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ORIGINAL ARTICLE

Claudin rather than occludin is essential for differentiation in rat incisor odontoblasts

M Hoshino[1], S Hashimoto[2,3], T Muramatsu[2,3], M Matsuki[2], H Ogiuchi[1], M Shimono[2,3]

[1]*Department of Oral and Maxillofacial Surgery, School of Medicine, Tokyo Women's Medical University, Tokyo, Japan;* [2]*Department of Pathology, Tokyo Dental College, Chiba, Japan;* [3]*Oral Health Science Center HRC7, Tokyo Dental College, Chiba, Japan*

Many morphological and developmental studies have demonstrated the characteristics of tight junctions (TJs) between odontoblasts. However, detailed localization of TJ-associated proteins in odontoblasts and their functions has not yet been clarified. To elucidate the relationship between the establishment of TJ structures and the differentiation of odontoblasts during early dentinogenesis, we studied the expression and localization of constituent proteins of TJs (claudin-1, occludin, ZO-1 and ZO-2) between odontoblasts in rat lower incisors using Western blotting, immunofluorescence and immunoelectron microscopy. When the expression of claudin-1 increases at the distal portion of mature odontoblasts, the TJs form complex networks of strands, and odontoblasts differentiated by developing distal membrane domains and by secreting specific molecules for mineralization. We conclude that the TJs of odontoblasts may play an important role in the differentiation of odontoblasts in rat lower incisors during early dentinogenesis.
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Keywords: tight junction; odontoblast; occludin; claudin; ZO-1; ZO-2

Introduction

Tight junctions (TJs) are located at the most distal portions of lateral membranes between adjacent epithelial cells, and are thought to function as boundaries between apical and basolateral plasma membrane domains to create and maintain their cellular polarity (Rodriguez-Boulan and Nelson, 1989). TJs also function as a primary barrier against diffusion of solutes through the paracellular pathway (Gumbiner, 1987, 1993; Schneeberger and Lynch, 1992). TJs are mainly composed of transmembrane proteins (including claudin and occludin) and cytoplasmic proteins (such as ZO-1, ZO-2 and ZO-3) (Tsukita *et al*, 1999). Claudin is an integral membrane protein of TJ-strands that is a member of a multigene family consisting of more than 20 proteins (Tsukita *et al*, 2001). The permeability barrier functions of TJs depend on the combination and ratio of different claudins (Tsukita *et al*, 2001). Occludin is a constituent protein of TJ-strands that contributes to TJ barrier functions (Furuse *et al*, 1993; McCarthy *et al*, 1996; Hirase *et al*, 1997). ZO-1 is a key cytoplasmic protein assembled at TJs that binds to claudin and occludin as well as to the actin cytoskeleton (Itoh *et al*, 1997; Fanning *et al*, 1998) while ZO-2 is another cytoplasmic protein that can also bind to the actin cytoskeleton and can form independent complexes with ZO-1.

Dentinogenesis is a sequential process that involves the differentiation of odontoblasts from undifferentiated ectomesenchymal cells to the dental papilla, establishing their cellular polarity and their ability to secrete dentin matrix proteins that subsequently mineralize (Nanci, 2003). During dentinogenesis, odontoblasts possess TJs that constitute the junctional complex (JC) with adherens junctions and desmosome-like structures analogous to epithelial cells (Sasaki *et al*, 1982). Many morphological and developmental studies have demonstrated the characteristics of TJs between odontoblasts (Iguchi *et al*, 1984; Calle, 1985; Sasaki and Garant, 1996). Ultrastructural studies have shown that TJs can be observed as multiple focal fusions (Sasaki *et al*, 1982; Iguchi *et al*, 1984). Furthermore, freeze-fracture studies have demonstrated strands of intramembrane particles at the most distal portions of odontoblasts (Sasaki *et al*, 1982; Iguchi *et al*, 1984; Calle, 1985; Arana-Chavez and Katchburian, 1997, 1998). However, the relationship between expression and localization of TJ-associated proteins and differentiation processes of odontoblasts during dentinogenesis is still unclear.

The objective of this study was to investigate the expression and localization of TJ-associated proteins,

Correspondence: Dr T Muramatsu, Department of Pathology, Tokyo Dental College, 1-2-2, Masago, Mihama-ku, Chiba 261-8502, Japan. Tel: +81 43 270 3782, Fax: +81 43 270 3784, E-mail: tmuramat@tdc.ac.jp
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including claudin-1, occludin, ZO-1 and ZO-2, between odontoblasts in rat lower incisors during early dentinogenesis.

