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Immunolocalization of the Factors Related to Wnt Signaling Pathway in Developing Rat Molar

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Abstract: In the Wnt/ β -catenin signaling pathway, Wnt signal is transmitted to glycogen synthase kinase-3 β (GSK-3 β) through Dishevelled (Dvl), GSK-3 β activity is inhibited, β -catenin phosphorylation is inhibited by the inactive-type GSK-3 β , and β -catenin is transferred to the nucleus where it interacts with lymphoid enhancing factor (LEF)/T-cell factor (TCF), a transcription factor, which is considered to induce and regulate gene expression. In tooth development, it has been reported that Wnt and LEF are expressed at the earliest stage and are related to tooth development, but there are few reports on the situation at a later stage, and there have been no reports on Dvl and GSK-3 β . In this study, we immunohistochemically examined the distribution of factors related to the Wnt/ β -catenin signaling pathway, Wnt10, Dvl, GSK-3 β , p-GSK-3 β (inactive GSK-3 β), and β -catenin, using serial sections of rat first molar germ to investigate the role of the Wnt signaling pathway in tooth germ development and tooth morphogenesis. Immunostaining for anti-Wnt10, anti-Dvl, anti-GSK-3 β , anti-p-GSK-3 β , and anti- β -catenin showed positive reactions at the inner enamel epithelium of tooth germ and weakly positive reactions at the dental papilla cells in contact with the inner enamel epithelium at embryonic day 19. At 8 days after birth, immunostaining for every antibody showed positive reactions for preameloblasts and odontoblasts and more clearly positive reactions for secretory ameloblasts and odontoblasts. These results suggest that Wnt10, Dvl, GSK-3 β , p-GSK-3 β , and β -catenin are distributed in inner enamel epithelium, secretory ameloblasts, and odontoblasts, and that the Wnt/ β -catenin signaling pathway via Dvl and p-GSK-3 β is involved in these cells. In addition, for each of the factors, differentiated secretory cells showed more clearly positive reactions than undifferentiated cells; therefore, we conclude that the Wnt10 signaling pathway may be involved in differentiation to ameloblasts and odontoblasts, as well as secretory functions of ameloblasts and odontoblasts.

Keywords: Dvl, GSK-3 β , β -catenin, Immunolocalization, Developing molar.

Introduction

Wnt signal is related to the formation of axis or organ in embryo and proliferation or differentiation of cells after birth; the signaling pathway is involved in the formation of complicated networks¹⁾. In the canonical Wnt/ β -catenin signaling pathway, when Wnt binds to its receptors, Frizzled and low-density-lipoprotein receptor-related protein (LRP) 5/6, Frizzled then interacts with Dishevelled (Dvl), Dvl controls glycogen synthase kinase-3 β (GSK-3 β) activation, and then inactive-type GSK-3 β inhibits phosphorylation of β -catenin, which prevents β -catenin destruction. Axin, which is a scaffold protein of β -catenin destruction complex, becomes unstable due to the inactive-type

GSK-3 β , then the complex is destroyed and β -catenin is released. As a result, it is considered that intracellularly accumulated β -catenin is transferred to the nucleus, interacts with lymphoid enhancing factor (LEF)/T-cell factor (TCF), a transcription factor, and induces or regulates gene expression^{1), 2), 3)}.

Regarding Wnt in tooth development, many studies have been reported on the early stage of tooth germ formation. It has been reported that Wnt-4, -6, -7b, and -10b localize in the dental epithelial thickenings and the epithelium of the bud stage tooth germs, Wnt-5a is distributed in the mesenchyme⁴⁾, and Wnt-10a is localized in the enamel knot at the early stage and in the underlying mesenchyme or odontoblasts at the late bell stage⁵⁾. With regard to β -catenin, it has been reported that the gene is expressed in the inner enamel epithelium and enamel knot, and Wnt regulates β -catenin activation in tooth development⁶⁾. It has

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also been reported from study on tooth germs in *LEF1*^{-/-} mice that intraepithelial Wnt10 mediates the transcriptional activation of the fibroblast growth factor (FGF) 4 gene in the epithelium via β -catenin and LEF1, FGF4 then signals to the subjacent mesenchyme, and FGF3 in mesenchyme in turn induces expression of Sonic hedgehog (Shh) in the epithelium⁷. Furthermore, it has been reported from study on Wnt signal inhibitor Dickkopf (*Dkk1*) that Wnt/ β -catenin signaling is activated at multiple stages of tooth development; Wnt signaling can initiate tooth development, the results of which can expand into the expression of multiple signaling factors, BMP-4 and Msh homeobox homolog (*Msx1/2*, among others⁸). Namely, it has been considered that the Wnt/ β -catenin pathway can initiate the formation of tooth germ in the dental lamina, and plays a key role in tooth morphogenesis, such as the formation of molar cusps. However, this was reported from investigations of Wnt or β -catenin, but there have been no reports on investigations of the intermediate factors in the Wnt signaling pathway. With regard to the stage after the late bell stage, there have been very few reports on investigations of the Wnt/ β -catenin signaling pathway.

