Analysis of Osteogenic Property in Dental Follicle Cells during Mouse Molar Development

HEUI-JUNG HWANG

Department of Medical Science

The Graduate School, Yonsei University

Analysis of Osteogenic Property in Dental Follicle Cells during Mouse Molar Development

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 requirement for the degree of master of Medical Science

HEUI-JUNG HWANG

December 2004

This certifies that the Master's Thesis of HEUI-JUNG HWANG is approved.

_____

HAN-SUNG JUNG

_____

YUN-JUNG YOO

_____

HYOUNG-WOO PARK

The Graduate School

Yonsei University

December 2004

ACKNOWLEDGMENTS

I would like to thank Professor Han-Sung Jung for invaluable advice and guidance.

My thanks also go to Professor Hee-Jin Kim, Dr. Hitoshi Yamamoto at Nihon University for help me on several experiments.

It is great pleasure to thank Dr. Jae-Young Kim, Sung-Won Cho, Jinglei Cai, Jong-Min Lee, Min-Jung Lee, Kyoung-Won Cho, Hyun-A Lee, Kyung-Seok Hu, Hyun-Do Park, Hyun-Ho Kwak for letting me collaborate on several experiments.

I am grateful to many people from the department of Oral Biology, College of Dentistry, Yonsei University, who made my MS. course enjoyable. Ju-Youn, Soo-Young, Hae, Hyun-Joo, Sun-Young, Du-Sik, Jeong Hee, Su-Hyun.

Special thanks to Professor Syng-Il Lee, Professor Yun-Jung Yoo for encouragement.

It is also great pleasure to thank E-Soon Kwon, Jin-il Kwon, Kyung-Ran Park.

Finally, special thanks go to my family for supporting me always.

Heui-Jung Hwang

TABLE OF CONTENTS

LIST OF FIGURES

Abstract

Analysis of Osteogenic Property in Dental Follicle Cells during Mouse Molar Development

HEUI-JUNG HWANG

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor HAN-SUNG JUNG)

In mice, dental mesenchymal cells comprise the dental papilla and dental follicle in the developing mammalian tooth bud. It is thought that dental follicle cells have the ability to differentiate into fibroblasts, cementoblasts, and osteoblasts. However, cellular differentiation and the effects of environmental factors are not known exactly.

In this study, the expression of BSP and OPN, using immunohistochemical markers for hard tissue, was not detected at E14 dental follicle, and positive BSP and OPN reactions were observed in the alveolar bone area at PN8. Despite the lack of hard tissue formation in the E14 dental follicles, dental follicle cells showed the potential to form hard tissue in the following experiment. In order to characterize this potential of dental follicle cells *in vitro*, E14 dental follicle cells were separated into single cells, then cultured. We assessed the expression of both *Runx2* and *Bsp* in the aggregated cells,

and determined to the ability of the cells to differentiate into osteoblasts and their potential to form bone. Furthermore, after the addition of bone inducers into the cultured dental follicle cells, including developing calvaria and BMP4, *Runx2* and *Bsp* expressed in the aggregated cells showed the effects of environmental factors on differentiation into osteoblasts. These results suggest that dental follicle cells have the ability to differentiate into osteoblasts as the result of interaction with environmental factors.

Key words: tooth development, dental follicle cells, oseteoblast, differentiation, environmental factor

Analysis of Osteogenic Property in Dental Follicle Cells during Mouse Molar Development

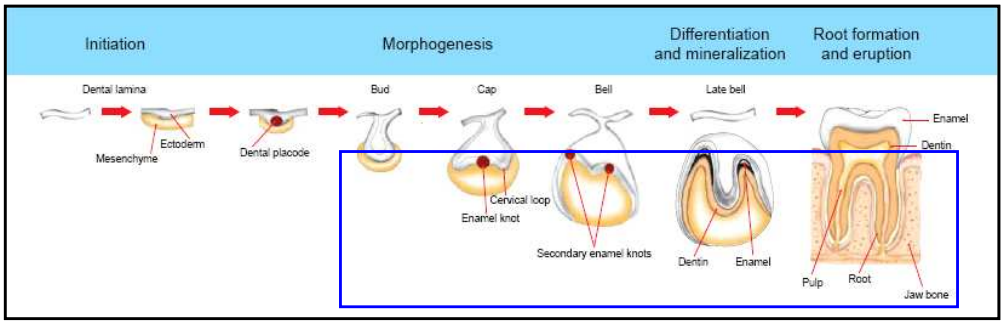
HEUI-JUNG HWANG

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor HAN-SUNG JUNG)

I. INTRODUCTION

Early tooth development resembles, both morphologically and in terms of the relevant molecules, the development of other ectodermal appendages such as hair and glands. Interactions between the ectoderm and the underlying mesenchyme constitute a central mechanism which regulates the morphogenesis of all such organs.[1] During the bud stage (Between Embryonic day E9.0 and E11.5) in mouse, the dental epithelium initiates tooth development, and the first morphological evidence of tooth development is the appearance of the dental lamina. At cap stage (E13.5), the epithelium has formed a bud, and the mesenchyme begins to condense. At E14, the structure of the developing tooth is characterized by the enamel organ, dental papilla, and dental follicle. At bell stage (E15.5), tooth shape is determined by epithelial folding. The dentin and enamel form the odontoblast

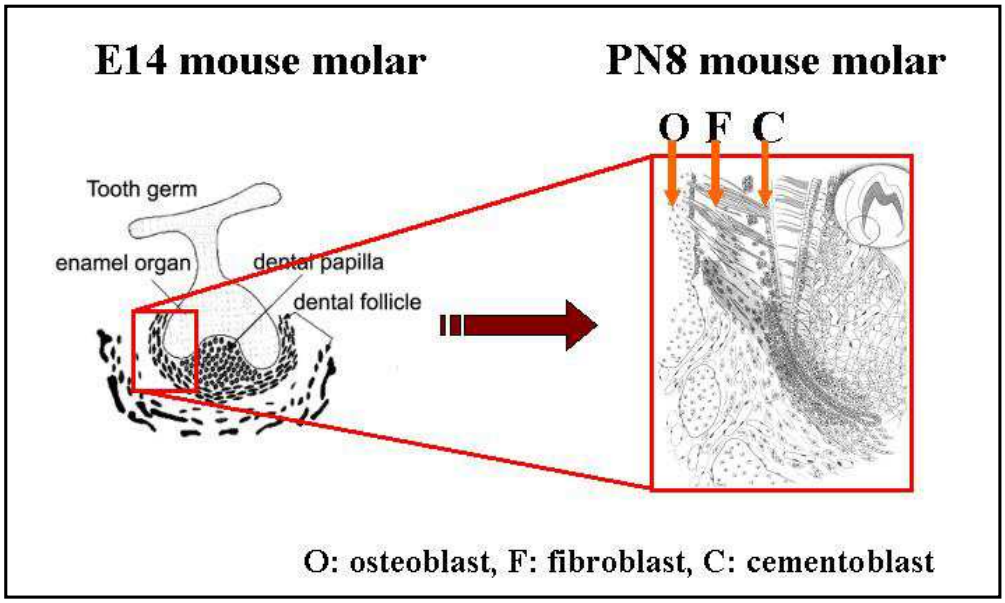


Journal of Cell Science 116, 1647-1648, 2003

Figure 1. Schematic diagram of tooth development. Tooth development begins with initiation and morphogenesis, followed by differentiation and mineralization. After the completion of crown formation, roots develop, and the tooth erupts into the oral cavity. The blue box shows the specific region for differentiation mechanism in control.