Materials and methods

Rat lower incisors were employed in this study to observe the complete sequence of early dentinogenesis and odontoblast differentiation in the same specimens. Based on the criteria of Takuma and Nagai (1971), we divided odontoblast differentiation into five stages – (a) differentiating papilla cell (PC), (b) pre-odontoblast (PO), (c) young odontoblast (YO), (d) old odontoblast (OO) and (e) short odontoblast (SO) (Figure 1A).

Animals and tissue preparations
Fifty adult male Sprague–Dawley rats, weighing 150–200 g each, were used in this study. The animals were deeply anesthetized with an intraperitoneal injection of sodium thiopental and were then killed. This study was conducted in accordance with the Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College.

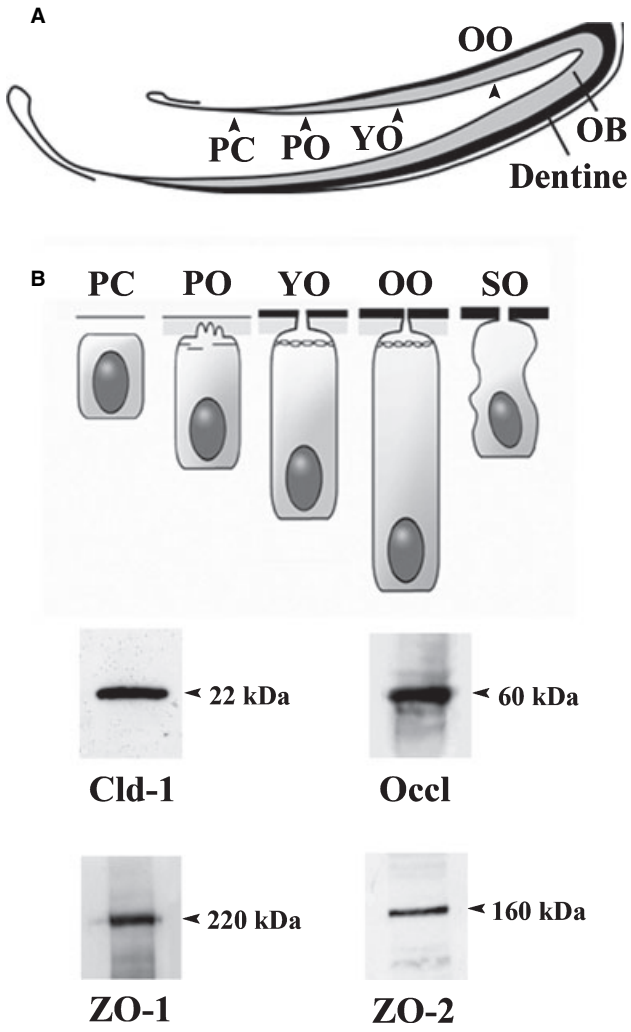

Figure 1 (**A**) Schematic representation of Takuma and Nagai's classification dividing odontoblast differentiation into five stages – (a) differentiating papilla cell (PC), (b) pre-odontoblasts (PO), (c) young odontoblast (YO), (d) old odontoblasts (OO) and (e) short odontoblast (SO) – on the basis of their cytological characteristics. (**B**) The single band of claudin-1, occludin, ZO-1, and ZO-2 was detected approximately at 22, 60, 220 and 160 kDa, respectively

Primary antibodies
Rabbit anti-occludin polyclonal antibody, rabbit anti-claudin-1 polyclonal antibody, rabbit anti-ZO-1 and ZO-2 polyclonal antibodies were all from Zymed Laboratories, Inc. (South San Francisco, CA, USA).

Western blotting analysis
For Western blotting analysis, dental pulp tissues including odontoblasts were obtained from lower incisors, homogenized at 4°C using a Teflon-glass homogenizer with a loose-fitting pestle, and total proteins were extracted. Fifty micrograms of total protein was separated on 7.5% ready gels (Bio-Rad Laboratories, Hercules, CA, USA) at room temperature and were transferred to polyvinylidene fluoride membranes and incubated overnight at 4°C with primary antibodies. The membranes were blotted at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Sigma, St Louis, MO, USA) for 1 h and were visualized using ECL^{TM} Western Blotting Detection Reagents (Amersham Biosciences UK, Ltd, Buckinghamshire, UK).