It has been reported that gene expression of β -catenin is positive in inner enamel epithelium, ameloblasts, and preodontoblasts, whereas it is negative in differentiated odontoblasts.⁶ However, in this reported study, matrix formation by the odontoblasts was unclear and the odontoblasts were not in the secretory stage during which matrix formation is active. In the active tooth morphogenesis stage, it has been reported that Wnt-10a regulates dentin sialophosphoprotein (DSPP) and is involved in odontoblast differentiation and tooth morphogenesis since Wnt-10a mRNA was found to be expressed in preodontoblasts and odontoblasts and it showed striking colocalization with DSPP expression⁹. On the other hand, it has been reported that Wnt-1 and β -catenin inhibit dental pulp stem cell differentiation *in vitro*⁹. Therefore, these reports have some conflicting results, and it was difficult to definitively conclude that the Wnt/ β -catenin signaling pathway is involved in odontoblast and ameloblast differentiation as well as matrix formation.

Therefore, in this study, to clarify whether the Wnt/ β -catenin signaling pathway is involved in ameloblast and odontoblast differentiation and matrix formation by these cells, we immunohistochemically investigated the distributions of factors involved in the Wnt/ β -catenin signaling pathway (*wnt10*, *Dvl*, *GSK-3 β* , β -catenin) at the early bell stage immediately before differentiation of ameloblasts and odontoblasts, as well as at the late bell stage by which time matrix formation by these cells is underway, using rat molar tooth germ.

Materials and Methods

Tissue preparation

The experiment was performed using Sprague–Dawley (SD) rats at embryonic day 19 and 8 days after birth, according to the Guidelines for the Handling of Experimental Animals of Tokyo Dental College.

The rats were fixed by perfusion or immersion in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde or 0.01 M periodic acid–0.075 M lysine–2% paraformaldehyde (PLP). Samples were fixed and decalcified with 10% ethylenediamine-tetraacetic acid (EDTA) or triethyl-ammonium EDTA in 80% ethanol¹⁰. The first molar tooth germ with surrounding tissues was removed, embedded in paraffin, and longitudinal sections 5 μ m in thickness were prepared. Some of the sections were treated with hematoxylin and eosin (H-E) staining.

Immunostaining

The paraffin sections were deparaffinized, and the antigen was activated and unmasked by irradiating the sections twice with 400-W microwaves in 0.01% citric acid buffer (pH 6.0) for 15 min. Free aldehyde radicals were removed by immersing the sections in 0.1% glycine PBS for 30 min, and the endogenous peroxidase activity was eliminated by immersion in 0.3% H₂O₂, 0.1% NaN₃, and PBS for 30 min. Blocking was performed for 1 h with normal goat or rabbit serum (1:10). The sections were incubated overnight at room temperature with the following diluted primary antibodies in 1% bovine serum albumin (BSA) PBS: Wnt10 (C-19) (anti-Wnt10b C-terminus, goat polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA; 1:50) (detectable Wnt10a and Wnt10b), *Dvl* (G-19) (anti-Dvl N-terminus, goat polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA; 1:50), *GSK-3 β* (H-76) (anti-GSK-3 β C-terminus, rabbit polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA; 1:50), p-GSK-3 β (Ser9) (anti-short amino-acid sequence-containing phosphorylated serine 9 of GSK-3 β ; GSK-3 β activity is negatively regulated by phosphorylation on Ser-9, goat polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA; 1:50), and β -catenin (E-17) (anti- β -catenin, amino acids 758–771, mouse monoclonal antibody; Chemicon International, Temecula, CA; 1:50). Samples were then treated with a secondary antibody: peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) (H+L) (Zymed LA, San Francisco, CA; 1:50), rabbit anti-goat IgG (H+L) (Zymed LA, San Francisco, CA; 1:50), or goat anti-mouse IgG (H+L) (MP Biomedicals LLC-Cappel Products, USA; 1:50), at room temperature for 1 h and color-developed with a Ni²⁺–Co²⁺–DAB solution¹¹.

For observations related to antibody reactions, negative control sections were produced by treating the sections with rabbit-IgG (1:50), goat-IgG (1:50), or mouse-IgG (1:50).