and ameloblast, respectively.[2,3] The dental papilla differentiates into dentin and pulp, while the dental follicle differentiates into the cementum, periodontal ligament, and alveolar bone.[4-6] The effects of various growth factors on tooth development have been studied in mouse embryonic tooth germs. Previously, the promotion of tooth morphogenesis and dental follicle cell differentiation were thought to be predicated on the stimulation of cell proliferation.[7] Epithelial-mesenchymal tissue interactions, which are ostensibly mediated by extracellular matrix molecules, constitute important regulators of tooth morphogenesis and differentiation.[8] It is generally understood that the genes which are induced or expressed in developing tissues can normally be considered good markers for cell determination and differentiation. Bone sialoprotein and osteopontin are major non-collagenous proteins in bone and other mineralized connective tissues, such as dentin, cementum, and calcified cartilage tissue.[9-13] Both bone sialoprotein and osteopontin are prominent bone-matrix proteins, and are associated with the formation and remodelling of the mineralized tissue matrix.[14]

Recent studies have demonstrated that bone sialoprotein mRNA is expressed almost exclusively in differentiated osteoblasts, odontoblasts, and cementoblasts.[12,14,15] The expression of bone sialoprotein, osteopontin and alkaline phosphatase in cells may reflect a specific trend toward osteoblastic differentiation. Alkaline phosphatase is also expressed constitutively by bone-forming cells, as well as some periodontal ligament cells, and is recognized as an enzyme marker for bone differentiation.[16-18] *Runx2*, a member of the RUNX family of transcription factors, exhibits a highly



Periodontology, 24, 9-27, 2000

Figure 2. Schematic diagram of dental follicle development from E14 to PN8 in mouse embryos. At E14, cap stage, dental follicle structure was observed around the tooth germ. Enamel organ, dental papilla, and dental follicle were examined with regard to morphological structure. Dental follicles differentiate into fibroblasts, osteoblasts, and cementoblasts at PN8 (Ten Cate *et al*, 1971). Fibroblasts, osteoblasts, and cementoblasts then differentiate into alveolar bone, periodontal ligament, and cementum, respectively.

restricted tissue expression pattern in bone. *Runx2* has been demonstrated to regulate several osteoblast-specific genes.[19-21] *Runx2* is a runt domain transcription factor that is essential for bone development and tooth morphogenesis.[22] Growth and differentiation factors (GDF) 5, 6, and 7 are members of the bone morphogenetic protein (BMP) family, which comprises a part of transforming growth factor (TGF-) superfamily.[23] GDF 5, 6, and 7 are known to play in the formation of tendon and ligament formation and are therefore probably involved in the formation of periodontal ligament. GDF gene expression in the periodontal ligament was first detected in cells associated with the initial process of periodontal ligament fiber bundle formation.[24] The developmental potency of the dental follicle has been studied in a variety of tooth transplantation experiments. However, the effects of environmental factors on cellular differentiation have yet to be elucidated. Therefore, there are many intriguing facets of this subject to be further explored at the tissue, cell and molecular levels.

In this study, the osteogenic property of dental follicle cells was examined the expression of several osteoblast differentiation. To confirm osteoblast differentiation during initial molar development in mouse, using the specific bone-forming markers BSP and OPN, this study sought to characterize the differentiation of both the mandibular bone and the dental follicle. In addition, we attempted to analyse this potential in cultured dental follicle cells *in vitro*. At E14, dental follicle cells were showed ability to differentiate into osteoblasts, as well as the ability to differentiate into alveolar bone. These activities were monitored according to the expression

of *Runx2* and *Bsp* gene markers in aggregated cells, using cell *in situ* hybridization. Our results indicate that dental follicle cells have the potential to differentiate into osteoblasts via direct interaction with environmental factors.

II. MATERIALS AND METHODS

1. Animals

Adult ICR mice were housed in a temperature-controlled room (22±1℃) under artificial illumination (lights on from 05:00 to 17:00), at 55% relative humidity, with free access to food and water. Mouse embryos were obtained from time-mated pregnant mice. The day on which a vaginal plug was confirmed was designated as embryonic day 0 (E0).

2. Histology

Samples taken from mice at days E14, E16, and E18 of embryonic development, and PN2, PN5, PN8, PN11 of the post-natal period, were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4℃, then embedded in paraffin. Specimens were cut to a thickness of 7 ㎛, and sections were then stained with both hematoxylin and eosin (H&E).

3. Immunohistochemistry

In order to conduct immunohistochemistry, the specimens were fixed with 4% PFA at 4℃ and embedded in paraffin. Specimens were cut to a thickness of 7 ㎛. The tissue sections were then deparaffinized and rehydrated. In order to nonspecific background staining due to endogenous

peroxidase, slides were incubated in hydrogen peroxide blocks for 15 minutes. The specimens were then washed twice in buffer. When required, the tissues were incubated in digestive enzymes. The specimens were then washed an additional 4 times in buffer. Apply ultra v blick and incubated for 60-70 minutes at room temperature in order to block reactive immunoglobulins. The samples were washed an additional 4 times in buffer. The specimens were then incubated for 10-15 minutes at room temperature with biotinylated goat anti-mouse antibody, then washed4 times in buffer. The washed samples were incubated for a further 10-15 minutes at room temperature with streptavidin peroxidase, and rinsed 4 times in buffer. 1-2 drops DAB chromogen was then added to 1 ml of DAB substrate, mixed by swirling, and applied to tissue. The tissues were then incubated for an additional 5-15 minutes, according to the desired stain intensity, and finally counter-stained and coverslipped using a permanent mounting medium.

4. Kidney Transplantation

After 2 hours of incubation in a 37℃ incubator, the four particles of divided dental follicle cells were carefully separated from the filter. Using a male adult mouse as the host, the dental follicle cells were transplanted into the kidney for 3 weeks. This *in vivo* culture method can result in the formation of fully-calcified bone.

