A critical role of *Wnt5a* during murine tooth development

Dae-Woon Kang

The Graduate School

Yonsei University

Department of Dentistry

A critical role of *Wnt5a* during murine tooth development

A Master's Thesis

Submitted to the Department of dentistry

and the Graduate School of Yonsei University

in partial fulfillment of the

requirements for the degree of

Master of dental science

Dae-Woon Kang

December 2007

This certifies that the Master's thesis

of Dae-Woon Kang is approved.

_____

Han-Sung Jung

_____

Byung-Jai Choi

_____

Hee-Jin Kim

The Graduate School

Yonsei University

December 2007

ACKNOWLEDGEMENTS

이 논문이 완성되기까지 많은 관심과 배려 그리고 세심한 가르침을 주신 정한성 교수님께 진심으로 감사 드립니다. 또한 대학원 진학뿐만 아니라 앞날에 대한 조언과 지도를 아끼지 않으신 김희진 교수님께도 진심으로 감사 드립니다.

논문의 작성과 심사에 많은 지도와 관심을 주신 최병재 교수님께도 무한한 감사의 말씀을 드리며 바쁜 와중에도 많은 도움과 힘이 되어준 구강생물학 교실의 조성원 교수, 허경석 교수와 징레이(Jinglei Cai) 선생님께도 깊은 감사를 드립니다.

끝으로 항상 큰 힘이 되어 주시는 양가부모님 그리고 사랑하는 아내 조정미와 아들 창현이에게도 사랑의 마음을 전하며 논문을 바칩니다.

2007 년 12 월

저자 씀

<TABLE OF CONTENTS>

<LIST OF FIGURES>

ABSTRACT

A critical role of Wnt5a during murine tooth development

Dae-Woon Kang

Department of Dentistry
The Graduate School, Yonsei University

(Directed by Professor Han-Sung Jung)

Murine tooth development involves a series of epithelial–mesenchymal interactions that are mediated by a complex molecular network. We have previously suggested that tooth size is determined by the dental mesenchymal factors. Numerous genes such as *Bmp2*, *Bmp4*, *Fgf3*, *Fgf10*, and *Lef1* are expressed in the dental mesenchyme. However, it has been reported that exogenous BMP4, NOGGIN, FGF3 and FGF10 has no effect on the alterations in tooth size at the cap stage tooth germ[2]. Among *Wnts* genes in tooth development, only *Wnt5a* is expressed in the dental mesenchyme. In the present study, we investigated the expression pattern of *Wnt5a* in developing tooth germs and the role of *Wnt5a* in the regulation of tooth size by treatment of

exogenous WNT5A on tooth germs at cap stage. As results, *Wnt5a* was expressed in both dental epithelium and dental mesenchyme from E14 to E17 overlapping partly with both *Shh* and *Bmp4* expressions in tooth germs. Moreover, WNT5A retarded the development of tooth germs by inducing cell death severely in the epithelium and mesenchyme of non-dental regions, but not severely in the dental region. In this dental region, the epithelial mesenchymal gene interactions among *Wnt5a*, *Fgf10*, *Bmp4*, and *Shh* may partly rescue the cell death in the WNT5A-treated tooth germ. Taken together, WNT5A-induced cell death inhibited overall development of tooth germ, which might result in smaller teeth with blunter cusps after 3 weeks. It is suggested that *Wnt5a* is involved in regulating cell death in non-dental regions, while *Wnt5a* in the dental region acts as a regulator of other genes concerning cell proliferation, cusp formation and determining tooth size.

Key words: WNT5A, tooth size, *Shh*, *Bmp4*, cell proliferation, cell death

A critical role of Wnt5a during murine tooth development

Dae-Woon Kang

Department of Dentistry
The Graduate School, Yonsei University

(Directed by Professor Han-Sung Jung)

I. INTRODUCTION

Tooth development is governed by the epithelial–mesenchymal interactions. Numerous signaling molecules localized in the epithelium, mesenchyme, or both with distinct patterns of expression interact with each other to mediate tissue interactions [18]. The molecular details of signaling networks have been dissected in great detail, particularly in four signal families, BMP, FGF, HH and WNT [10].

Various members of the BMP family exhibit a complex expression pattern throughout odontogenesis [1,28]. Members of the FGF family are necessary for tooth initiation and for tooth morphogenesis to proceed beyond the bud stage [11]. Especially, *Fgf10* stimulates cell proliferation in the dental epithelium, but is expressed only in the dental mesenchyme during active morphogenesis at the cap stage [10,11]. *Shh*, another secreted signaling factor, influences the growth and patterning of the tooth. Deletion of *Shh* causes a cap-stage tooth rudiment and a smaller tooth germ [6]. Wnt signaling is also important in organogenesis [16]. Six Wnt genes such as *Wnt3, -4, -6, -7b, -10a*, and *-10b* and one Wnt receptor *MFz6* are observed in or around the dental epithelium [14,20], while *Wnt5a* and agonists/antagonists *Mfrzb1* and *Mfrp2* are

expressed in a graded proximodistal manner in dental mesenchyme [20]. Especially, *Wnt5a* and *Mfrzb1* are strongly expressed in the dental papilla mesenchyme at the cap stage [20].

We have recently reported that tooth size is determined by the dental mesenchyme, suggesting that this is regulated by intrinsic mesenchymal factors [2]. Numerous genes such as *Bmp2*, *Bmp4*, *Fgf3*, *Fgf10*, *Lef1*, and *Wnt5a* are expressed in the dental mesenchyme. However, exogenous treatment with BMP4, NOGGIN, FGF3, and FGF10 has no effect on the alterations in tooth size [2], although retarded development and smaller molar teeth have been detected following the addition of Mfrzb1 at E10.5 and E11.5 [21]. The expressions of *Wnt5a* and *Mfrzb1* were highly coincident in the mesenchyme from the dental lamina to the cap stage [21]. Based on this overlap between the expressions of a ligand and possible antagonist in mesenchyme, it was suggested that Mfrzb1 acts as a continuous "sink" for Wnt proteins. On the other hand, targeted mutation of *Wnt5a* in mice has been shown to produce craniofacial defects [26] (specifically, reduced outgrowth of the facial processes), but the role of *Wnt5a* as a mesenchymal factor in the tooth remains unknown.

This study investigated the expression pattern of the *Wnt5a* and the effect of additional WNT5A protein on tooth germs at the cap stage. The possible interactions between WNT5A and key signaling factors (i.e., *Bmp4*, *Fgf10*, and *Shh*) were examined in explants after WNT5A treatment. We carried out further *in vitro* analyses to demonstrate the role of *Wnt5a* in dental epithelium and mesenchyme respectively.

II. MATERIALS AND METHODS

Animals

Adult ICR mice were housed in a temperature-controlled room (22℃) under artificial illumination (lights on from 05:00 to 17:00) and at 55% relative humidity with assess to food and water *ad libitum*. The embryos were obtained from time-mated pregnant mice. Embryonic day 0 (E0) was designated as the day on which a vaginal plug was confirmed. Embryos at E11 to E17 were used in this study.