Results

H-E staining

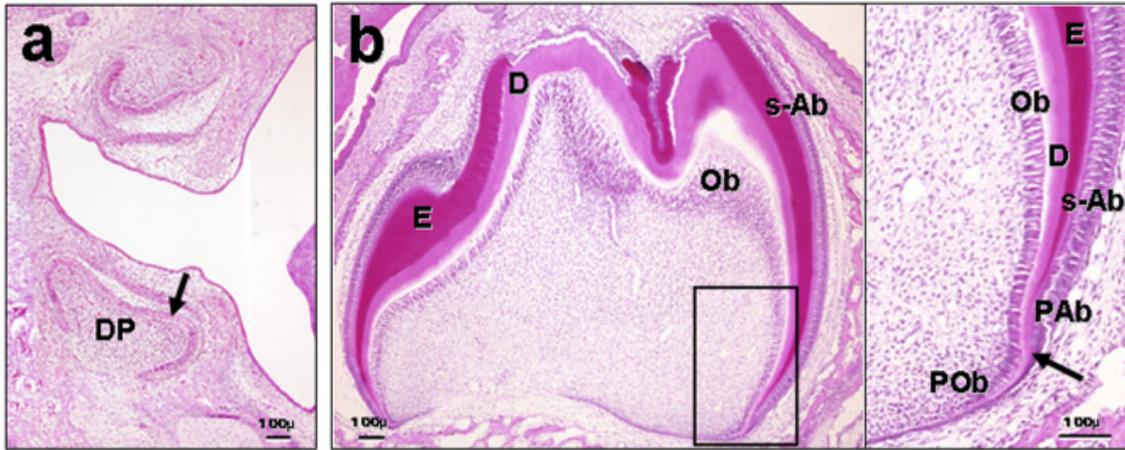


Figure 1. Hematoxylin and eosin (H-E) staining.

a: Rat first molar germ on embryonic day 19. The germ was in the early bell stage before calcification. Dense mesenchymal cells before differentiation to odontoblasts were distributed in the dental papilla (DP) along the inner enamel epithelium (arrow) before differentiation to ameloblasts. Bars 100 μ m.

b: Rat maxillary first molar germ 8 days after birth. Enamel (E) and dentin (D) have formed in the cusp. Near the apical end (arrow), differentiation from preodontoblasts (POb) and preameloblasts (PAb) into odontoblasts (Ob) and secretory ameloblasts (s-Ab) was observed. Bars 100 μ m. The boxed areas are enlarged to the right of the panel.

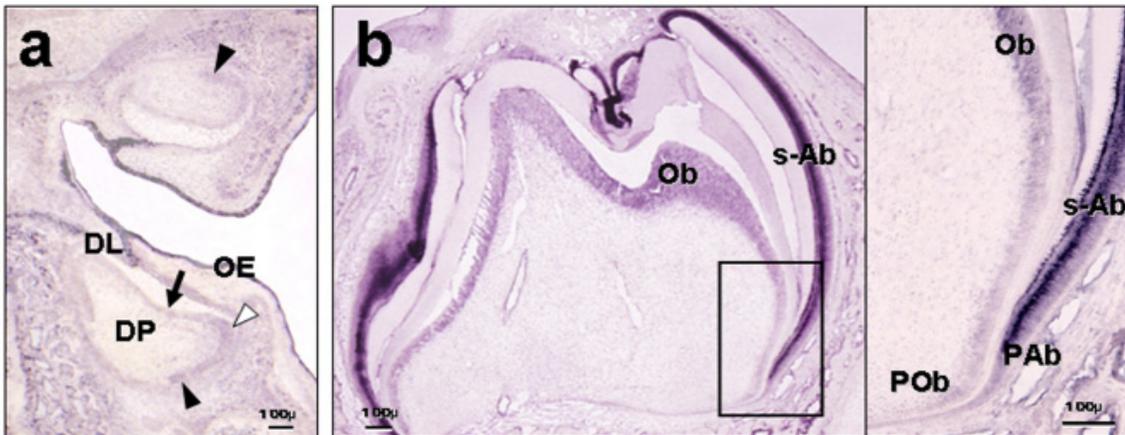


Figure 2. Immunohistochemical observation of Wnt10

a: Rat first molar germ on embryonic day 19. Positive reactions were observed in the inner enamel epithelium (arrow), cervical loop (black arrowhead), and secondary enamel knot (white arrowhead) in tooth germ, along with the oral epithelium (OE) and the dental lamina (DL). More weakly positive reactions were observed in dental papilla (DP) cells in contact with the enamel epithelium. Bars 100 μ m.

b: Rat maxillary first molar germ 8 days after birth. Clearly positive reactions were observed at the odontoblasts (Ob) and secretory ameloblasts (s-Ab). More weakly positive reactions were observed in the preodontoblasts (POb) and preameloblasts (PAb). Bars 100 μ m. The boxed areas are enlarged to the right of the panel.

The germ on embryonic day 19 was in the early bell stage before calcification. The inner enamel epithelium showed a high columnar cell layer before differentiation to ameloblasts. In addition, dense mesenchymal cells before differentiation to odontoblasts were distributed in the dental papilla along the inner enamel epithelium (Fig. 1a). In the first molar germ on 8 days after birth, tall columnar secretory-stage ameloblasts and odontoblasts were distributed with enamel or dentin in the cusp. In near the apical end of the dental neck, differentiation from preodontoblasts and preameloblasts into odontoblasts and

secretory-stage ameloblasts was observed (Fig. 1b).