5. Cell culture

In order to culture the dental follicle cells, mouse dental follicles were isolated from the first mandibular molars of E14 mice, then separated into single cells. The dental follicle cells were then cultured in DMEM (Dulbecco's minimum essential medium) containing 10% fetal bovine serum at 37℃ in a humidified atmosphere containing 5% CO_2. In general, dental follicle cells were suitable for cell attachment onto 4-well Petri dishes(SPL labware, Germany) after 3 days in culture.

6. Di.I. application and Fate Mapping

Di.I (1,19-dioctadecyl-3,3,39,39-tetramethyl indocar-bocyanine perchlorate; Molecular Probes, Eugene, OR) was used as a cell tracer in the observation of cell migration during mouse molar development. A 0.3% w/v Di.I in DMSO (dimethyl sulfoxide) was washed for microinjection. The Di.I injection was performed using 10 cm borosilicate capillary pipettes (Sutter Instruments, BF120-94-10), pulled with a Sutter Instrument Flaming Brown micropipette puller, filled by capillary action. Using an electrical device, the lipophilic carbocyanine dye was introduced to the cell membranes adjacent to the injection site. The exact position of the dye could be determined using a fluorescent microscope (LEICA, MZ FL III). Injections were directed into the dental follicle.

7. Beads implantation

Affigel Blue beads (BioRad) with 150 ㎛-diameters were dried, then soaked in 0.5 mg/ ml of human recombinant NOGGIN (Regeneron). In order to determine levels of osteogenic activity, single NOGGIN-soaked beads were implanted into cultured dental follicles at E14. The cultured dental follicle cells were used after bead implantation.

8. Cell *in situ* hybridization

Dental follicle cells in culture were then fixed in 4% PFA, and washed three times in DEPC (dethyl pyrocarbonate)-PBS (phosphate buffered saline). To 25 ml 0.1 M triethanolamine, pH 8.0, 62.5 ㎕ acetic anhydride was added, and quickly mixed until completely dispersed. The culture was incubated in this mixture for 10 minutes at room temperature. The cultures were then washed in 1 x SSC for 5 minutes, and treated with 0.2 M HCl in DEPC-water for 10 minutes, and washed twice in DEPC-PBS (dethyl pyrocarbonate-phosphate buffered saline) for 5 minutes. Pre-hybridization solution was added, and this mixture was incubated for 6 hours at room temperature. The pre-hybridization solution was removed, and probes were added at a final concentration of between 1 and 2㎍/㎖. This was allowed to hybridize overnight at 60℃. The next day, the cultures were rinsed in 0.2 x SSC, and washed in 0.2 x SSC at 60℃ for 1 hour. The cultures were adjusted to room temperature in 0.2 x SSC for 5 minutes, then

blocked in 20% sheep serum in PBT for at least one hour at room temperature. The culture was then incubated overnight with anti-digoxygenin antibody (coupled to alkaline phosphatase) diluted to a final concentration of 1:1000 in 20% sheep serum in PBT, and rinsed three times in PBT. The cultures were washed four times in PBT for 10 minutes, and then washed twice in alkaline phosphatase buffer at room temperature for 10 minutes. For every 10 ml of alkaline phosphatase buffer used, 4.5 ul of NBT and 3.5 ul of BCIP was added, and developed in the dark, the duration of which depended on the abundance of the RNA. When the reaction had proceeded far enough, the sample was washed in PBT, and ultimately fixed in 4% formamide.

III. RESULTS

1. Morphological findings

In order to understand the precise development of the dental follicle in mouse first molar tooth development, frontal wax sections of the E14 to PN11 mouse teeth were stained with hematoxylin and eosin. At E14 (cap stage), the structure of tooth was observed to consist of the enamel organ, dental papilla, and dental follicle (Fig. 3-A). At E16 (late cap stage), the mandibular bone manifested around the dental follicle (Fig. 3-B). At E18 (bell stage), the mandibular bone was observed near the dental follicle (Fig. 3-C). Dental follicle cells thinned at PN2 (Fig. 3-D). At PN5, between the dentin and mandibular bone, a cell layer developed (Fig. 3-E). At PN8, this cell layer appeared as a thick line (Fig. 3-F). At PN11 and PN14, we observed periodontal ligament- like and alveolar bone tissue (Fig. 3-G).

2. Ossification of the mandibular bone by immunohistochemistry

In order to determine the relationship between morphological changes in the bone and the expression patterns of bone sialoprotein (BSP) and osteopontin (OPN). BSP and OPN expression pattern during the formation of the developing mandibular bone in the mouse first molar from E14 to PN11 were examined immunohistochemistry after frontal section. BSP and OPN are expressed in bone and other mineralized connective

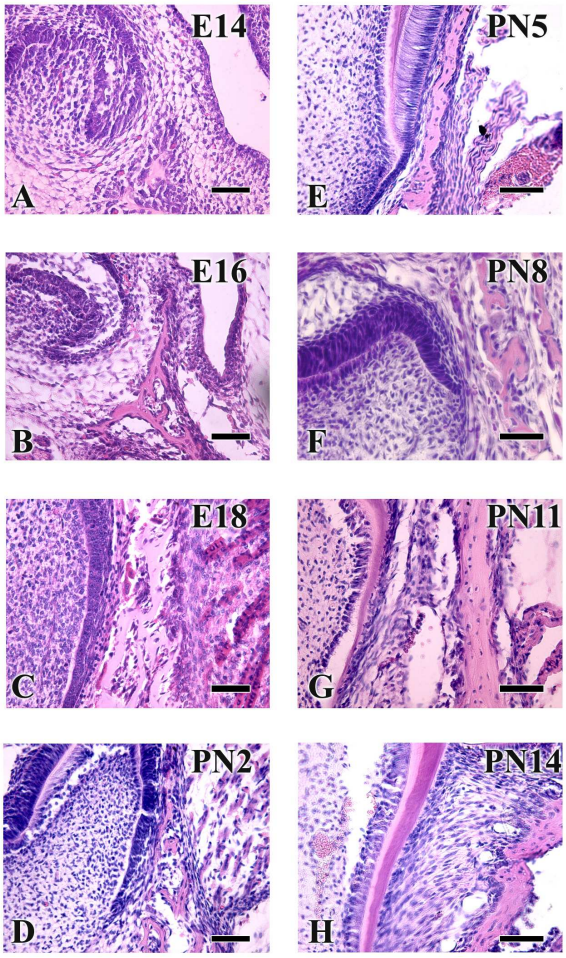


Figure 3. Histology of tooth morphogenesis of mouse first molar. A, B, C: Embryonic day 14 (E14) day cap stage frontal sections (A) E16 early bell stage frontal sections (B), E18 bell stage frontal sections (C). D, E, F, G, H: Postnatal (PN) stage frontal sections. PN2 stage frontal sections (D), PN5 stage frontal section (E), PN8 stage frontal sections (F), PN11-14 stage frontal sections (G-H). (Scale bar: (A-H) 50 ㎛)