Immunofluorescence microscopy
For immunofluorescence, the lower incisors were extracted from rat mandibles and were embedded in 10% agarose gels and stored at 4°C. The extruded incisors were carefully cut at the apical side and were divided into two parts with agarose gel. The most apical part of each piece was embedded in OCT compound (Tissue-Tek®; Sakura Finetechnical Co., Ltd, Tokyo, Japan) and was quickly frozen in liquid nitrogen; thereafter, 8-μm frozen longitudinal sections were prepared. The frozen sections were rinsed in 0.01 M phosphate-buffered saline (PBS) and were subsequently incubated for 10 min with 10% goat serum in PBS. The sections were then incubated with primary antibodies (diluted 1:100) at room temperature for 2 h. After washing in PBS, the specimens were incubated with Alexa fluor®488 anti-rabbit IgG antibody (1:75 dilution; Molecular Probes, Inc., Eugene, OR, USA) in PBS at 4°C for 1 h. The specimens were observed and photographed using a conventional fluorescence microscope, Axioplan 2 (Carl Zeiss Jena GmbH, Jena, Germany).

Immunoelectron microscopy
For immunoelectron microscopy, the animals were killed by perfusion with fixative solution containing 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The extracted lower incisors were dissected into small pieces, dehydrated with graded ethanol and embedded in LR white resin (London Resin, Hampshire, UK) at 4°C with UV light. Ultrathin sections (90–95 nm in thickness) were prepared, and were immediately incubated in 0.01 M

PBS with 0.1 M glycine and 10% bovine serum albumin (PGB). They were then incubated at 37°C for 2 h with primary antibodies at a 1:50 dilution in PGB. After rinsing in PGB, sections were incubated at room temperature for 1 h with anti-rabbit gold colloidal particles diluted 1:75 in PGB. Finally, post-staining with 6% uranyl acetate and Reynold's lead was performed. The specimens were examined and photographed using a transmission electron microscope (H-7100, Hitachi Co, Tokyo, Japan).

Results

Western blotting analysis
Western blotting analysis revealed distinct single bands of claudin-1, occludin, ZO-1 and ZO-2, approximately at 22, 60, 220 and 160 kDa, respectively, in the apical part of the dental pulp (Figure 1B).

Immunofluorescence microscopy
We observed odontoblasts in each stage of early differentiation, such as PC, PO and YO (Figure 2A), according to Takuma and Nagai's classification (Figure 1A). That classification is based on the cytological characteristics of odontoblasts in different stages of development and maturation.

Expression of TJ-associated proteins (claudin-1, occludin, ZO-1 and ZO-2) was clearly detected at most distal portions of odontoblasts corresponding to the JC. However, expression patterns of these four proteins in differentiating odontoblasts were different (Figure 2B).

Expression of claudin-1
Immunoreactivity for claudin-1 was relatively weak at the most distal portion of odontoblasts in the early stage of differentiation (Figure 2Ba) and gradually increased its intensity as the differentiation of odontoblasts proceeded (Figure 2Bb). Expression of claudin-1 was clearly detected at distal portions of odontoblasts corresponding to the distal JC (Figure 2Bc), and formed network structures in the advanced stage (Figure 2Bd).

Expression of occludin
Immunolabeling for occludin was clearly observed at the most distal site of odontoblasts in the early stage of differentiation (Figure 2Be). As the differentiation of odontoblasts proceeded, immunoreactivity for occludin decreased (Figure 2Bf), and eventually disappeared in the advanced stage (Figure 2Bg).

Expression of ZO-1
Positive immunoreaction for ZO-1 was strongly detected at the most distal portion of odontoblasts in the early stage (Figure 2Bh), and did not change and could be clearly recognized as the differentiation of odontoblasts proceeded (Figure 2Bi,j), and formed network structures as observed in claudin-1 (Figure 2Bk).

Expression of ZO-2
Immunoreactivity for ZO-2 was found at the most distal portion of odontoblasts, although it was relatively weak in the early stage (Figure 2Bl). ZO-2 gradually increased in intensity of staining as the differentiation of odontoblasts proceeded (Figure 2Bm), and was clearly detected at the distal portions of odontoblasts, corresponding to the distal JC in the advanced stage (Figure 2Bn). ZO-2 also formed network structures as shown in claudin-1 (Figure 2Bo).