Immunostaining

Anti-Wnt10: Clearly positive reactions were observed at the oral epithelium and the dental lamina on embryonic day 19. In the early bell stage tooth germ, the positive reactions were observed in the inner enamel epithelium, cervical loop, and secondary enamel knot. In addition, more weakly positive reactions were observed in dental papilla cells in contact with the inner enamel epithelium than in the epithelium (Fig. 2a). In the

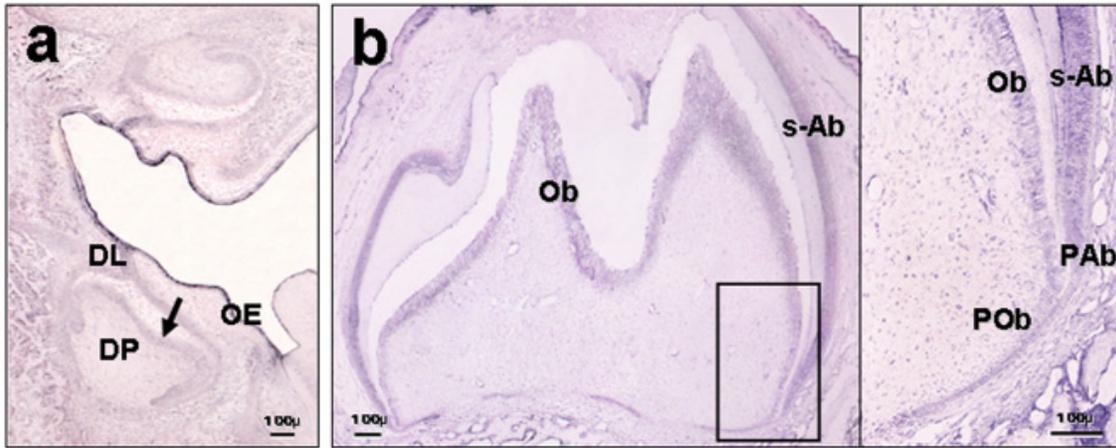


Figure 3. Immunohistochemical observation of Dvl

a: Rat first molar germ on embryonic day 19. Positive reactions were observed in the inner enamel epithelium (arrow) along with the oral epithelium (OE) and the dental lamina (DL). In addition, the most weakly positive reactions were observed in dental papilla (DP) cells in contact with the enamel epithelium. Bars 100 μ m.

b: Rat maxillary first molar germ 8 days after birth. Positive reactions were shown in the odontoblasts (Ob) and secretory ameloblasts (s-Ab). The preodontoblasts (POb) and preameloblasts (PAb) showed weaker reactions than odontoblasts and secretory ameloblasts. Bars 100 μ m. The boxed areas are enlarged to the right of the panel.

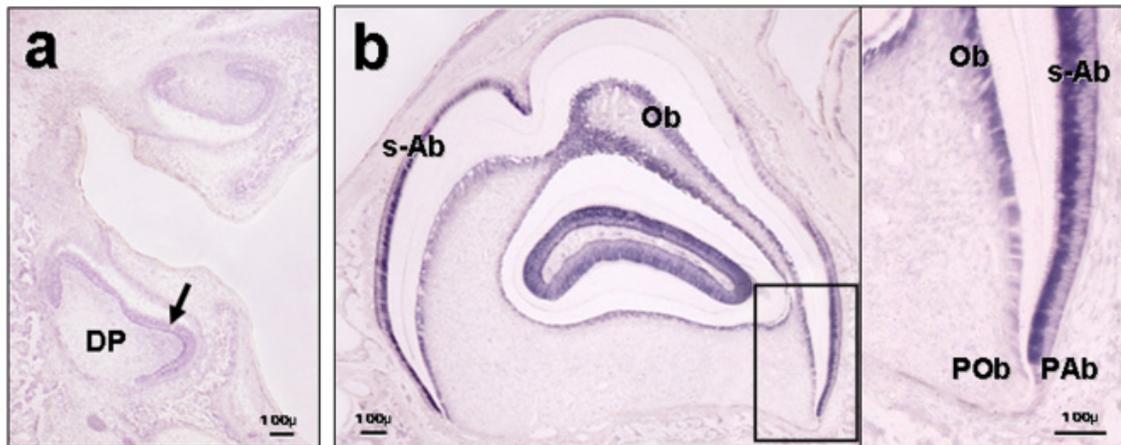


Figure 4. Immunohistochemical observation of GSK-3 β

a: Rat first molar germ on embryonic day 19. The immunostaining showed positive reactions in the inner enamel epithelium (arrow) and weakly positive reactions in dental papilla (DP) cells in contact with the epithelium. Bars 100 μ m.

b: Rat maxillary first molar germ 8 days after birth. Clearly positive reactions were observed in the odontoblasts (Ob) and secretory ameloblasts (s-Ab). The reactions in the preodontoblasts (POb) and preameloblasts (PAb) were weak and tended to be clearer with differentiation into odontoblasts or secretory ameloblasts. Bars 100 μ m. The boxed areas are enlarged to the right of the panel.

first molar germ on 8 days after birth, clearly positive reactions were observed in the odontoblasts and secretory ameloblasts. In addition, more weakly positive reactions than these were observed in the preodontoblasts and preameloblasts (Fig. 2b).