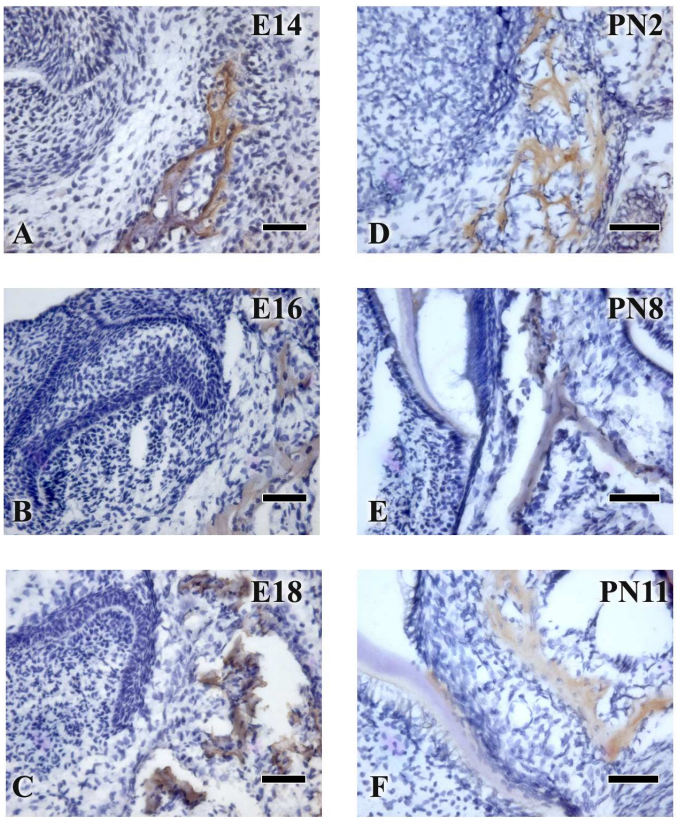


Figure 4. The immunohistochemical expression of bone sialoprotein (BSP) in the developing alveolar bone at E14, E16, E18, PN2, PN8 and PN11. To determine osteogenic activities, we examined the expression of several osteoblast differentiation-specific markers including BSP, using immunohistochemistry. The majority of bone-like tissue stained positive for BSP. At E14 (A),BSP was expressed on the buccal side of the tooth germ. At E16 (B), BSP was expressed on both the lingual and buccal sides of the tooth germ. At E18 (C), BSP was expressed around the entirety of the tooth germ. At PN2, BSP was expressed adjacent to the dentin (D). At PN8, BSP was expressed adjacent to the dentin and root dentin (E). At PN11, BSP was expressed around the periodontal ligament (F). (Scale bar: (A-F) 50 ㎛)

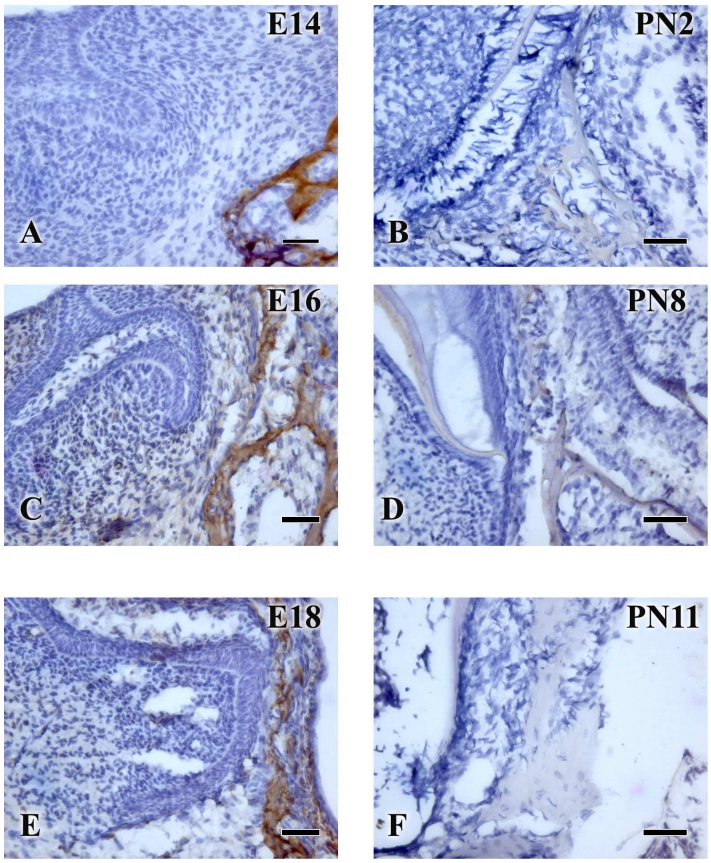


Figure 5. The immunohistochemical expression of osetopontin (OPN) in the developing alveolar bone at E14, E16, E18, PN2, PN8 and PN11. To determine osteogenic activities, we examined the expression of several osteoblast differentiation-specific markers OPN, using immunohistochemistry. The majority of bone-like tissue stained positive for OPN. At E14 (A), OPN was expressed on the buccal side of the tooth germ. At E16 (B), OPN was expressed on both the lingual and buccal sides of the tooth germ. At E18 (C), OPN was expressed around the entirety of the tooth germ. At PN2, BSP was expressed adjacent to the dentin (D). At PN8, OPN was expressed adjacent to the dentin and root dentin (E). At PN11, OPN was expressed around the periodontal ligament (F). (Scale bar: (A-F) 50 ㎛)

tissues. In general, the patterns of expression of BSP and OPN were similar. BSP and OPN are known markers for osteoblasts, osteocytes and bone. At E14, BSP and OPN were expressed on the buccal side of the tooth germ (Fig. 4, 5-A). At E16, BSP and OPN were expressed on both the lingual and buccal side of the tooth germ (Fig. 4, 5-B). At E18, BSP and OPN were expressed around whole the tooth germ (Fig. 4, 5-C). At PN2, BSP and OPN were expressed adjacent to the dentin (Fig. 4, 5-D). At PN8, BSP and OPN were expressed adjacent to both the dentin and root dentin (Fig. 4, 5-E). At PN11, BSP and OPN were expressed around the entirety of the periodontal ligament (Fig. 4, 5-F).

3. Kidney transplantation

To examine differentiation potential of the dental follicle cells, E14 mesenchymal cells were used in kidney transplantation for *in vitro* culture. After micro-dissecting the tooth germ in an E14 mouse mandible, the tooth germ was separated into the epithelium and mesenchyme. The remaining mesenchymal cells were separated into lingual side, buccal side, dental papilla and dental papilla with dental follicle, then transplanted into a kidney capsule for 3 weeks. The lingual side cells, buccal side cells, dental papilla cells, and dental papilla cells with dental follicle cells were calcified into bone tissue (Figs. 6). Prior to E14, dental mesenchymal cells exhibit osteogenic properties. As a result, dental mesenchymal cells maintain the potential for differentiation at E14.