Preparation of tissues and *in vitro* organ culture

The molar tooth germs of E14 mice were dissected and incubated in Dispase II (Roche, Germany) at 1.2 U/ml in PBS for 20 min. The tooth germs were washed in DMEM with 10% FBS, and dental epithelium was separated from the dental mesenchyme. The molar tooth germs, dental epithelium and dental mesenchyme were cultured with Trowell-type organ culture method using 10% FBS contained DMEM respectively.

Bead implantation

Affigel-blue beads (BioRad) were washed with PBS and then incubated in WNT5A (500 μg/ml) and PBS control. All beads were incubated at room temperature (RT) for at least 1 h. Three experimental groups were carried out as follows: 1) Beads were implanted into the mesenchyme of both sides (Fig. 1) or the buccal side (Fig. 3) of E14 tooth germs using fine forceps; 2) Beads were placed on the top of the dental mesenchyme (Figs. 4A, B, D and E); 3) Beads were placed on the top of the dental epithelium (Figs. 4C, F). The bead implanted explants were cultured at 37 ℃ for 48 h.

Immunohistochemistry

The tooth germs implanted protein soaked beads were fixed after culture for 48 h in 4% paraformaldehyde (PFA) in PBS overnight at 4℃, embedded in Optimal Cutting Temperature (O.C.T) (Tissue-Tek, Cat. NO 4583, USA) compound using conventional methods and then cut to a thickness of 7 μm. Sections were incubated overnight at 4℃ with a mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA, Neo Markers, USA), as the primary antibody. After being washed with PBS, the sections were reacted with a goat anti-mouse immunoglobulin secondary antibody and the streptavidin-peroxidase at RT for 10 min each. Finally, the antibody binding to the sections was visualized using a Diaminobenzodine tetrahydrochloride (DAB) reagent kit (Zymed, Cat No 00-2014, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL, In Situ Cell Death Detection Kit, POD, USA) was used for cell death detection in individual cells of the histological sections of bead implanted explants. The TUNEL procedures were carried out as described previously (Yamamoto et al., 2004).

Transplantation of bead implanted explants into the renal subcapsular layer

To calcify the bead implanted tooth germs, they were cultured for 48 h *in vitro* and transplanted into the renal subcapsular layer of adult ICR male mice. After 3 weeks, the host mice were sacrificed to obtain the calcified teeth.

Isolation of *Wnt5a*

cDNA fragments of *Wnt5a* of mouse were amplified by reverse transcriptase-PCR from the ICR mouse, using specific primers were 5'-CCACTACGGGATTAAGCCTGA-3' (forward) and 5'-

CTAGCGTCCACGAACTCCT-3' (reverse). Primers were designed according to the published sequences of *Wnt5a* genes (NM_009524). Product size was 546 bp and inserted into the *EcoRI* sites of pGEM-T easy vector (Promega, USA).

Whole-mount *in situ* hybridization

Bead implanted cultured tooth germs, mandibles at E11 to E13, and tooth germs at E14 to E17 were fixed overnight in 4% PFA. *In situ* hybridization was carried out by treating the specimens with 20 μg/ml proteinase K for 3 to 6 min at RT. The anti-sense RNA probes were labeled with digoxygenin (BMS, Korea). After *in situ* hybridization, the specimens were cryosectioned at a thickness of 10 μm.

Quantitative real-time PCR (RT-qPCR)

RNA was extracted from the bead implanted explants using total RNA isolation following the manufacturer's recommendations and further purified using the Total RNA Mini Kit (RBC, Korea). For cDNA synthesis, reverse transcription of RNA was performed using the RevertAid^{TM} M-MLV Reverse Transcriptase (Fermentas, Canada). Real-time PCR was performed using a Thermal Cycler Dice^{TM} Real Time System and SYBR Premix EX Taq^{TM} (Takara, Japan) according to the manufacturer's instructions. For RT-qPCR, the protocol included an initial incubation of the reaction mixture for 1 min at 95°C. The amplification program consisted of 40 cycles with a 95°C denaturation for 20 secs and a 55–60°C annealing and extension for 45 secs. The RT-qPCR for each sample was performed in triplicate and the amount of the RT-qPCR products were normalized using GAPDH as an internal control. Detection of the fluorescent product was carried out either at the end of the extension period or after an additional 2 secs step at 2°C below the melting temperature of the product. The data were analyzed with the Thermal Cycler

Dice™ Real Time System analysis software. To determine the relative level of gene expression, we used the comparative ΔΔCt method, previously described by Livak and Schmittgen. The test was employed to determine significant changes at the 99% confidence level ($p < 0.01$).

III. RESULTS

Effect of exogenous WNT5A during tooth development

Beads soaked in PBS and in WNT5A protein were implanted into E14 tooth germs, respectively (Figs. 1A, C). After 48 h in culture, WNT5A-treated tooth germs showed abnormal shape (Fig. 1D), which was different from that of PBS-treated tooth germs (Fig. 1B). The size of WNT5A-treated tooth germs (*N*=98/126) were smaller than that of PBS-treated tooth germs (*N*=115/115) (Figs. 1B, D). PBS-treated tooth germs had developed to the bell stage (Fig. 1E), whereas WNT5A-treated tooth germs showed retarded growth, being at the cap stage (Fig. 1F). Furthermore, the oral epithelium was absent around WNT5A-treated beads (Fig.1F). Whilst a few apoptotic cells were detected in both the epithelium and mesenchyme of PBS-treated tooth germs (Fig. 1G), WNT5A-treated tooth germs showed greatly increased numbers of apoptotic cells in the mesenchyme around the implanted beads (Fig. 1H), but only a few apoptotic mesenchymal cells being in contact with the dental epithelium (Fig. 1H). This result shows that WNT5A induces the epithelial and mesenchymal cell death. The level of cell proliferation was also compared between two groups. Few positive reactions for cell proliferation were detected around WNT5A-treated beads (Figs. 1I, J). However, the level of cell proliferation was not much difference between two groups. In addition, the specimens in which beads were implanted only on the buccal side showed the same morphology, cellular proliferation, and cell death during tooth development as those of the specimens with beads on both sides (data not shown).

Bead-implanted tooth germs were also transplanted into a kidney capsule and incubated for 3 weeks. Calcified teeth were formed from both WNT5A-treated tooth germs (*N*=10/10) and PBS-treated tooth germs (*N*=11/11) (Figs. 1K－N). Most of WNT5A-treated tooth germs (*N*=8/10) as

well as PBS-treated tooth germs (*N*=11/11) formed two teeth (the first and second molars), the larger of which, was compared with the tooth size from PBS-treated tooth germs. One tooth germ (*N*=1/8) formed calcified tooth that was as large as the first molars from PBS-treated tooth germs, while the other seven WNT5A-treated tooth germs (*N*=7/8) formed calcified teeth whose mesiodistal and buccolingual lengths were two-thirds those from PBS-treated tooth germs (Figs. 1K, M). Another two WNT5A-treated tooth germs (*N*=2/10), which formed only one tooth each, showed one tooth as large as the first molars from PBS-treated tooth germs and the other one was two-thirds those from PBS-treated tooth germs in the mesiodistal and buccolingual lengths. This result shows that the exogenous WNT5A resulted in smaller teeth by inducing cell death in both the epithelium and mesenchyme.