Immunoelectron microscopy
Ten nanometre colloidal gold particles indicating claudin-1, occludin, ZO-1 and ZO-2 were discernible by immunoelectron microscopy at the JC of odontoblasts (Figure 3). Claudin-1 was localized at cell-to-cell contacts observed at the most distal part of the JC which corresponded to TJs (Figure 3A), while occludin and ZO-1 were diffusely distributed through the JC including both tight and adherens junctions (Figure 3B,C), ZO-2 was also detected at cell-to-cell contacts localized at the most distal portion of the JC, which might suggest their localization in TJs (Figure 3D).

Discussion

Tight junctions are well-developed structures that are observed in odontoblasts as well as in epithelial cells (Sasaki *et al*, 1982; Iguchi *et al*, 1984; Calle, 1985). Freeze fracture replicas demonstrate that TJs between odontoblasts are observed as small maculae or fasciae occludens rather than belt-like zonulae (Sasaki *et al*, 1982; Iguchi *et al*, 1984; Arana-Chavez and Katchburian, 1997). It has been demonstrated that radial bundles of collagen fibers run through distal JC without interruption in the intercellular spaces between adjacent odontoblasts, and penetrate the collagen networks of the pre-dentin (Salomon *et al*, 1991; Bishop and Yoshida, 1992; Tabata *et al*, 1994, 1995). The structures of TJs in odontoblasts are different from those in epithelial cells, implying functional differences of TJs between epithelial cells and odontoblasts. Recently, TJ-associated proteins (claudin-1, occludin, ZO-1 and ZO-2) have been identified and are believed to reflect cell-specific regulation and functional demands. However, the expression and localization of TJ-associated proteins in odontoblasts have not yet been characterized. Therefore, we investigated the expression and localization of claudin-1, occludin, ZO-1 and ZO-2 in odontoblast layers of rat lower incisors.

In the process of forming epithelial cell-to-cell adhesions, primordial spot-like junctions are formed at the tips of thin cellular protrusions radiating from adjacent cells (Takai and Nakanishi, 2003). ZO-1 assembles at these spot-like junctions and recruits other TJ-associated proteins, including claudin and occludin (Ando-Akatsuka *et al*, 1999; Asakura *et al*, 1999). Furthermore, ZO-1 is associated with the formation of polarization in epithelial cells (Itoh *et al*, 1997; Fanning *et al*, 1998). Our results show that expression of ZO-1 is intensely observed from the early stage of odontoblasts (PCs), which suggests that ZO-1 may be necessary for initiation of the assembly of TJs in rat incisor odontoblasts.