Anti-Dvl: The immunostaining at embryonic day 19 showed positive reactions in the oral epithelium, the dental lamina, and the inner enamel epithelium. Mostly weakly positive reactions were observed in dental papilla cells in contact with the inner enamel epithelium (Fig. 3a). The immunostaining at 8 days after birth showed positive reactions in the odontoblasts and secretory

ameloblasts. The reactions in the preodontoblasts and preameloblasts were weak and tended to appear more clearly with differentiation into odontoblasts or secretory ameloblasts (Fig. 3b).

Anti-GSK-3 β : In the first molar germ on embryonic day 19, positive reactions were observed at the inner enamel epithelium. Dental papilla cells in contact with the epithelium showed weakly positive reactions (Fig. 4a). In the germ on 8 days after birth, clearly positive reactions were observed in the odontoblasts and secretory ameloblasts. The preodontoblasts and preameloblasts

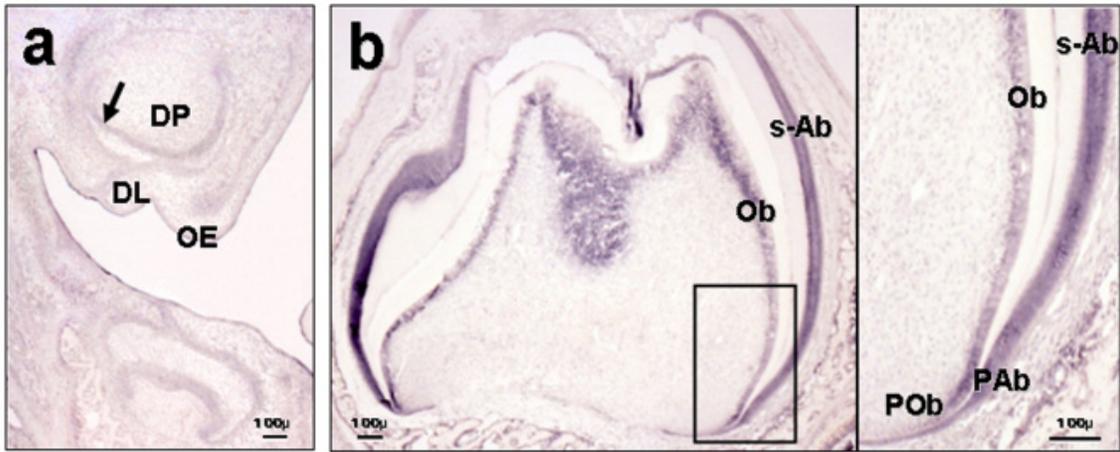


Figure 5. Immunohistochemical observation of p-GSK-3 β

a: Rat first molar germ on embryonic day 19. Positive reactions were observed in the inner enamel epithelium (arrow), the oral epithelium (OE), and the dental lamina (DL). In addition, weak reactions were observed in dental papilla (DP) cells in contact with the enamel epithelium. Bars 100 μ m.

b: Rat maxillary first molar germ 8 days after birth. The immunostaining revealed clearly positive reactions in the odontoblasts (Ob) and secretory ameloblasts (s-Ab). In the preodontoblasts (POb) and preameloblasts (PAb), weaker reactions were observed. Bars 100 μ m. The boxed areas are enlarged to the right of the panel.

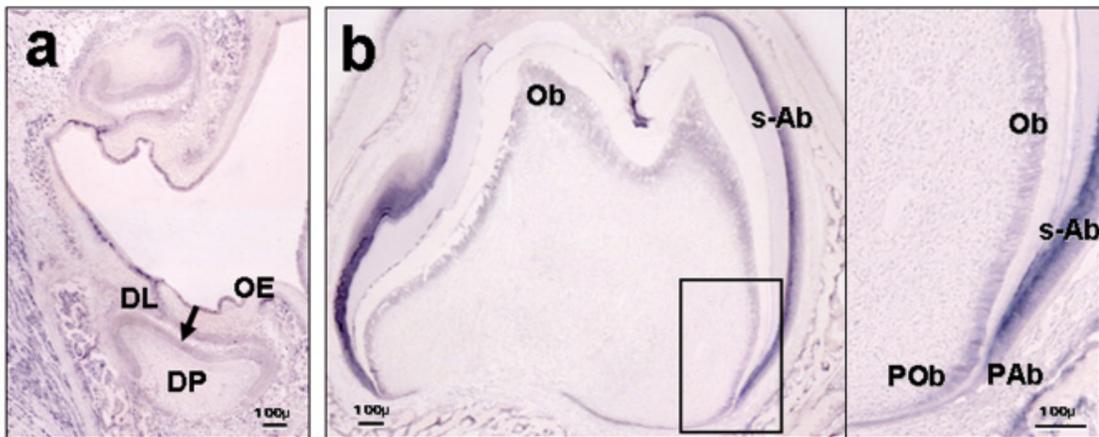


Figure 6. Immunohistochemical observation of β -catenin

a: Rat first molar germ on embryonic day 19. Positive reactions were observed at the inner enamel epithelium (arrow) along the oral epithelium (OE) and the dental lamina (DL). Dental papilla (DP) cells in contact with the epithelium showed weakly positive reactions. Bars 100 μ m.