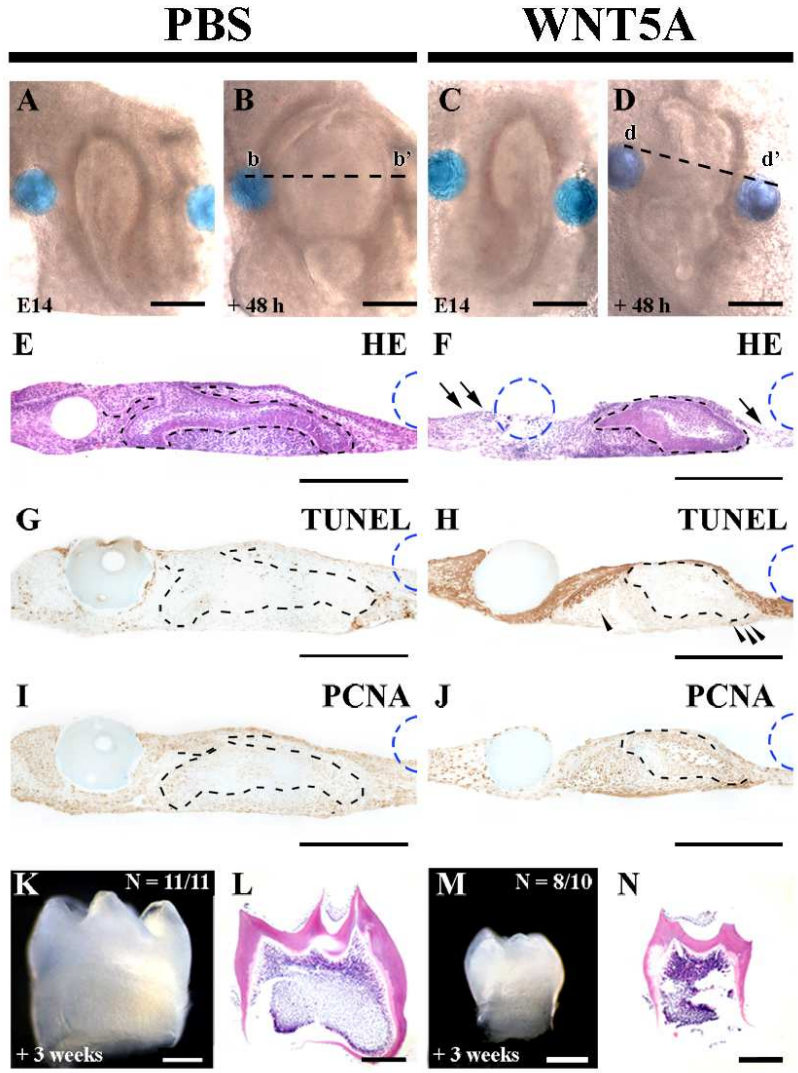


Figure 1. Tooth development from E14 tooth germs treated with exogenous WNT5A protein. (A, B) Upper view of E14 tooth germs implanted affigel-blue beads soaked in PBS (control) before culture (A) and after culture for 48 h (B). (C, D) E14 tooth germs with WNT5A-treated beads (500 μg/ml) before culture (C) and cultured for

48 h (D). (E, F) Hematoxylin and Eosin staining of frontal sections of (B, D). (E) PBS-treated tooth germs develop to the bell stage after 48 h. (F) Retarded growth of WNT5A-treated tooth germs are still at the cap stage, Oral epithelia of which were absent around WNT5A-treated beads (arrows). (G, H) TUNEL studies on frontal sections of (B, D), which indicated apoptotic cells locate in the place as visualized by dark brown. (G) A few TUNEL positive spots locate in the epithelium and mesenchyme of control explants. (H) Great increased TUNEL labeled mesenchymal cells are observed in the area around the beads in WNT5A-treated tooth germs. However, few TUNEL labeled cells are detected in the dental region (arrowheads). (I, J) Cell proliferation assay on frontal sections of (B, D), which indicated the proliferating cells locate in the place as visualized by brown. (I) PCNA positive cells locate in both epithelium and mesenchyme of control explants. (J) No much difference is detected on the number of PCNA-positive cells in WNT5A-treated tooth germs. (K, L) Teeth are formed from the tooth germs cultured with control beads in the kidney capsule after 3 weeks. (M, N) Smaller teeth are detected from the explants treated with WNT5A protein. Black dotted lines bb' and dd' in panels B and D indicate the section line in panels E-J. Blue dotted circles in panels E-J indicate beads. Dental epithelium is outlined by black dotted line in panels E-J. Scale bar: 250 μm.

Expression of *Wnt5a* in the developing molar tooth germs

Wnt5a expression was detected by whole-mount *in situ* hybridization in developing tooth germs from E11 to E17 (Fig. 2). During the initiation of tooth development (E11–E12) and mesenchymal cell condensation (E13), *Wnt5a* was expressed strongly in the mesenchyme (Fig. 2A–C). At the cap stage (E14), *Wnt5a* expression was strongly localized to the primary enamel knot (PEK), and also detected in the stellate reticulum (SR), dental follicle, and dental papilla (Fig. 2D). At the late cap and early bell stage (E15), the expression of *Wnt5a* was intense in the stratum intermedium (SI) cells and dental papilla, and weak in other parts of the dental organ and the dental follicle mesenchyme (Fig. 2E). At the bell stage (E16), *Wnt5a* was observed strongly at the tips of dental papillae, secondary enamel knots (SEKs), SI cells, and surrounding dental mesenchyme, and weakly in part of the inner dental epithelium (IDE) and SR cells (Fig. 2F). However, *Wnt5a* showed similar weaker expression in the IDE and SI cells at the late bell stage (E17), but it continued to be strongly expressed in the cuspal areas of the dental papilla (Fig. 2G).