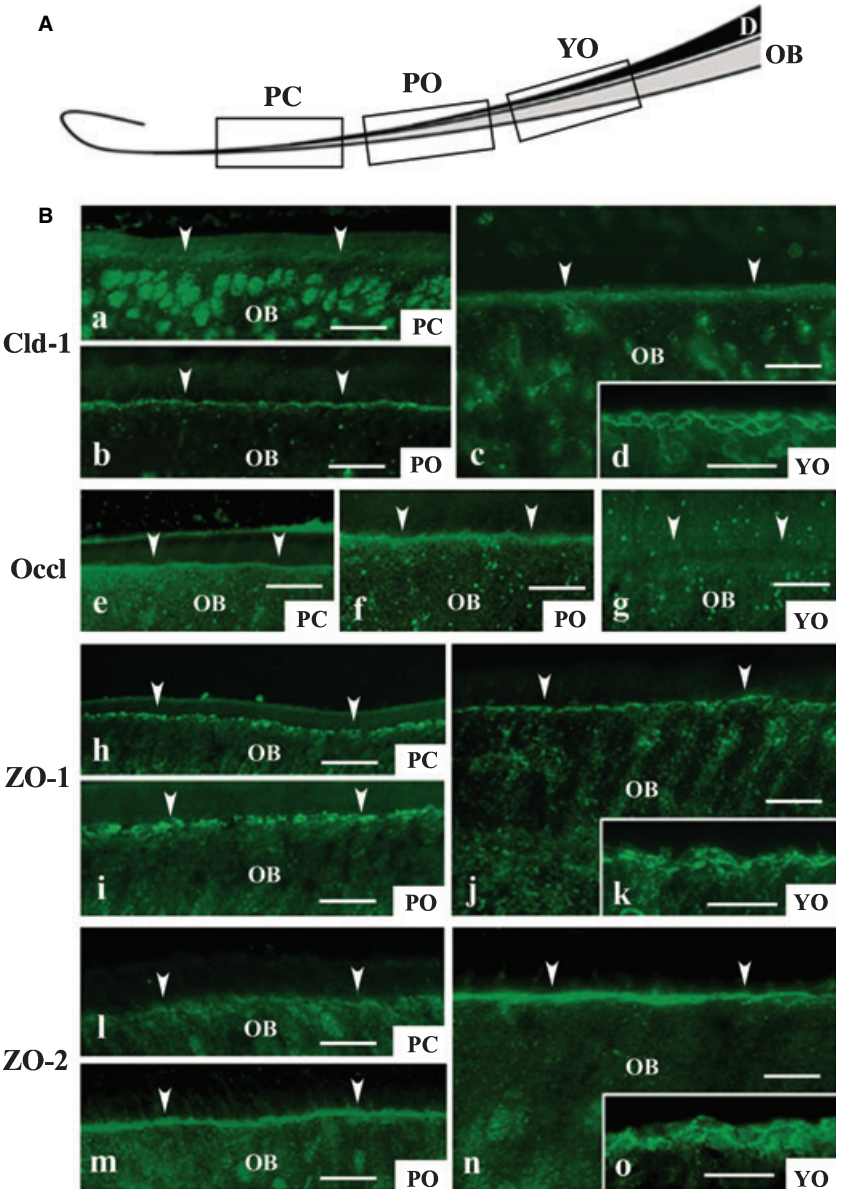

Figure 2 (**A**) Schematic representation of early stages of odontoblast differentiation in a rat lower incisor, showing observation area of each stages – differentiating papilla cell (PC), pre-odontoblasts (PO), and young odontoblast (YO) – by immunofluorescence microscopy. (**B**) Light micrograph showing immunolabeling for claudin-1, occludin, ZO-1, and ZO-2 in early dentinogenesis in rat lower incisors. Immunolabeling for claudin-1 (a–d). (a) Light micrograph showing relatively weak reaction for claudin-1 (arrowheads) at the most distal portion of PC. (b) Immunolabeling for claudin-1 (arrowheads) gradually increased as differentiation progressed in PO. (c) In more advanced stage (YO), clear reaction of claudin-1 (arrowhead) is observed at the most distal part of odontoblasts corresponding to the distal junctional complex (JC). (d) At higher magnification, immunoreaction for claudin-1 showing the mesh network structures is evident in YO. Immunolabeling for occludin (e–g). (e) Light micrograph showing clear expression of occludin (arrowhead) at the most distal portion of PC. (f) Although immunoreaction for occludin (arrowheads) is also detected in PO, it gradually reduces its intensity as differentiation proceeds. (g) Immunoreaction for occludin eventually appears to be absent in YO (arrowheads). Immunolabeling for ZO-1 (h–k). (h) Light micrograph showing intense labeling for ZO-1 (arrowheads) at the most distal portion of PC corresponding to the distal JC. (i, j) Clear reaction for ZO-1 (arrowheads) is observed through the differentiation process from PC to YO. (k) At higher magnification, immunolabeling for ZO-1 is detected as mesh network-like structures similar to that of claudin-1 in YO. Immunolabeling for ZO-2 (l–o). (l) Light micrograph showing relatively weak reaction for ZO-2 (arrowheads) at the most distal portion of PC. (m, n) Positive reaction is gradually increased and clear reaction for ZO-2 (arrowheads) is observed at the most distal portion of PO and YO. (o) At higher magnification, immunolabeling for ZO-2 is also recognized as mesh network-like structures similar to that of claudin-1 in YO. (D, dentin) Bars = 20 μm (a, b, c, e, f, g, h, i, j, l, m, n), bars = 10 μm (d, k, o)

Freeze-fracture studies have shown that TJs between odontoblasts appear as short rows of fused particles during early mantle dentin mineralization and become more complex, eventually forming networks of fused particles in advanced mineralization (Iguchi *et al*, 1984; Sasaki and Garant, 1996; Arana-Chavez and Katchburian, 1997). This suggests that TJs in odontoblasts is associated with dentin mineralization. Our results also show that immunoreaction of claudin-1 and ZO-2 is relatively weak in PCs, and gradually increases in