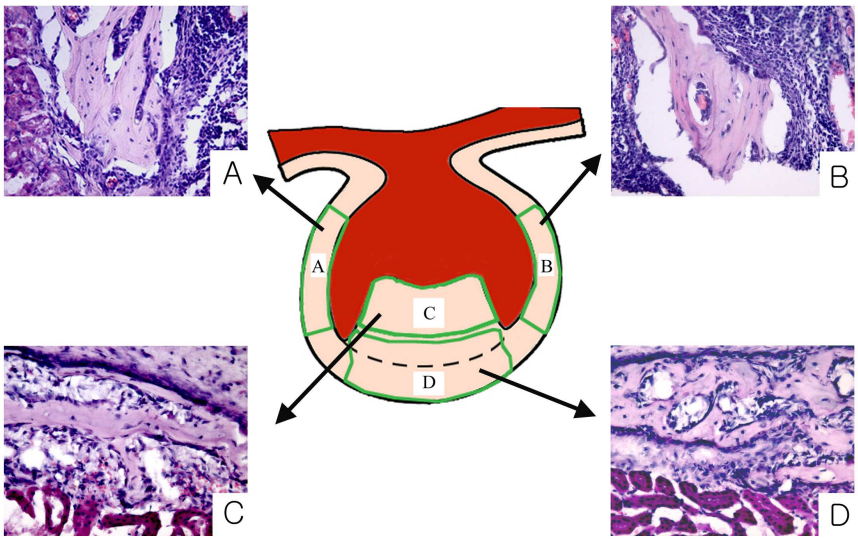


Figure 6. Differentiation potential of the dental follicle cells at E14 *in vivo*. Calcified tissues were obtained after the transplantation of dental follicle cells into a kidney capsule. A: lingual side, B: buccal side, C: dental papilla, D: dental papilla + dental follicle.

4. Cell culture

Mouse dental follicles were isolated from the first mandibular molar at E14. In order to conduct the *in vitro* culture, the E14 tooth germs were micro-dissected using a tungsten needle. The tooth germ was separated into the epithelium and the mesenchyme, and then the epithelium and dental papilla were removed. After the epithelium was separated, we carefully dissected out the dental papilla.

5. Alkaline phosphatase activity

In order to characterize bone differentiation, we assessed alkaline phosphatase activity. 3 days (Fig. 8-A) and 7days (Fig. 8-B) after the inception of cell culture, the cultures were stained for alkaline phosphatase activity. Alkaline phosphatase activity is recognized as an enzyme marker for bone differentiation. After 3 days (C) and 7 days (D), the culture specimens exhibited increased alkaline phosphatase activity.

6. Gene expression of cultured dental follicle cells

In order to determine the expression patterns of signalling molecules, E14 dental follicles were used to conduct another cell culture experiment. After 3 days and 7 days of cell culture, *in situ* hybridization was

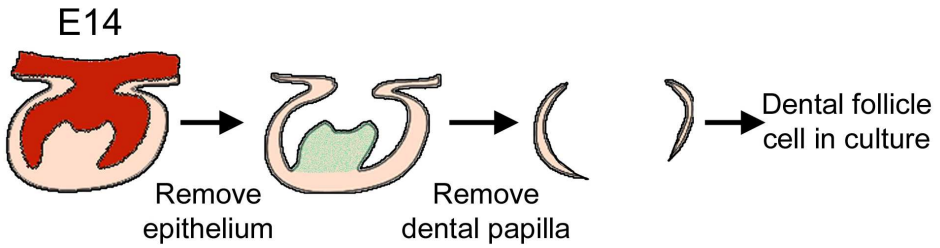


Fig 7. Dental follicle cells in culture at E14. For the culture of dental follicle cells, mouse dental follicles were isolated from the first mandibular molars of mice at E14, and then made into single cells. The dental follicle cells were cultured in Dulbecco's minimum essential medium, containing 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2.

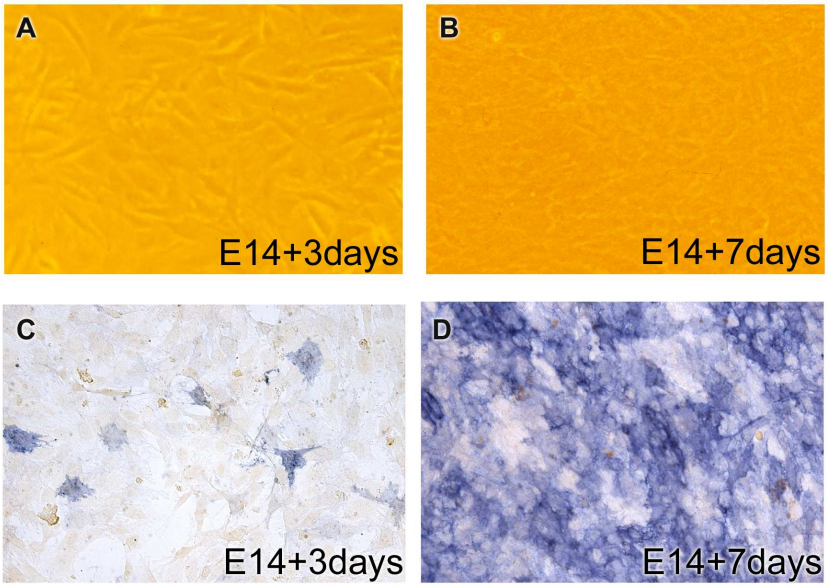


Figure 8. Dental follicle cells in culture at E14 after 3 (A) and 7 days (B). Alkaline phosphatase activity in cultured dental follicle cells at E14 for 3 days (C) and 7 days (D). Cells at 7 days in culture showed higher alkaline phosphatase activity than did cells at 3 days in culture.

carried out with the cultured cells. *Runx2* and *Bsp* expression were detected in aggregated dental follicle cells (Fig. 9-A, B). These expression patterns were higher than in the 3 day aggregated dental follicle cells.

7. Bead and bone tissue implantation

In order to characterize the potential differentiation of dental follicle cells, BMP4-soaked beads and developing calvaria (E14) were implanted on a cultured dental follicle dish. PBS beads were implanted as a control. After bead implantation, *Bsp* and *Runx2* expression patterns were examined using cell *in situ* hybridization. After BMP4 bead implantation, *Bsp* and *Runx2* expression were found to be stronger around the bead-implanted dish (Fig. 10). In the developing calvaria sample, *Bsp* and *Runx2* expressions were detected after 3 weeks (Fig. 11). In general, both *Bsp* and *Runx2* were expressed in all samples, indicating that osteogenic potential could be induced by surrounding factors.