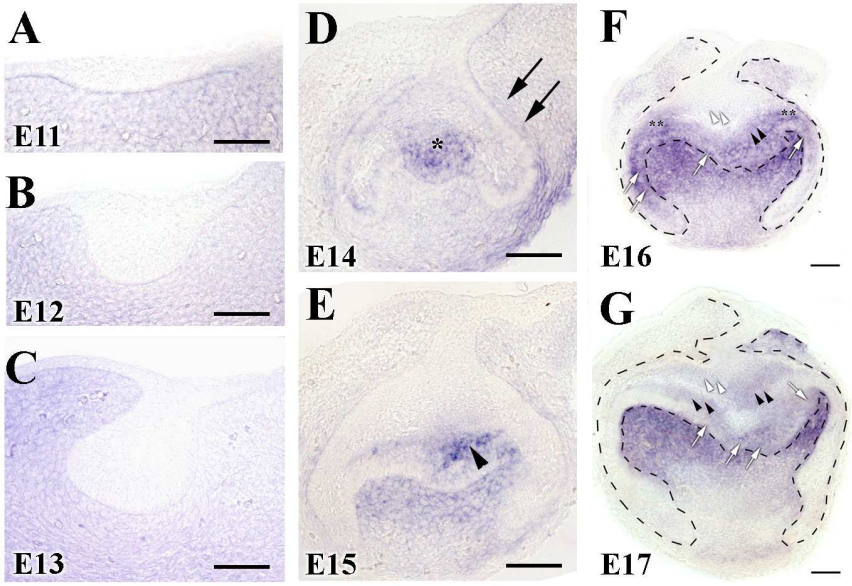


Figure 2. Expression of *Wnt5a* in developing tooth germs. (A-C) *Wnt5a* is expressed in the mesenchyme from E11-E13. (D) At E14, *Wnt5a* is expressed strongly in the primary enamel knot (PEK, *), weakly in the dental organ and surrounding dental mesenchyme (black arrows). (E) At E15, *Wnt5a* is detected strongly in the stratum intermedium (SI) cells and dental papilla, weakly in dental mesenchyme around dental follicle. (F) At E16, *Wnt5a* is intense at the tips of dental papilla, secondary enamel knots (SEKs, **) and SI cells, weakly in the inner dental epithelium (IDE) and stellate reticulum (SR) cells. (G) At E17, *Wnt5a* is specially detected in the cuspal areas of dental papilla and shows weaker widespread expression in the dental organ, especially in the SI cells. The *Wnt5a* expressions in the SI and SR cells were indicated by black arrowheads and white arrowheads respectively in panels E, G and F.

The dental epithelium was outlined by black dotted lines and the IDE expressed with *Wnt5a* was pointed out by white arrows in panels F and G. Scale bar: 50 μm.

Expression of *Wnt5a* and the key factors of tooth patterning in WNT5A-treated tooth germs

To understand the mechanisms underlying the effects of exogenous WNT5A protein on tooth size, we used whole-mount *in situ* hybridization to investigate the expression patterns of three key factors (*Shh*, *Bmp4*, and *Fgf10*) during tooth development after treatment with WNT5A protein for 48 h. Strong *Bmp4* expression was detected at the enamel knots and the cuspal area of the dental papilla in both WNT5A-treated and PBS-treated tooth germs (Figs. 3E, M). The expression level of *Bmp4* mRNA was stronger in WNT5A-treated than that in PBS-treated tooth germs (Figs. 3A, E, I, and M). The expression of *Fgf10* showed a similar spatial distribution and expression level in WNT5A-treated tooth germs and PBS-treated tooth germs (Figs. 3C, G, K, and O). Interestingly, dramatic alterations of tooth germs in term of both the spatial distribution and expression level of *Shh* expression were found after WNT5A treatment. *Shh* expression was found in the SEKs, and had spread to the rest of the IDE, SI cells, and SR cells of PBS-treated tooth germs (Figs. 3B, F). However, in WNT5A-treated tooth germs, *Shh* was expressed as a long strip mesiodistally, and the expression level of *Shh* was markedly increased (Figs. 3J, N). The expression of *Wnt5a* was also investigated in both tooth germ groups. It was no higher in WNT5A-treated tooth germs (Figs. 3L, P), and actually a little lower than that in PBS-treated tooth germs (Figs. 3D, H). The expression pattern of *Wnt5a* in PBS-treated tooth germs was similar to that of E16 tooth germs, while it was expressed in the PEK and at the tip of dental papilla in WNT5A-treated tooth germs (Fig. 3P).

The relative abundances of *Bmp4*, *Fgf10*, *Shh*, and *Wnt5a* transcripts were also examined. As shown in Fig. 3Q, *Bmp4*, *Fgf10* and *Shh* mRNA was more abundant in WNT5A-treated tooth germs than those of PBS-treated tooth germs. Especially, *Shh* was 143% higher, and *Bmp4* and *Fgf10* were increased by nearly 66% and 26%, respectively. However, *Wnt5a* mRNA was decreased by nearly 42% in WNT5A-treated tooth germs. All these results were consistent with the results from whole-mount *in situ* hybridizations.

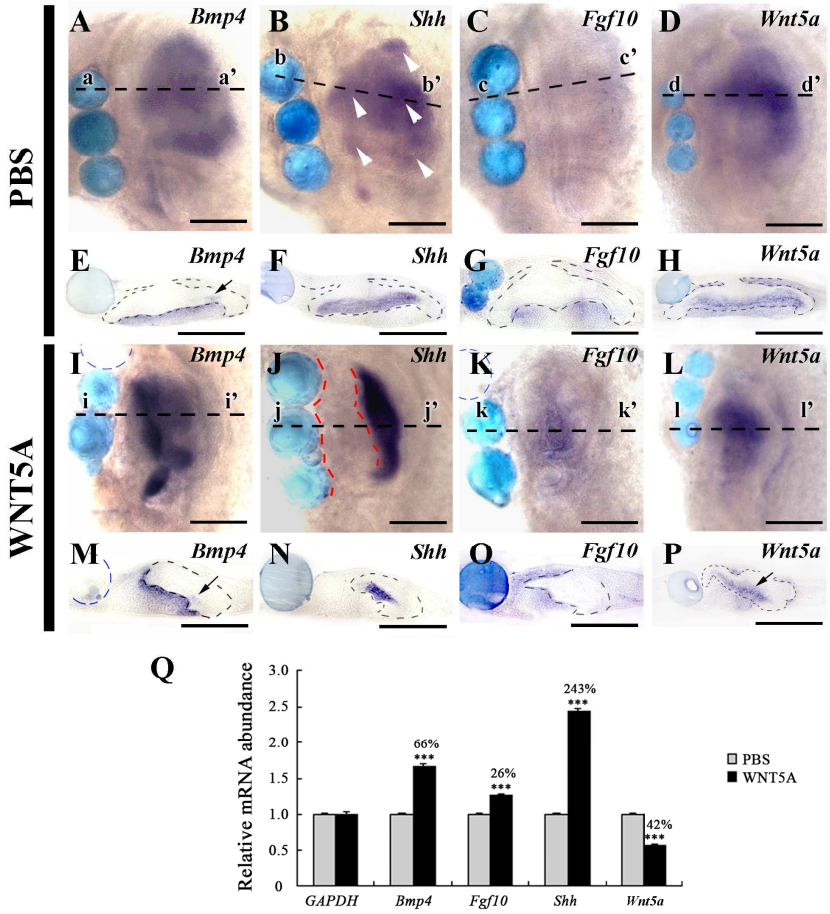


Figure. 3 Expression of *Bmp4*, *Shh* and *Fgf10* in PBS-treated and WNT5A-treated tooth germs by whole-mount *in situ* hybridization and relative abundance by RT-qPCR. (A-H) PBS-treated tooth germs (control) and (I-P) WNT5A-treated tooth germs are cultured for 48 h. Panels (E-H and M-P) show frontal sections of panels (A-D and I-L) respectively. (A, E) *Bmp4* is expressed in the SEKs (black arrows) and dental mesenchyme in controls. (I, M) Expression level of *Bmp4* is increased in WNT5A-treated tooth germs. (B) Five secondary enamel knots are distinguished in the first molar tooth germ by the