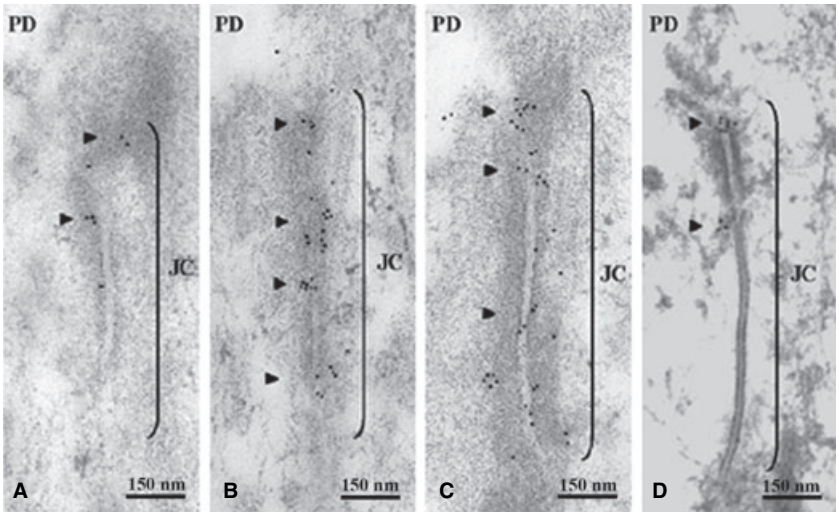

Figure 3 Immunoelectron microscopic localization of claudin-1, occludin, ZO-1 and ZO-2 in PO. (**A**) Immunoelectron microscopic image showing 10 nm gold particles indicating claudin-1 were localized at cell–cell contact point observed at the most distal part of the distal JC that might correspond to TJs of odontoblasts (arrowheads). (**B**) Immunoelectron microscopic image showing 10 nm gold particles indicating occludin were diffusely distributed through the distal JC of odontoblasts (arrowheads). (**C**) Immunoelectron microscopic image showing 10 nm gold particles indicating ZO-1 were diffusely distributed through the distal JC of odontoblasts (arrowheads). (**D**) Immunoelectron microscopic image showing 10 nm gold particles indicating ZO-2 were localized at cell–cell contact point observed at the most distal part of the distal JC that might correspond to TJs of odontoblasts (arrowheads). PD, pre-dentin; JC, junctional complex

intensity at the distal portions of odontoblasts as they differentiate (PO and YO). Furthermore, the intensity of staining increased with dentin mineralization. These results suggest that claudin-1 and ZO-2 are involved in dentin mineralization.

Immunohistochemically, the localization of TJ-associated proteins was investigated at the distal portion of odontoblasts, which corresponds to the distal JC. Furthermore, our immunoelectron microscopic findings indicate that ZO-1 and occludin are diffusely distributed in the JC, while claudin-1 and ZO-2 are localized at the most distal part of the JC, which may correspond to TJs. Particularly, claudin-1 and ZO-2 showed intermittent accumulation of gold particles at cell-to-cell contacts of opposing cells. These results support previous studies that used freeze-fracture replicas to show that the distal ends of plasma membranes of odontoblasts show numerous TJs formed by fused particles and grooves, and that branched and continuous rows of fused particles constitute TJs of the focal or macular type (Sasaki *et al*, 1982; Iguchi *et al*, 1984; Calle, 1985; Arana-Chavez and Katchburian, 1997, 1998).

Saitou *et al* (1998) showed that occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells, and function as a primary barrier against diffusion of a low molecular mass tracer through the paracellular pathway, which suggests that expression of occludin may not be necessary for the assembly of claudin-1 at the TJ area. Furthermore, in tracer studies using lanthanum nitrate, some studies have shown that the tracer can permeate freely between the odontoblasts from the pulp tissues to the pre-dentin (Tanaka, 1980; Iguchi *et al*, 1984). Earlier reports imply that occludin may not affect TJ strand formation or the barrier function of intercellular permeability between odontoblasts. Our results show that occludin disappears in YO, the stage where between-cell calcium transport is frequently observed. This suggests that expression of occludin may not be necessary for assembly of claudin-1 at TJ area, and disappearance of occludin may not affect TJ strand formation and barrier function of intercellular permeability between odontoblasts.