b: Rat maxillary first molar germ 8 days after birth. The immunostaining showed clearly positive reactions in the secretory ameloblasts (s-Ab), and positive reactions in the odontoblasts (Ob), preodontoblasts (POb), and preameloblasts (PAb). Bars 100 μ m. The boxed areas are enlarged to the right of the panel.

showed weaker reactions than odontoblasts and secretory ameloblasts (Fig. 4b).

Anti-p-GSK-3 β : The immunostaining at embryonic day 19 revealed positive reactions in the oral epithelium, the dental lamina, and the inner enamel epithelium. In addition, weak reactions were observed in dental papilla cells in contact with the inner enamel epithelium (Fig. 5a). The immunostaining at 8 days after birth revealed clearly positive reactions similarly to those for anti-GSK-3 β in the odontoblasts and secretory ameloblasts. In addition, weaker reactions were observed in the preodontoblasts

and preameloblasts (Fig. 5b).

Anti- β -catenin: In the first molar germ on embryonic day 19, clearly positive reactions were observed at the inner enamel epithelium with the oral epithelium and the dental lamina. Dental papilla cells in contact with the epithelium showed weakly positive reactions (Fig. 6a). In the germ on 8 days after birth, clearly positive reactions were observed in the secretory ameloblasts. The odontoblasts, preodontoblasts, and preameloblasts also exhibited positive reactions (Fig. 6b).

In the control group, no specific reactions were observed.

Table 1 Immunohistochemical distribution of Wnt10, Dvl, GSK-3 β , p-GSK-3 β , and β -catenin during rat tooth development

	Wnt10	Dvl	GSK-3 β	p-GSK3 β	β -catenin
Inner enamel epithelium	+	+	+	+	+
Dental papilla cells (Contact with the enamel epithelium)	±	±	±	±	±
Preameloblast	+	±	+	+	+
Ameloblast(secretory stage)	++	+	++	++	++
Preodontoblasts	+	±	+	+	+
Odontoblast	++	+	++	++	+

Dvl: Dishevelled, GSK-3 β : glycogen synthase kinase-3 β , p-GSK-3 β : phosphorylated GSK-3 β (inactive GSK-3 β).
 ++, Strong immunoreactivity; +, moderate immunoreactivity; ±, weak immunoreactivity.

On the basis of these results, Wnt10, Dvl, GSK-3 β , p-GSK-3 β , and β -catenin showed similar distribution patterns in the inner enamel epithelium, preameloblasts, secretory-stage ameloblasts, dental papilla cells (in contact with the enamel epithelium), preodontoblasts, and odontoblasts. Concerning ameloblasts, their distribution increased with differentiation from inner enamel epithelium into secretory-stage ameloblasts. Regarding odontoblasts, their distribution increased with differentiation from dental papilla cells in contact with the enamel epithelium into odontoblasts (Table 1).

Discussion

Wnt/ β -catenin signaling pathways in ameloblasts (secretory-stage) and progenitors

In the early stage of tooth germ, Wnt-4, -6, -7b, and -10 are distributed in the epithelium, and Wnt-10b in particular is distributed in the inner and outer enamel epithelium except enamel knot at the cap stage, and is distributed in the inner enamel epithelium at the early bell stage⁴. It has also been reported that the distribution increases in the cervical loop¹². On the other hand, Wnt-10a is distributed in enamel knot and it has been considered that Wnt-10a is involved in the morphogenesis of tooth crown⁵. In this study, anti-Wnt10, detectable as both Wnt10a and Wnt10b, showed positive reactions in the inner enamel epithelium including cervical loop and second enamel knot in embryonic day 19 rat. This finding agrees with the above-mentioned reports^{5, 12}, confirming that Wnt-10 signal induces the proliferation or the regulation of enamel epithelium cells and is involved in morphogenesis of tooth crown. In mouse incisor at embryonic day 18, it has been reported that Wnt-10a is expressed in differentiating ameloblast and is associated with ameloblast differentiation¹³. In this rat molar tooth germ 10 days after birth, Wnt10 showed clearly positive reactions in ameloblasts, at the secretory stage; therefore, it is considered that Wnt10 may be involved in differentiation to ameloblasts and secretion of enamel matrix.