diffused *Shh* expression (white arrowheads). (J) In WNT5A-treated tooth germs, stronger expression of *Shh* is detected as a strip, which might indicate the PEK. A distance is detected to be maintained between the beads and *Shh* expression (between red dotted lines). (C, G, K and O) *Fgf10* is expressed in the dental mesenchyme in both groups. (D, H) *Wnt5a* is expressed in SEKs and the tips of dental papilla in controls. (L, P) *Wnt5a* is detected in the PEK (black arrow) and the tip of dental papilla in WNT5A-treated tooth germs. The intensity of expression is not increased but weakly decreased. (Q) After bead implantation for 48 h, the ratios of the relative mRNA abundance in WNT5A-treated tooth germs were calculated and shown as the black bars. White bars represent the relative mRNA abundance in controls and were arbitrarily set as 1. Each result is the mean of at least 9 independent experiments. ***$P < 0.001$. Black dotted lines in panels A-D and I-L indicate the section line in panels E-H and M-P respectively. Blue dotted circles in panels I, K and M indicate beads. Dental epithelium is outlined by black dotted line in panels E-H and M-P. Scale bar: 250 μm.

Expression of the key factors of tooth development in WNT5A-treated dental tissues

To determine the relationship between WNT5A and the key molecules in the dental mesenchyme such as *Bmp4* and *Fgf10*, the dental mesenchyme was isolated from the dental epithelium and treated with WNT5A protein, then was examined by whole-mount *in situ* hybridization after for 48 h of *in vitro* culture. *Bmp4* mRNA was detected in both WNT5A-treated and PBS-treated dental mesenchyme (Figs. 4A, D), with it being present as a pronounced halo around WNT5A-treated beads (Fig. 4D). No clear expression of *Fgf10* mRNA was evident in either WNT5A-treated or PBS-

treated dental mesenchyme (Figs. 4B, E). The results for the epithelium-free mesenchymal explants were also consistent with the RT-qPCR results. *Bmp4* was increased by nearly 73%, whereas the expression of *Fgf10* was not altered (Fig. 4G). This result means that WNT5A induces the expression of *Bmp4* directly, but not induce that of *Fgf10* directly.

To determine whether exogenous WNT5A contributed to the increased expression of *Shh*, WNT5A was treated on the dental epithelia, which were separated from dental mesenchyme, and then *Shh* expression was examined by whole-mount *in situ* hybridization after for 48 h of *in vitro* culture. After 48 h *in vitro* culture, WNT5A-treated beads on the center of the dental epithelium were displaced out of the dental epithelium, while PBS-treated beads were remained on the center of the dental epithelium. Weak *Shh* expression was detected throughout PBS-treated dental epithelia (Fig. 4C), whereas *Shh* expression was markedly increased in WNT5A-treated dental epithelia (Fig. 4F).

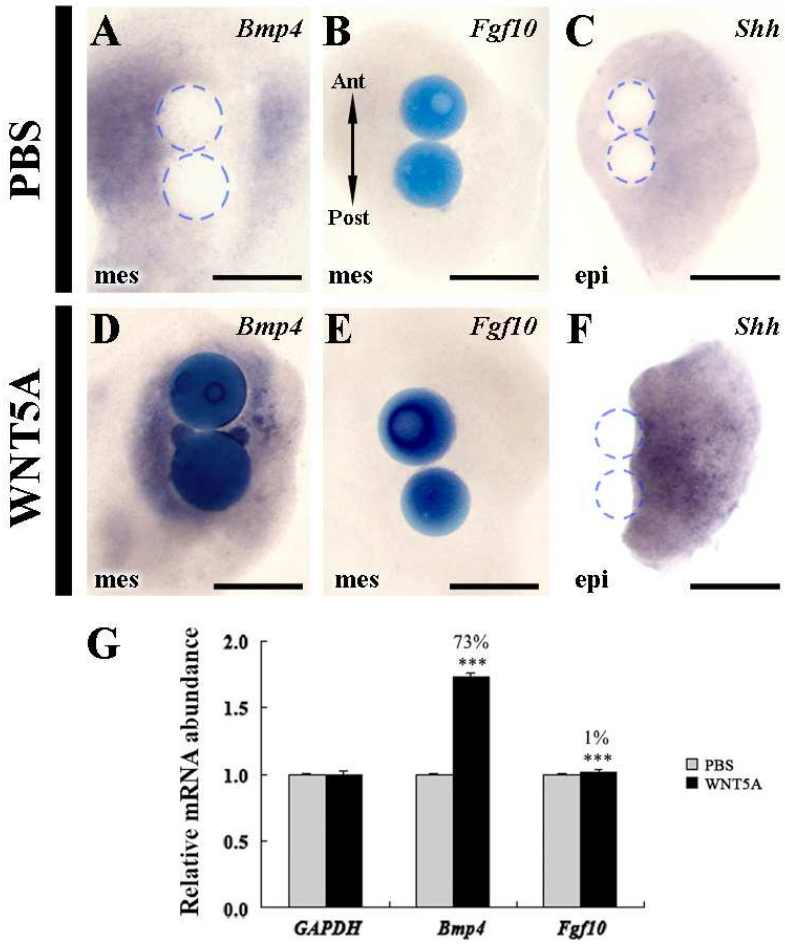


Figure4. Expression of *Bmp4* and *Fgf10* in the epithelium-free mesenchymal explants and *Shh* in the mesenchyme-free epithelial explants after bead implantation and relative abundance by RT-qPCR. (A) The expression of *Bmp4* can be still observed in the PBS-treated epithelium-free mesenchymal explants after for 48 h of *in vitro* culture. (D) The expression level of *Bmp4* is increased markedly in a halo surrounding WNT5A-treated beads. (B, E) The expression of *Fgf10* is lost in the epithelium-free mesenchymal explants in both

groups. (C) Diffused and weak *Shh* expression is remained throughout the mesenchyme-free epithelial explants after for 48 h of *in vitro* culture. (F) Stronger expression of *Shh* is detected in the WNT5A-treated mesenchyme-free epithelial explants. (G) After bead implantation for 48 h, the ratios of the relative mRNA abundance in WNT5A-treated dental mesenchyme were calculated and shown as the black bars. White bars represent the relative mRNA abundance in PBS-treated dental mesenchyme and were arbitrarily set as 1. Each result is the mean of at least 6 independent experiments. ***$P < 0.001$. Blue dotted circles in panels A and C indicate beads. The two-way arrow in panel B indicates the orientation of the dental tissues (epithelium or mesenchyme). Ant: anterior; Post: posterior; epi: epithelium; mes: mesenchyme. Scale bar: 250 μm.

