall, J., 2002. A gene network model accounting for development and evolution of mammalian teeth. Proc. Natl. Acad. Sci. U. S. A. 99, 8116–8120.

Salazar-Ciudad, I., Jernvall, J., Newman, S.A., 2003. Mechanisms of pattern formation in development and evolution. Development 130, 2027–2037.

Sasaki, T., Ito, Y., Xu, X., Han, J., Bringas Jr., P., Maeda, T., Slavkin, H.C., Grosschedl, R., Chai, Y., 2005. LEF1 is a critical epithelial survival factor during tooth morphogenesis. Dev. Biol. 278, 130–143.

Schmitt, R., Lesot, H., Vonesch, J.L., Ruch, J.V., 1999. Mouse odontogenesis in vitro: the cap-stage mesenchyme controls individual molar crown morphogenesis. Int. J. Dev. Biol. 43, 255–260.

Sengel, P., 1976. Morphogenesis of skin. In: Abercrombie, M., Newth, D.R., Torrey, J.G. (Eds.), Developmental and Cell Biology Series. Cambridge Univ. Press, pp. 1–269.

te Welscher, P., Zuniga, A., Kuijper, S., Drenth, T., Goedemans, H.J., Meijlink, F., Zeller, R., 2002. Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. Science 298, 827–830.

Wang, Y.H., Upholt, W.B., Sharpe, P.T., Kollar, E.J., Mina, M., 1998. Odontogenic epithelium induces similar molecular responses in chick and mouse mandibular mesenchyme. Dev. Dyn. 213, 386–397.

Yamamoto, H., Kim, E.J., Cho, S.W., Jung, H.S., 2003. Analysis of tooth formation by reaggregated dental mesenchyme from mouse embryo. J. Electron. Microsc. 52, 559–566.

Yamamoto, H., Cho, S.W., Song, S.J., Hwang, H.J., Lee, M.J., Kim, J.Y., Jung, H.S., 2005. Characteristic tissue interaction of the diastema region in mice. Arch. Oral Biol. 50, 189–198.