Regarding Dvl, which interacts with Frizzled receptor of Wnt, it has been reported that Dact2, which binds to Dvl and regulates the Wnt signaling pathway, distributed in enamel epithelium and preameloblasts¹⁴, but there have been no previous reports on Dvl and tooth development. In this study, anti-Dvl showed a reaction identical to that of Wnt10 in the inner enamel epithelium, preameloblasts, and secretory-stage ameloblasts, confirming that Wnt signal transmission is mediated by Dvl in these cells. GSK-3 β phosphorylates β -catenin in β -catenin destruction complex including Axin, and β -catenin is degraded through the ubiquitin-proteasome pathway. When activation of GSK-3 β is inhibited by Wnt signaling, β -catenin accumulates intracellularly, is transferred to the nucleus, and interacts with a transcription factor (LEF/TCF), which is considered to induce and control gene expression¹¹. Therefore, it has been considered that the activation or inactivation of GSK-3 β has a role as the change-over switch by which Wnt signaling is or is not communicated downstream. However, in tooth development, there have been no reports on GSK-3 β . In this study, both anti-p-GSK3 β (inactive-type and GSK-3 β activity is negatively regulated by phosphorylation) and anti-GSK3 β (reacts with any of GSK3 β) exhibited positive reactions in the inner enamel epithelium and ameloblasts, and the reactions tended to be clearer with differentiation from preameloblasts into secretory-stage ameloblasts. The reactions for the two antibodies showed similar patterns, confirming that the reaction by anti-GSK-3 β was mainly with the inactive-type p-GSK3 β . In addition, these immune reactions showed the same patterns in the above reactions in Wnt10 and Dvl. Therefore, in inner enamel epithelium, preameloblasts, and secretory-stage ameloblasts, it is considered that the Wnt signaling through Dvl regulates GSK-3 β activity and inhibits β -catenin destruction.

With regard to β -catenin, which accumulates intracellularly, is transferred to the nucleus, binds to a transcription factor, and induces gene expression, it has been reported that β -catenin gene is expressed in inner enamel epithelium and enamel knot at the early stage of tooth germ, and is expressed in ameloblasts at a

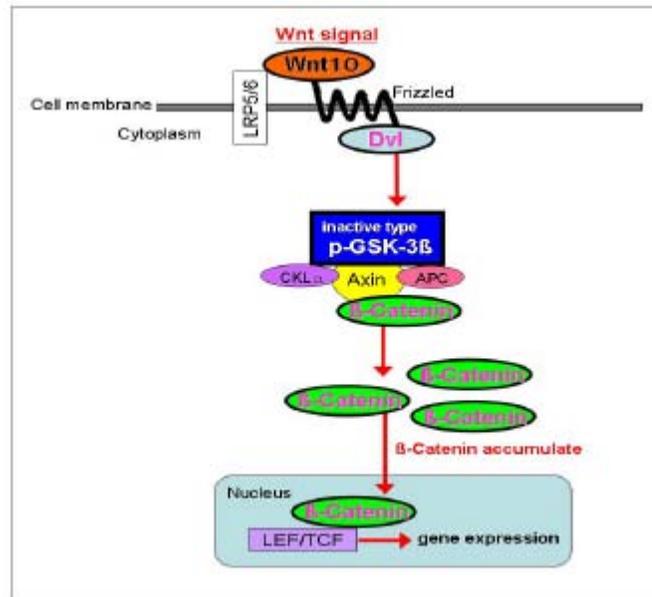


Figure 7 Proposed model of the Wnt10/ β -catenin signaling pathway in ameloblasts and odontoblasts. We present the Wnt10/ β -catenin signaling pathway in ameloblasts and odontoblasts. Wnt10 signaling through Dishevelled (Dvl) regulates glycogen synthase kinase-3 β (GSK-3 β) activation, inactive-type GSK-3 β prevents β -catenin destruction, intracellular β -catenin is transferred to the nucleus and interacts with lymphoid enhancing factor (LEF)/T-cell factor (TCF), a transcription factor, which induces gene expression. This may be involved in the differentiations to ameloblasts or odontoblasts as well as the secretory functions of ameloblasts and odontoblasts¹⁹.

later stage of tooth germ⁶. In this study, the distribution of anti- β -catenin was observed in inner enamel epithelium, preameloblasts, and secretory-stage ameloblasts, and showed similar gene expression patterns to those of the above-mentioned report. In addition, the reaction pattern of anti- β -catenin agreed with these reaction patterns for antibodies of Wnt10, Dvl, GSK-3 β , and p-GSK-3 β , confirming that Wnt10 signaling acts on a transcription factor (LEF/TCF) through Dvl, GSK-3 β , and β -catenin, and is involved in gene expression in the above cells.