IV. DISCUSSION

Wnt5a is expressed in both the dental epithelium and dental mesenchyme during tooth development

Gene targeting has been used to analyze the functional roles of Wnt pathway in many mammalian organs, such as *Wnt1* in midbrain and cerebellum, *Wnt4* in kidney, *Wnt5a* in lung, and *Wnt7a* in limb [13,15,17,22]. Sarkar and Sharpe suggested a role for Wnt signaling in tooth development by showing that the addition of Mfrzb1 (a Wnt antagonist) at the dental lamina stage leads to retarded development and smaller molar teeth. [21] It was also suggested that the expression of *Mfrzb1* is consistent with that of *Wnt5a* during early tooth development [20]. We previously suggested that tooth size is determined by mesenchymal factors [2]. *Mfrzb1* and *Wnt5a* are known to be expressed in the dental mesenchyme, with the latter being the only one among 19 Wnt genes to be expressed therein [20]. However, in the present study, we found that *Wnt5a* is expressed not only in the dental mesenchyme but also in the dental epithelium during tooth development (Fig. 2). Interestingly, *Wnt5a* is also expressed in the PEK and dental organ at the cap stage. *Wnt5a* continued to be strongly expressed in the cuspal areas of the dental papilla and SI cells. It has been reported that *Wnt5a* was also expressed in a distinct cluster of epithelial cells at the tip of the cusp next to the IDE cells, dental mesenchyme, dental follicle, dental pulp, and some parts of ameloblasts at PN3 [14]. These results indicate that *Wnt5a* is first expressed in the mesenchyme, and then also appears in the dental epithelium at the cap stage, especially in the PEK. Its expression is maintained in both the dental epithelial and dental mesenchymal tissues at the subsequent stages. Therefore, it is suggested that *Wnt5a* plays a role in ensuring correct epithelial–mesenchymal interactions during tooth development. Additionally, the fact that *Wnt5a* expression on the SI cells which are known to express high

alkaline-phosphatase activity suggests that *Wnt5a* is involved in enamel formation.

WNT5A inhibits tooth development and produces smaller teeth by inducing cell death at the cap stage

Our results after treatment with exogenous WNT5A in E14 tooth germs are consistent with the results of additional Mfrzb1 in the early stage tooth germs [21] from the viewpoint of (1) inhibition of tooth development and (2) calcified teeth being smaller after incubation in the kidney capsule for 3 weeks. We investigated the mechanisms underlying these results by examining cellular proliferation and cell death. TUNEL analysis showed significant differences in the mesenchyme around WNT5A-treated beads compared with PBS-treated beads. Moreover, the oral epithelia were absent around WNT5A-treated beads. These results indicate that WNT5A induces cell death in both the epithelium and mesenchyme of the non-dental regionss. However, only a few apoptotic cells was found in the dental region, which suggests that certain signaling pathways protect the dental region from severe cell death (Fig. 5A). On the other hand, it has been reported that *Wnt5a* is required for the proliferation of the limb bud and its progenitor cells [26] and that *Wnt5a* regulates cell proliferation in the developing of external genitalia [24]. It was also suggested that the role of *Wnt5a* signaling varies between tissues, such as cell proliferation being decreased in limb but increased in lung in *Wnt5a* mutant mice [13,24,26]. However, in the present study, the cell proliferation in tooth germs was not changed by exogenous WNT5A treatment. It indicates that exogenous WNT5A casted the predetermined balance between cell death and cell proliferation during tooth development. Together with these results on cell death and cell proliferation, it is suggested that WNT5A induces cell death, inhibiting the development and growth of tooth germs and resulting in smaller teeth (Fig. 5B). Moreover, the cusps of

WNT5A-treated teeth were smaller and much blunter than those of PBS-treated teeth. Since *Wnt5a* showed strong expression in the IDE and the tip of dental papilla at the bell stage, it is also suggested that *Wnt5a* is related to cusp formation.

Cross-talk of WNT5A, *Bmp4*, *Fgf10*, and *Shh* between the mesenchyme and epithelium might regulate tooth development

Bmp4 is expressed in the epithelium at the initiation stage [25] and shifts to the dental mesenchyme thereafter at the early bud stage [3]. The mesenchymally expressed *Bmp4* mimics its effect on the early dental epithelium by inducing morphological changes and the expression of *Msx1*, *Msx2*, *Lef1*, and *Bmp4* itself [3,12,30]. In the present study, the PBS-treated dental mesenchyme showed *Bmp4* expression after 48 h of *in vitro* culture. This result means that *Bmp4* expression can be maintained without dental epithelium and still stimulated by the mesenchymal genes. Moreover, *Bmp4* expression was not detected around beads in non-dental regions of WNT5A-treated tooth germs, while the level of *Bmp4* expression was higher in WNT5A-treated dental mesenchyme than that of PBS-treated mesenchyme. Additionally, it has been reported that the expression of *Bmp4* in the PEK acts as an inducer of cell death in the dental epithelium [9]. However, in our study, a number of TUNEL-positive reactions were observed in the non-dental mesenchyme, which was not detected *Bmp4* expression. These data suggest that *Bmp4* is not related to cell death in the mesenchyme of non-dental regions.

Shh was reported to be involved in pattern formation of several organs including teeth [6]. Moreover, *Shh* is believed to be involved in cell proliferation of the early dental epithelium by activating the expression of its receptor *Ptc* and a transcription factor *Gli1* in teeth [8,30]. *Shh* was suggested to stimulate epithelium and mesenchyme proliferation by a paracrine and

autocrine signaling pathway at the cap stage [4]. *Shh* may exert a direct mitogenic effect in regulating proliferation within dental epithelium at the bell stage, especially on preameloblast and SI cells, at least in part, by promoting *cyclin D1* transcription to control the G1-to-S transition [7]. In the present study, diffused and weak expression of *Shh* was detected in the PBS-treated mesenchyme-free epithelial explants, while WNT5A induced strong expression of *Shh* in the mesenchyme-free epithelial explants. These results suggest that *Shh* expression in the dental epithelium is governed by mesenchymal factors and that *Wnt5a* functions as an inducer of *Shh* expression. *Bmp4* was reported to function upstream of *Shh* in tooth development [3,30]. Therefore, it is possible to think that the expression level of *Shh* might be increased not only by WNT5A directly but also by *Bmp4* which was activated by exogenous WNT5A (Fig.5A). The closed relationship between *Shh* and *Wnt5a* has been reported in hair follicle morphogenesis, suggesting that *Wnt5a* is a target of SHH is supported by both WNT5A and SHH being capable of regulating proliferation [5,19,23,26]. In the present study, the expression patterns of *Wnt5a* and *Shh* also show overlapped and closed spatial distributions in the dental epithelium during tooth development. This expression correlation indicates the presence of an interaction between *Shh* and *Wnt5a*. Therefore, it is suggested that *Wnt5a* as well as *Shh* is an effector in cell proliferation within dental epithelium and mesenchyme [3,7].