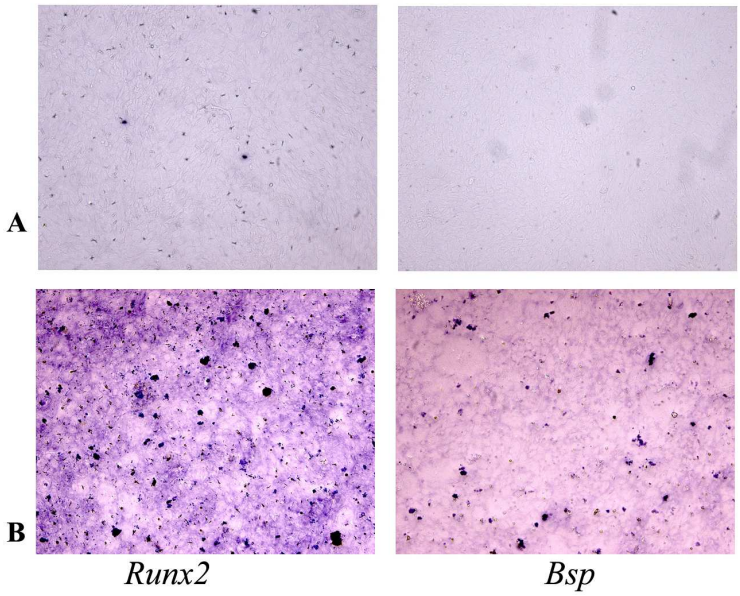
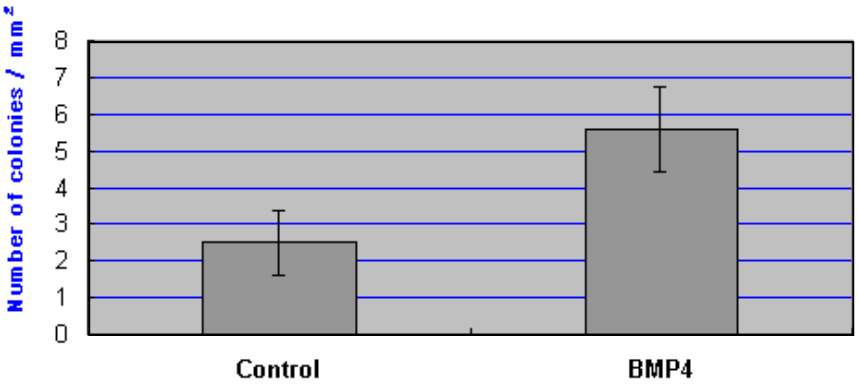
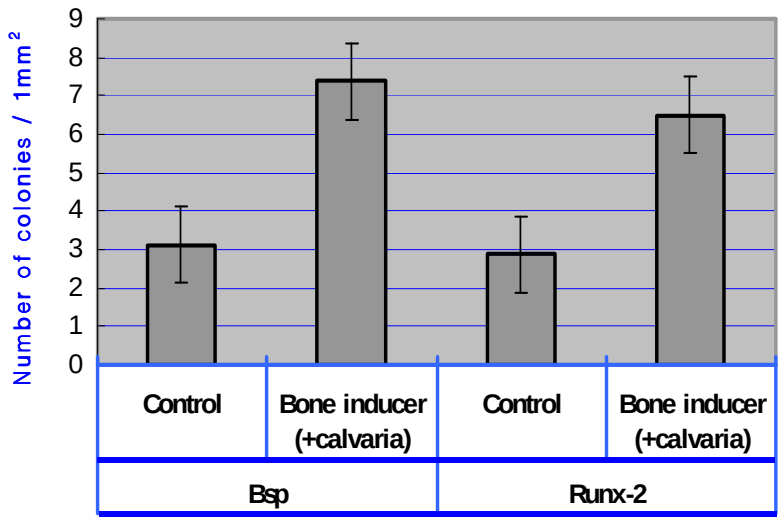


Fig 9. Gene expression pattern after dental follicle cells were cultured for 3 days (A) and 7 days (B) at E14. *Runx2* and *Bsp*, expression patterns were observed after *in situ* hybridization on cultured cells. These expression patterns appeared in aggregated cells at 7 days. Gene expression at 7 days (B) was higher than at 3 days.



	Control	BMP4
MEAN	2.5	5.58
(±) S.D.	0.89	1.17

Fig 10. Number of *Bsp*-expressed colonies were determined in aggregated cells 48 hours after implantation of BMP4 protein (100 ㎍/㎖)-soaked beads. After BMP4 bead implantation, *Runx2* and *Bsp* expression stronger near the bead-implanted dish.



	Bsp		*Runx-2*	
	Control	**Bone inducer (+calvaria)**	**Control**	**Bone inducer (+calvaria)**
MEAN	**3.13**	**7.38**	**2.88**	**6.5**
(±) S.D.	**0.83**	**1.6**	**1.25**	**0.93**

Figure 11. Number of colonies expressing *Runx-2* and *Bsp* were counted in aggregated cells, which were cultured with bone tissue for 3 weeks. After bone tissue implantation, signalling molecules levels were examined using cell *in situ* hybridization. In the culture dishes with bone tissue, *Runx-2* and *Bsp* expression were detected in more aggregated colonies.

DISCUSSION

1. Differentiation of dental follicle cells

These spacing patterns of teeth are developed by epithelium-mesenchyme interactions, and the early development of the tooth involves the development of other epithelial appendages, such as feather buds and mammary glands.[25,26] Mesenchymal cells in the developing tooth, so-called dental mesenchymal cells, are derived from two different origins: the cranial neural crest (CNC) and the non-CNC.[27] CNC-derived cells migrate, proliferate and ultimately differentiate into odontoblasts, cementoblasts, fibroblasts, osteoblasts and chondroblasts, etc.[28-31] Tooth bud formation is one of the best examples of asynchronous development. From the dental lamina to the individual tooth, each tooth establishes its own identity during development in its proper position in relation to both the maxilla and the mandible. At E18 (late bell stage), the dental follicle surrounds each tooth germ, which is located between each tooth germ and its bony compartment. At PN11, tooth structure have already shown dentin, enamel, cementum, alveolar bone, and periodontal ligaments. In addition, immunohistochemistry for osteoblast was examined to study the differentiation of the mandibular bone. BSP and OPN are major non-collagenous proteins occurring in bone and other mineralized connective tissues.[9-13] Both BSP and OPN are prominent bone-matrix proteins that are related to function in the formation and remodeling of the mineralized tissue matrix.[14] In order to characterize distance changes during tooth formation, we assessed the expression of the specific bone forming markers, BSP and OPN. The expression patterns of BSP and OPN

expression were examined by using immunostaining (Figs. 4 and 5). During embryonic development, distance changes in the dental follicle and mandibular bones narrowed, as the tooth germ and mandibular bone grew. At PN2, dental follicle cells and alveolar bone were almost attached. At PN5, the dentin and alveolar bone begin to separate. Dental follicle cells, however, could not be used to precisely determine differentiation time during initial molar development in mice. In the case of H-E staining, dental follicle cells did not shown for differentiation that how the cells differentiate during tooth development. However, as a result, it seems that dental follicle cell begin to differentiate first, as the result of interaction between the dental follicle and mandibular bone.