It has been reported that Wnt10 signals mediate the transcriptional activation of the FGF4 gene via LEF1 and β -catenin in the odontogenic epithelium, FGF4 induces FGF3 in mesenchyme, and FGF3 induces Shh in epithelium with other mesenchymal factors, suggesting that β -catenin is involved in these inductions by epithelial-mesenchymal interaction⁷. In addition, it has been reported that Shh signaling, which was induced by the epithelial-mesenchymal interaction, regulates cell proliferation and differentiation in dental epithelium, and controls polarization of secretory-stage ameloblasts by Smoothed (Smo), which is a factor in the Shh signaling pathway, in mutant mouse¹⁵. These reports suggest that Wnt10 signaling was communicated to β -catenin, involved in proliferation and differentiation of enamel epithelium by epithelial-mesenchymal interaction, and involved in the secretion of enamel protein by ameloblast polarization. In our study, the antibody-positive reactions of Wnt10, Dvl, p-GSK-3 β , and β -catenin were observed in the secretory-stage

ameloblasts. Therefore, it has been suggested that the Wnt signaling through Dvl, GSK-3 β , and β -catenin may be involved in secretory functions by direct action on ameloblasts in addition to epithelial-mesenchymal interaction.

Wnt/ β -catenin signaling pathways in odontoblasts and progenitors

In tooth germ mesenchyme, gene expressions of Wnt-5a and Wnt-10a have been reported^{4, 5, 16}. For Wnt-10a in particular it has been reported that the expression was observed in enamel knot in early tooth germ, then shifted to the underlying mesenchymal cells, and was observed in preodontoblasts and odontoblasts at a later stage⁵. In addition, regarding odontoblast differentiation, Wnt-10a gene expression is involved in dentin calcification and is correlated with increased gene expression of DSPP, especially in dentin, so it has been reported that Wnt-10a induces DSPP expression and is involved in odontoblast differentiation⁵. The anti-Wnt-10 in this study showed clear positive reactions in dental papilla cells in contact with enamel epithelium, preodontoblasts, and odontoblasts, and showed stronger reactions according to the differentiation. These findings confirmed that Wnt-10 is involved in odontoblast differentiation, as well as the secretion of matrix proteins, such as DSPP, by odontoblasts.

It has been reported that Dvl-binding protein, Dact3, is expressed in dental mesenchyme, including preodontoblasts. In

addition, Dact3 is considered to regulate the Wnt signaling pathway in preodontoblasts against Dact2 regulation in enamel epithelium and preameloblasts¹⁴. The anti-Dvl showed positive reactions not only in enamel epithelium but also in dental papilla cells, preodontoblasts, and odontoblasts, with a similar tendency to Wnt10. Accordingly, it is suggested that Wnt signal transmission is mediated by Dvl at the downstream phase in the above mesenchymal cells, and Dact is considered to be involved in the transmission for this regulation.

For GSK-3 β and p-GSK-3 β , there have been no reports on mesenchymal cells in tooth germ, but in preosteoblasts, the same as in mesenchymal cells, it has been reported in cell culture that GSK-3 β phosphorylation was induced by Wnt, the proportion of p-GSK-3 β /GSK-3 β increased, and as a result the levels of β -catenin increased¹⁷. Antibodies against both GSK-3 β and p-GSK-3 β similarly reacted in dental papilla cells, preodontoblasts, and odontoblasts, and these reaction patterns agreed with the reaction patterns of Wnt-10, Dvl, and β -catenin. Therefore, it has been suggested that the reaction of anti-GSK-3 β in the above mesenchymal cells was mainly by p-GSK-3 β , inactive-type GSK-3 β , and Wnt-10 signaling through Dvl regulation of GSK-3 β activation; it is considered that, as a result, β -catenin accumulation increases.

Regarding β -catenin in tooth germ mesenchymal cells, it has been reported that β -catenin gene expression was positive in preodontoblasts, whereas it was negative in differentiated odontoblasts⁶, and β -catenin inhibits dental pulp stem cell differentiation⁹. However, in a recent study, it has been reported that a β -catenin reporter gene, BAT-gal (β -catenin-activated transgene driving expression of nuclear β -galactosidase reporter), was expressed in odontoblasts in the mouse incisor¹⁸. In this study, positive reactions of anti- β -catenin were observed in dental papilla cells in contact with enamel epithelium, preodontoblasts, and odontoblasts; therefore, it has been suggested that Wnt10 signaling passed through β -catenin, and was involved in differentiation of the above cells. Furthermore, it has been considered that this Wnt10/ β -catenin signaling pathway is involved in dentin calcification via DSPP.

In summary, we hypothesize that, in ameloblasts and odontoblasts, Wnt10 signaling through Dvl regulates GSK-3 β activation, inactive-type GSK-3 β prevents β -catenin destruction, and intracellular β -catenin is transferred to the nucleus and interacts with a transcription factor (LEF/TCF), which induces gene expression (Fig. 7). As a result, it is considered that the Wnt10/ β -catenin signaling pathway may be involved in the differentiations to ameloblasts and odontoblasts, as well as the secretory functions of ameloblasts and odontoblasts.

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