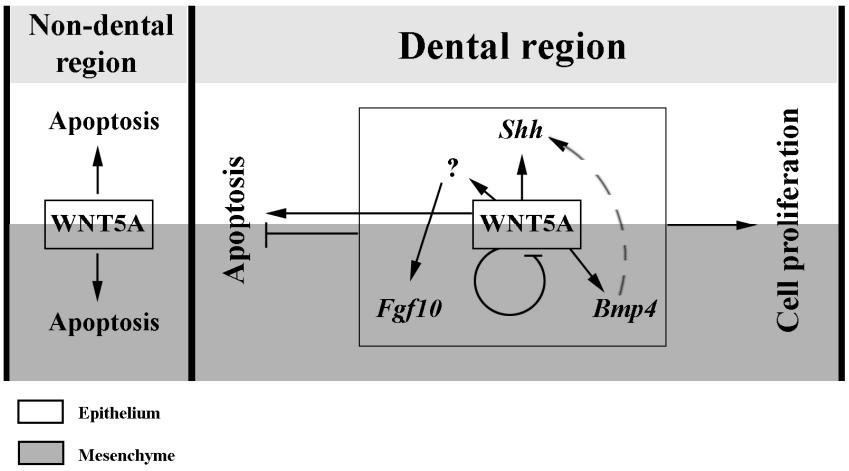
FGF10 was suggested to have redundant functions in developing tooth by regulating tooth morphogenesis at the cap stage by stimulating epithelial cell proliferation in the cervical loops [11]. However, locally applied FGF10 does not influence *Shh* expression; nor does SHH affect *Fgf10* expression [11]. Meanwhile, the slight increase in *Fgf10* expression was not related to the increased expression of *Shh* in this study. The expression of *Fgf10* somehow depends on epithelial mediators, as evidenced by the expression of *Fgf10* being lost in the epithelium-free mesenchyme in the present study (Fig.5A). Therefore, it is suggested that *Fgf10* is induced by epithelial molecules

affected by WNT5A and would function to partly maintain the dental epithelial proliferation in WNT5A-treated tooth germs.

In addition, *Wnt5a* mRNA expression was decreased after WNT5A treatment, suggesting a negative feedback loop of WNT5A was occurred (Fig.5A). This negative feedback loop may be important for regulating the survival and activity of dental region to prevent from cell death. However, this feedback loop regulated by WNT5A itself or by certain negative feedback regulator has to be further elucidated.

During organ development, interactions between epithelium and mesenchyme, communications between neighboring cells of the same tissue are crucial for growth and differentiation. These sequential and reciprocal tissue interactions are mediated by diffusible growth factors [18], which has been confirmed or remains to be elucidated by the new data present here on tooth development.

A)



B)

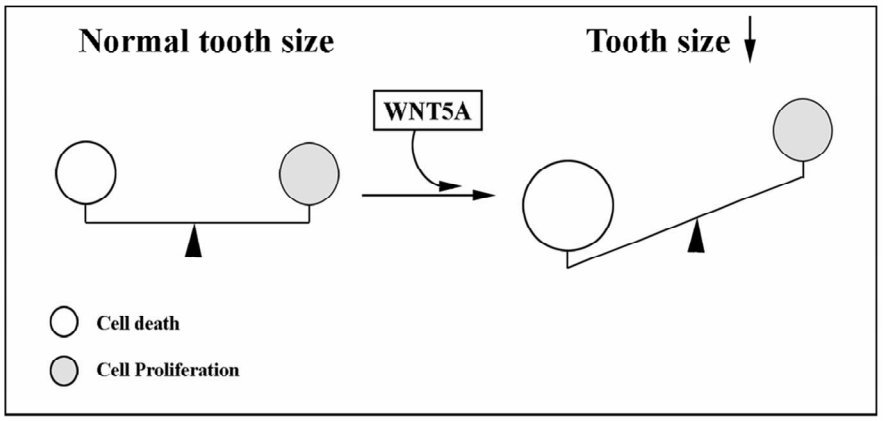


Figure 5. WNT5A induces cell death and breaks the balance between cell proliferation and cell death, which is partly rescued by a *Wnt5a*-involved epithelial–mesenchymal interations on tooth size determination. (A) WNT5A induces cell death in both the dental and non-dental regions. The WNT5A-induced cell death is partly

rescued by the epithelial–mesenchymal interactions in the dental region. In the epithelial–mesenchymal interactions, of the expressions of *Shh* in the epithelium, and *Bmp4* and *Fgf10* in the mesenchyme are induced by exogenous WNT5A directly or indirectly. Especially, *Shh* is suggested to be possibly induced by WNT5A directly, or through *Bmp4* (dotted arrow, Chen et al., 1996). *Fgf10* expression is mediated by certain epithelial factor. However, it can not be rescued by exogenous WNT5A. Moreover, a negative feedback loop of WNT5A is occurred. These epithelial–mesenchymal interactions may be involved in maintenance of cell proliferation for tooth formation in the dental region. Thus, WNT5A-induced cell death in the dental and non-dental regions might result in small teeth. It is suggested that *Wnt5a* acts as a regulator concerning tooth size determination. (B) Normal tooth size is maintained by a balance between cell death and cell proliferation. The balance is altered by exogenous WNT5A with increased apoptotic reactions, resulting in a decrease (arrow) on tooth size.

V. CONCLUSION

In conclusion, exogenous WNT5A induces the cell death, which leads to retarded growth of tooth germs and forms smaller teeth with blunter cusps (Fig. 5B). However, WNT5A-induced cell death was partly rescued by the epithelial–mesenchymal interaction in the dental region. The rescue from cell death and the maintenance of cell proliferation in the dental region may be mediated through the induction of *Fgf10*, *Bmp4*, and *Shh* by WNT5A directly or indirectly (Fig.5A). Taken together, it is suggested that *Wnt5a* evokes cell death in non-dental regions, while *Wnt5a* in the dental region acts as a regulator of other genes concerning cell proliferation, cusp formation and tooth size determination.

REFERENCES

1. Aberg, T., Wozney, J., and Thesleff, I., 1997. Expression patterns of bone morphogenetic proteins (bmps) in the developing mouse tooth suggest poles in morphogenesis and cell differentiation. Dev. Dyn. 230,383-396.
2. Cai, J., Cho, S.W., Kim, J.Y., Lee, M.J., Cha, Y.G., Jung, H.S., 2007. Patterning the size and number of tooth and its cusps. Dev. Biol. 304,499-507.
3. Chen, Y., Bei, M., Woo, I., Satokata, I., Maas, R., 1996. Msx1 controls inductive signaling in mammalian tooth morphogenesis. Development 122,3035-3044.
4. Chen, Z., Zhang, L., Wang, Z.F., Sun, Z.J., Zhang, Q., Fan, B., 2003. Expression of Shh, Ptc1, Ptc2 mRNA in the cap stage of mouse molar. Zhonghua Kou Qiang Yi Xue Za Zhi 38,93-95
5. Chiang, C., Swan, R.Z., Grachtchouk, M., Bolinger, M., Litingtung, Y., Robertson, E.K., Cooper, M.K., Gaffield, W., Westphal, H., Beachy, P.A., Dlugosz, A.A., 1999. Essential role for Sonic hedgehog during hair follicle morphogenesis. Dev. Biol. 205,1-9.
6. Dassule, H.R., Lewis, P., Bei, M., Maas, R., McMahon, A.P., 2000. Sonic hedgehog regulates growth and morphogenesis of the tooth. Development 127, 4775-4785.
7. Gritli-Linde, A., Bei, M., Maas, R., Zhang, X.M., Linde, A., McMahon, A.P., 2002. Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. Development 129,5323-5337.
8. Hardcastle, Z., Mo, R., Hui, C.C., Sharpe, P.T., 1998. The Shh signalling pathway in tooth development: defects in Gli2 and Gli3 mutants. Development 125,2803-2811.