2. Potential of dental mesenchymal cells

In order to determine the differentiation potential of dental mesenchymal cells at E14 *in vitro*, mesenchymal cells were transplanted into a kidney capsule for 3 weeks (Fig 6). After 3 weeks, fully calcified tissues were obtained. This indicates that calcified mesenchymal cells were have differentiation potential of dental mesenchymal cells at E14 *in vitro.* In order to determine the differentiative potential of dental follicle cells during mouse initial molar development, we cultured dental follicle cells at E14 (Fig 7). At E14, the distance between the mandible and the tooth germ was largest during embryogenesis, and thus the differentiation of dental follicle cells could be clearly observed. In the construction of the *in vitro* culture, the E14 tooth germs were initially microdissected using a tungsten needle. The dental follicle cells, after 3 days of culturing, were suitable for cell attachment onto 4-well dishes, and the 7 day cultured dental follicle cells filled the dishes.

ALPase activity began to appear on the cultured dish from E14 at 3 days (Fig 8). ALPase is constitutively expressed by bone-forming cells and some periodontal ligament cells, and has been recognized as an enzyme marker for bone differentiation.[16-18] This result suggests that dental follicle cells cultured for 3 days maintain the potential to differentiate into osteoblasts.

3. *Runx2, Bsp* expression in cultured dental follicle cells

Runx2 is a runt domain transcription factor which is essential for bone development and tooth morphogenesis.[22] In this study, the osteogenic properties of dental follicle cells were assessed with regard to the expression of the osteoblast differentiation-specific markers, *Runx2* and *Bsp,* using *in situ* hybridization. In order to characterize the potential of dental follicle cells cultured *in vitro,* E14 dental follicle cells were evaluated in terms of their potential to differentiate into osteoblasts, as well as their potential to differentiate into alveolar bone, using the monitoring of *Runx2* and *Bsp* expression in aggregated cells (Fig 9). These results were consistent with existing data suggesting that dental follicle cells maintain the ability to differentiate into osteoblasts: however, the specific factors relevant to this differentiation were not identified in prior studies.[32,33]

4. Osteogenic property of cultured dental follicle cells

In order to characterize the osteogenic property of dental follicle cells, BMP4 soaked beads and calvaria were implanted into cultured dental follicle dishes. BMPs are potent factors which regulate osteoblast differentiation, and may be involved in terminal osteoblastic differentiation

and bone formation.[34] The expression of the BMP gene was observed in the mesenchymal cells, chondroblasts, and osteoblasts. Developing calvaria at E14, has osteoblasts and an osteogenic front.[35] This suggests active bone formation. We predict that the gradients of this formation might be identical, but the determination of gradation is outside the scope of the current study, and so was not addressed. Calvaria are well known to be a model system of intramembranous ossification during embryogenesis, and calvaria exhibit the ability to induce bone formation.[35] In the experiment involving BMP4-soaked beads, PBS beads were implanted as a control. After bead implantation, signalling molecules were examined using cell *in situ* hybridization. After the implantation of BMP4 beads, *Runx2* and *Bsp* expression were found to be more robust near the bead-implanted dish (Fig. 10). In the experiment adding added calvaria, gene expression was detected in the cultured aggregated cells with bone tissue for a period of 3 weeks (Fig. 11). These results suggest that dental follicle cells have the potential to differentiate into osteoblasts as the result of interaction with environmental factors. *Bsp* in dental follicle cells activated using cell *in situ* hybridization and aggregated cells exhibits a greater extent of osteogenic potential than does *Runx2*.

CONCLUSION

In mice, dental mesenchymal cells consist of the dental papilla and dental follicle in developing mammalian tooth buds. Dental follicle cells are believed to have the ability to differentiate into fibroblasts, cementoblasts, and osteoblasts. The role of the dental follicle was clarified by studies of the development of tooth buds known to be unable to form mineralized tissue. I examined to analyze the osteogenic properties of dental follicles in the molars of mice. According to the results of immunohistochemical study of BSP and OPN, the ossification of the mandible surrounding the dental follicle cells is detected. This indicates that changes in the thickness of the dental follicle cells might be fundamental to their own differentiation. When E14 mesenchymal cells and dental mesenchymal cells were transplanted into kidney capsule, hard tissue formation was observed. Cytochemical examination of the expression of alkaline phosphatase in the cultured follicular cells revealed strong enzyme activity in the cultured dental follicle cells. E14 dental follicle cells exhibited definite potential to differentiate into osteoblasts, and their osteogenic properties were found to involve the expression of *Runx2* and *Bsp* in aggregated cells, thereby characterizing this potential in cultured dental follicle cells *in vitro*. After the addition of bone inducers, including developing calvaria and BMP4-soaked beads, into the cultured dental follicle cells, *Runx2* and *Bsp* were expressed in the aggregated cells, demonstrating the effects of environmental factors on differentiation into osteoblasts. This also indicates that the E14 cultured dental follicles

retained ossification potential. Therefore our results suggest that dental follicle cells have osteogenic properties which are dependent on interactions with environmental factors. Greater insight into the development of periodontal tissue would lead to for a greater clinical understanding of periodontal regeneration. Further study is, therefore, needed, in order to obtain knowledge and understanding regarding dental follicle cells, and their capacity in terms of specific differentiation.

REFERENCE

1. Thesleff I. Epithelial-mesenchymal signalling regulating tooth morphogenesis. J of Cell Sci 2003;116:1647-1648

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

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

4. Ten Cate A, Mills C, Solomon G. The development of the periodontium. A transplantation and autoradiographic study. Anat Rec 1971;170(3):365-379

5. Ten Cate AR, Mills C. The development of the periodontium: the origin of alveolar bone. Anat Rec 1972;173(1):69-77

6. Thesleff I. Does epidermal growth factor control tooth eruption. J Dent Child 1987;54:321-329

7. Zeichner-David M, Oishi K, Su Z, Zakatchenko V, Chen LS, Arzate H, Bringas P Jr. Role of Hertwig's epithelial root sheath cells in tooth root development. Dev Dyn 2003;228(4):651-663

8. Thesleff I. Interaction between the extracellular matrix and the cell surface

determine tooth morphogenesis and the cellular differentiation of the dental mesenchyme. Ontogenez 1989;20(4):341-349

9. Fisher LW, McBride OW, Termine JD, Young MF. Human bone sialoprotein. J Biol Chem 1990;26:2347-2351

10. Shapiro HS, Chen J, Wrana JL, Zhang Q, Blum M, Sodek J. Characterization of porcine bone sialoprotein: primary structure and cellular expression. Matrix 1993;13:431-440