As the expression of claudin-1 increases at the distal portion of mature odontoblasts (YO), TJs form a complex network of strands and membrane cholesterol and rigidity of the distal plasma membrane increase; these events may indicate the establishment of a distal membrane domain (Gumbiner, 1990). At the same stage, odontoblasts become to be polarized, and reorganized cell organelles, matrix vesicle formation ceases, and finally secrete calcium-binding molecules, such as phosphoproteins, to ensure the progression of mineralization (Inage and Toda, 1988; Begue-Kirn *et al*, 1998), and in fact dentin matrix begins to mineralize. As mineralization of dentin matrix starts almost simultaneously with the formation of TJs (Arana-Chavez and Katchburian, 1997), we surmise that TJs between odontoblasts may participate in determining the direction of mineral secretion and establishing the distal membrane domain (Figure 4).

Conclusion

Tight junction-associated proteins, such as claudin-1, occludin, ZO-1and ZO-2, between odontoblasts of rat lower incisors demonstrated different patterns of expression. We conclude that TJs of odontoblasts in rat lower incisors may play an important role in the differentiation

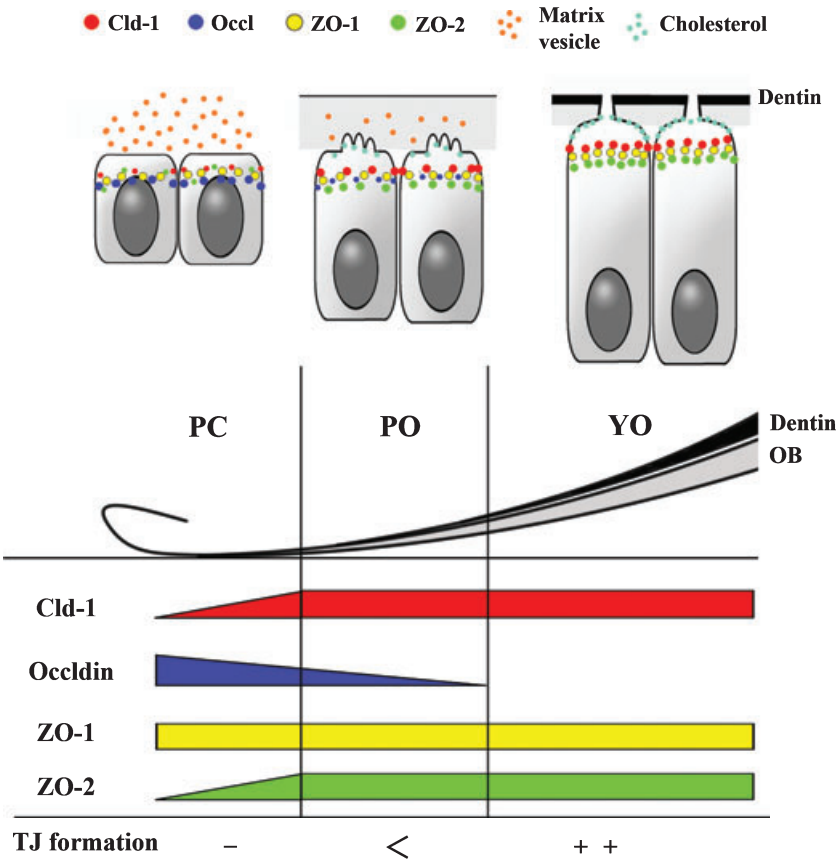

Figure 4 Summary of tight junction (TJ)-associated protein expression and odontoblast differentiation including establishment of distal membrane domain, ability or disability of molecule secretion, and TJ formation, in relation to dentin mineralization. In the early stage of odontoblasts (differentiating papilla cell, PC), immunoreaction for claudin-1 and ZO-2 is relatively weak, and the TJs form short rows of fused particles. Matrix vesicle releasing and mantle dentin formation is rich. On the other hand, expression of occludin is clear in this stage. As the expression of claudin-1 and ZO-2 increases at the distal portion of mature odontoblasts (young odontoblast, YO), the TJs form a complex network of strands, and membrane cholesterol and rigidity of the distal plasma membrane increase, which indicates the establishment of distal membrane domain. Immunoreaction for occludin is gradually reduced and eventually appears to be absent (YO). At the same stage, the odontoblasts become polarized, matrix vesicle secretion ceases, and dentin matrix begins to mineralize to form the circum dentin. Immunolabeling for ZO-1 is clearly observed through the differentiation process from PC to YO

of odontoblasts in early dentinogenesis, especially in determining the direction of mineral secretion and establishing the distal membrane domain.

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