9. Jernvall, J., Aberg, T., Kettunen, P., Keranen, S., Thesleff, I., 1998. The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with cell death in the mouse tooth enamel knot. Development. 125:161-169.
10. Jernvall, J., Thesleff, I., 2000. Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech. Dev. 92,19-29.
11. Kettunen, P., Laurikkala, J., Itaranta, P., Vainio, S., Itoh, N. Thesleff, I., 2000. Associations of FGF-3 and Fgf-10 with signaling networks regulating tooth morphogenesis. Dev. Dyn. 219,322-332.
12. Kratochwil, K., Dull, M., Farinas, I., Galceran, J., Grosschedl, R., 1996. Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. Genes Dev, 10,1382-1394.
13. Li, C., Xiao, J., Hormi, K., Borok, Z., Minoo, P., 2002. Wnt5a participates in distal lung morphogenesis. Dev. Biol. 248,68-81.
14. Luukko, K., Loes, S., Furmanek, T., Fjeld, K., Kvinnsland, I.H., Kettunen, P., 2003. Identification of a novel putative signaling center, the tertiary enamel knot in thepostnatal mouse molar tooth. Mech. Dev. 120,270-276.
15. McMahon, A.P., Bradley, A., 1990. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 62,1073-1085.
16. Miller, J.R., 2002. The Wnts. Genome Biol. 3,REVIEWS3001.
17. Parr, B.A., McMahon, A.P., 1995. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. Nature 374,350-353.
18. Pispa, J., Thesleff, I., 2003. Mechanisms of ectodermal organogenesis. Dev. Biol. 262, 195-205.
19. Reddy, S., Andl, T., Bagasra, A., Lu, M.M., Epstein, D.J., Morrisey, E.E., Millar, S.E., 2001. Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a

target of Sonic hedgehog in hair follicle morphogenesis. Mech. Dev. 107,69-82.

20. Sarkar, L., Sharpe, P.T., 1999. Expression of Wnt signaling pathway genes during tooth development. Mech. Dev. 85,197-200.
21. Sarkar, L., Sharpe, P.T., 2000. Inhibition of Wnt signaling by exogenous Mfrzb1 protein affects molar tooth size. J. Dent. Res. 79,920-925.
22. Stark, K., Vainio, S., Vassileva, G., McMahon, A.P., 1994. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature 372,679-683.
23. St-Jacques, B., Dassule, H.R., Karavanova, I., Botchkarev, V.A., Li, J., Danielian, P.S., McMahon, J.A., Lewis, P.M., Paus, R., McMahon, A.P., 1998. Sonic hedgehog signaling is essential for hair development. Curr. Biol. 8,1058-1068.
24. Suzuki, K., Bachiller, D., Chen, Y.P., Kamikawa, M., Ogi, H., Haraguchi, R., Ogino, Y., Minami, Y., Mishina, Y., Ahn, K., Crenshaw, E.B. 3rd., Yamada, G., 2003. Regulation of outgrowth and cell death for the terminal appendage: external genitalia development by concerted actions of BMP signaling. Development 130,6209-6220.
25. Vainio, S., Karavanova, I., Jowett, A., Thesleff, I., 1993. Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. Cell 75,45-58.
26. Yamaguchi, T.P., Bradley, A., McMahon, A.P., Jones, S., 1999. A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development 126,1211-1223.
27. Yamamoto, H., Cho, S.W., Kim, E.J., Kim, J.Y., Fujiwara, N., Jung, H.S., 2004. Developmental properties of the Hertwig's epithelial root sheath in mice. J. Dent. Res. 83,688-692.

28. Yamashiro, T., Tummers, M., Thesleff, I., 2003. Expression of bone morphogenetic proteins and Msx genes during root formation. J. Dent. Res. 82,172-176.
29. Zhang, Y., Zhao, X., Hu, Y., St Amand, T., Zhang, M., Ramamurthy, R., Qiu, M., Chen, Y., 1999. Msx1 is required for the induction of Patched by Sonic hedgehog in the mammalian tooth germ. Dev. Dyn. 215**,**45-53.
30. Zhang, Y., Zhang, Z., Zhao, X., Yu, X., Hu, Y., Geronimo, B., Fromm, S.H., Chen, Y.P., 2000. A new function of BMP4: dual role for BMP4 in regulation of Sonic hedgehog expression in the mouse tooth germ. Development 127,1431-1443.

ABSTRACT (in Korean)

쥐 치아 발생에 있어 *Wnt5a* 유전자의 주요 역할

쥐 치아 발생은 일련의 상피와 간엽의 상호작용으로 일어나며 이는 복잡한 분자들의 네크워크에 의해 중재된다. 치아크기는 치아 간엽요소들에 의해 결정된다고 알려져 있고 *Bmp2*, *Bmp4* , *Fgf3*, *Fgf10*, and *Lef1* 등 많은 유전자들이 치아 간엽조직에서 발현된다. 그러나 외부에서 주입되는 BMP4, NOGGIN, FGF3 그리고 FGF10 단백질은 모상기 치배에서 치아의 크기에 영향을 주지 않았다고 보고되었다.

치아 발육에 있어 *Wnt* 유전자들중 *Wnt5a* 만이 치성간엽조직에서 발현되는데 본 연구에서는 치아발육중 치배에서의 *Wnt5a* 유전자의 발현양상을 알아보고 치아의 크기를 조절하는 함에 있어 *Wnt5a* 유전자의 역할을 알아보기 위해 모상기의 치배에 WNT5A 단백질을 투여하였다.

실험결과 *Wnt5a* 유전자는 배아기 14 일부터 17 일까지의 기간 동안 간엽뿐만 아니라 상피에서도 발현되었고 이는 *Shh* 및 *Bmp 4* 가 치배에서 발현되는 부위와도 일부 일치한다. 또한 WNT5A 는 dental region 에서는 심하지 않았으나 non-dental region 의 상피와 간엽 모두에서 심하게 세포사멸을 유도함으로써 치배의 발육을 늦췄다. 이러한

dental region 에서의 결과는 *Wnt5a*, *Fgf10*, *Bmp4*, and *Shh* 유전자들의 상피간엽간 상호작용으로 인해 세포사멸이 억제된 결과인 듯하다. WNT5A 에 의해 유도된 세포사멸은 전체적인 치배의 발육을 저해시키고 이는 3 주후에 크기가 작고 교두가 뭉뚝한 형태의 치아를 형성하게 하였다.

결국 *Wnt5a* 유전자는 non-dental region 에서 세포의 사멸을 조절하는데 관여하는 반면 dental region 에서는 세포증식, 교두형성, 치아크기결정등과 연관된 유전자들의 regulator 의 역할을 하는 것 같다.

핵심되는 말: *WNT5A, Shh*, *Bmp4*, 치아 크기, 세포증식, 세포사멸