11. Chen J, Shapiro HS, Wrana JL, Reimers S, Heersche JN, Sodek J. Localization of bone sialoprotein (BSP) expression to sites of mineralized tissue formation in fetal rat tissues by in situ hybridization. Matrix 1991;11:133-143

12. Chen J, Shapiro HS, Sodek J. Developmental expression of bone sialoprotein mRNA in rat mineralized conncetive tissue. J Bone Mineral 1992;8:987-997

13. Bianco P, Fisher LW, Young MF, Termine JD, Robey PG. Expression of bone sialoprotein (BSP) in developing human tissues. Calcif Tissue Int 1991;49:421-426

14. Chen J, McCulloch CA, Sodek J. Bone sialoprotein in developing porcine dental tissue: cellular expression and comparison of tissue localization with osteopontin and osteonection. Arch Oral Biol 1993;38(3):241-249

15. Hultenby K, Reinholt FP, Norgard M, Oldberg A, Wendel M, Heinegard D. Distribution and syn-thesis of bone sialoprotein in metaphyseal bone of young rats show a distinctly different pattern from that of osteopontin. Eur J Cell Biol 1994;63:230-239

16. Tenenbaum, Heersche. Differentiation of osteoblasts and formation of mineralized bone in vitro. Calcif Tissue Int 1982;34(1):76-89

17. Arceo N, Sauk JJ, Moehring J, Foster RA, Someman MJ. Human periodontal cells initiate mineral-like nodules in vitro. J Periodontol 1991;62(8):499-503

18. Hou LT, Yaeger JA. DNA content and alkaline phosphatase expression in cells of different gingival overgrowths. J Oral Pathol Med 1995;24(3):97-102

19. Gitelman SE, Kirk M, Ye JQ, Filvaroff EH, Kahn AJ, Derynck R. Vgr-1/BMP-6 induces osteoblastic differentiation of pluripotential mesenchymal cells. Cell Growth Differ 1995;6:827-836

20. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1:a transcriptional activator of osteoblast differentiation. Cell 1997;89:747-754

21. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997;89:755-764

22. Aberg T, Wang XP, Kim JH, Yamashiro T, Bei M, Rice R, Ryoo HM,

Thesleff I. *Runx2* mediates FGF signaling from epithelium to mesenchyme during tooth morphogenesis. Dev Biol 2004;270:76-93

23. Storm EE, Huynh TV, Copeland NG, Jenkins NA, Kingsley DM, Lee SJ. Limb alterations in brachypodism mice due to mutations in a new member of the TGFbeta-superfamily. Nature 1994;368(6472):639-643

24. Sena K, Morotome Y, Baba O, Terashima T, Takano Y, Ishikawa I. Gene expression of growth differentiation factors in the developing periodontium of rat molars. Journal of Dental Research. J Dent Res 2003;82:166-171

25. Hogan BL, Yingling JM. Epithleium/mesenchymal interactions and branching morphogenesis of the lung. Cur Opin Dev 1998;8:481-486

26. Pispa J, Thesleff I. Mechanisms of ectodermal organogenesis. Dev Biol 2003;262:195-205

27. Chai Y, Jiang X, and Ito Y. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 2000;127:1671-1679

28. Noden DM. The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissue. Dev Biol 1983;96:144-165

29. Lumsden AG. Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth. Development 1988;103:155-169

30. Noden DM. Cell movements and control of patterned tissue assembly during craniofacial development. J Craniofac Genet Dev Biol 1996;176:151-165

31. Imai H, Osumi-Yamashita N, Ninomiya Y, Kazuhiro E. Contribution of early-emigrating midbrain crest cells to the dental mesenchyme of mandibular molar teeth in rat embryos. Dev Biol 1991;11(4):192-213

32. Hou LT, Liu CM, Chen YI, Wong MY, Chen KC, Chen J, Thomas HF. characterization of dental follicle cells in developing mouse molar. Arch Oral Biol 1999;44:759-770

33. Arzate H, Aguilar-Mendoza ME, Esponda Aguilar C, Portilla Roverson J. Bovine cementum extract influences murine dental follicle cells in vitro. Arch Med Res 1997;28:470-413

34. Wozney JM, Rosen V, Celeste AJ, Mitsoc LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. Novel regulators of bone formation: molecular clones and activities. Science 1988;242:1528-1534

35. Rice DPC, Kim HJ, Thesleff I. Detection of gelatinase B expression reveals osteoclastic bone resorption as a feature of early calvarial bone development. Bone 1997;21:479-486

Abstract (IN KOREAN)

생쥐 치아의 발생동안 치낭세포의 골형성 분석

(지도 교수 : 정 한 성)

연세대학교 대학원

의과학과

황 희 정

마우스에서 치아 간엽세포는 발생중인 포유류의 치아 싹에서 치유두와 치아주머니 세포로 구성되어 있다. 치아주머니세포는 섬유모세포, 시멘트모세포, 뼈모세포로 분화할 수 있는 능력을 가지고 있는 것으로 알려져 있다. 그러나 치아주머니의 세포의 세포분화와 주위 상호작용에 대하여는 정확하게 알려져 있지 않다.

면역화학적방법을 시행한 결과, 경조직의 표지자로 알려진 BSP 와 OPN이 14일된 흰쥐 배아의 치아주머니 세포에서 나타나지 않았고, 생후 8일된 흰쥐의 치조골로 예상되는 부분에서 관찰되었다. 14일된 흰쥐 배아의 치아주머니 세포에서 경조직 형성되지 않았음에도 불구하고 이 세포들이 골 형성의 잠재력을 가지는 것을 다음과 같은 방법으로 확인할 수 있었다. 이러한 치아주머니 세포의 골 형성 잠재능력을 확인하기 위해 14일된 마우스 배아 아래턱의 치아주머니 세포들을 단일세포로 만들어 체외배양을 하였다. 뼈 형성과 치아 발생에 필수적인 유전자 Runx2와 초기 시기 뼈 형성에 나타나는 BSP이 모여 있는 치아주머니 세포들에서 반응이 나타나는 것으로 보아 뼈모세포로 어느정도

분화되기 시작했고, 뼈를 형성하는 잠재능력을 가지고 있다는 것을 알 수 있었다. 치아주머니세포에 주위 상호인자로써 발생중인 머리덮개 뼈와 BMP4 첨가하여 *Runx2* 와 *Bsp* 로 확인하여 본 결과 모여진 세포들에서 반응이 나타나는 것을 확인할 수 있었다. 이러한 결과들은 주변 환경의 상호작용과 함께 치아주머니 세포들은 뼈모세포로 분화할 수 있는 잠재력을 가지고 있음을 나타내는 것이다.

핵심되는 말: 치아발생, 치낭세포, 조골세포, 분화, 환경적 요인