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Dynamic assembly of tight junction-associated proteins ZO-1, ZO-2, ZO-3 and occludin during mouse tooth development

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Summary. Tight junctions might play a role during tissue morphogenesis and cell differentiation. In order to address these questions, we have studied the distribution pattern of the tight junction-associated proteins ZO-1, ZO-2, ZO-3 and occludin in the developing mouse tooth as a model. A specific temporal and spatial distribution of tight junction-associated proteins during tooth development was observed. ZO-1 appeared discontinuously in the cell membrane of enamel organ and dental mesenchyme cells. However, endothelial cells of the dental mesenchyme capillaries displayed a continuous fluorescence at the cell membrane. Inner dental epithelium first showed an evident signal for ZO-1 at the basal pole of the cells at bud/cap stage, but ZO-1 was accumulated at the basal and apical pole of preameloblast/ameloblasts at late bell stage. Surprisingly, in the incisor ZO-1 decreased as the inner dental epithelium differentiated, and was re-expressed in secretory and mature ameloblasts. On the contrary, ZO-2 was confined to continuous cell-cell contacts of the enamel organ in both molars and incisors. The lateral cell membrane of inner dental epithelial cells was specifically ZO-2 labeled. However, ZO-3 was expressed in oral epithelium whereas dental embryo tissues were negative. In addition, occludin was hardly detected in dental tissues at the early stage of tooth development, but was distributed continuously at the cell membrane of endothelial cells of ED19.5 dental mesenchyme. In incisors, occludin was detected at the cell membrane of the secretory pole of ameloblasts. The occurrence and relation during tooth development of tight junction proteins ZO-1, ZO-2 and occludin, but not ZO-3, suggests a combinatory assembly in tooth morphogenesis and cell differentiation.

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Introduction

The developing rodent tooth is a good model to study organogenesis, including initiation, morphogenesis and cell differentiation. It is regulated by inductive and reciprocal interactions between epithelial and mesenchymal cells (Thesleff and Sharpe, 1997). The first morphological sign of tooth development is a thickening of the oral epithelium, which subsequently buds into the underlying neural crest-derived mesenchyme. This mesenchyme becomes surrounded by the dental epithelium and forms the dental papilla. The mesenchymal odontoblasts and the epithelial ameloblasts differentiate terminally and deposit the organic matrix of dentin and enamel, respectively (Ten Cate, 1998).

Cell-cell contacts in tooth epithelium and mesenchyme may play a role in histomorphogenesis and cell differentiation. The contacts between odontoblasts are found at the junction between the cell body and the odontoblast process in the form of a modified junctional complex. This complex is formed by fascia adherens, fascia and/or maccula occludens, and gap junctions (reviewed by Sasaki and Garant, 1996). Adherens junctions (AJ) in the enamel organ and secretory ameloblasts have been reported (Sasaki et al., 1984; Nishikawa et al., 1990). Changes in the distribution of AJ, and desmosomal and hemidesmosomal proteins during development of molars have been published (Fausser et al., 1998).

Studies have speculated on the role of cell junctions in remodeling and cell differentiation in organogenesis (Hubber et al., 1996; Balda and Matter, 1998; Mitic and Anderson, 1998). Tight junctions (TJ, zonula occludens)

are comprised of a diversity of peripheral and transmembrane proteins (Stevenson and Keon, 1998; Tukuse and Furuse, 1998, 1999; Balda and Matter, 2000; Zahraoui et al., 2000), which might play a role in regulating the development and normal function of the cells (Balda, 1998). The TJ was described as a well-developed intercellular adhesion apparatus in simple epithelial and endothelial cells, which is located at the most apical part of their lateral membranes (Farquhar and Palade, 1963). In this region the plasma membrane of adjacent cells forms a series of contacts that occlude the extracellular space. The TJ seals the intercellular space and is responsible for the separation of apical and basolateral fluid compartments of epithelia and endothelia. The TJ functions as a diffusion barrier to plasma membrane lipids and proteins, which helps to define apical and basolateral membrane domains of polarized cells (Wong and Gumbinr, 1997; Mitic and Anderson, 1998). TJ proteins, which have been reported, include occludin, claudin, junctional adhesion molecule (JAM) and the proteins ZO-1, ZO-2 and ZO-3 (ZO, zonula occludens), which couple occludin and claudin to the cytoplasmic plaque. Actin filaments, cingulin, 7H6, Rab3B, Rab8, Rab 13, symplekin, AF-6, ASIP/Par3, Sec 6 and Sec8 are also found at the TJ (for a recent review see Zaharaoui et al., 2000).

Occludin (Furuse et al., 1993) is the major transmembrane protein localized at the TJ. This protein concentrates within the TJ fibrils, to confer cell-cell adhesion and to function in the permeability barrier.

Claudin has been described as a family of at least 16 proteins that are responsible for TJ-specific obliteration of the intercellular space in epithelial and endothelial cell sheets (Furuse et al., 1998, Kubota et al., 1999; Tsukita and Furuse, 2000). Some results have provided direct evidence of the pivotal role of the claudin family in generating the paracellular physical barrier of tight junctions necessary for spermatogenesis and normal CNS system (Gow et al., 1999).

Junctional adhesion molecule (JAM) (Martin-Padura et al., 1998) is a member of the Ig superfamily, which behaves as a component of endothelial and epithelial junctions, and influences the paracellular transmigration of monocytes. A plaque of cytoplasmic proteins under the junction may be responsible for scaffolding the transmembrane proteins, creating a link to the perijunctional actin cytoskeleton and transducing regulatory signals that control the paracellular barrier (Dejana et al., 2000).

Zonula occludens 1 (ZO-1) is a member of a family of putative signal-transduction proteins termed MAGUK (Membrane-associated guanylate kinase homologues) localized at cell-cell contacts (Mitic and Anderson, 1998; Gonzalez-Mariscal et al., 2000). It has been suggested that ZO-1 may anchor occludin at the membrane and instruct its polymerization. The TJ-associated membrane protein ZO-1 (Stevenson et al., 1986) has been localized in epithelial cells and occasionally colocalized with cadherins in cells lacking TJs, such as fibroblasts and cardiac muscle cells (Itoh et al., 1991, 1993; Horwarth et al., 1992; Tsukita et al., 1992). A recent report has described that ZO-1 function may contribute to epithelial/mesenchymal transition (Reichert et al., 2000). ZO-1 binds claudins, occludin, ZO-2, ZO-3 cingulin and actin (Cordenonsi et al., 1999; Witchen et al., 1999).

ZO-2 was identified as a ZO-1 binding protein by immunoprecipitation (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994). Both ZO-1 and ZO-2 may contribute to the structural organization of components mediating particular signal transduction (Fanning et al., 1996). New findings show that CASK/LIN-2, a MAGUK protein required for EGF receptor localization and signaling, has a transcription regulation function (Hsueh et al., 2000). In addition, ZO-3 has been reported as a novel member of the MAGUK protein family that directly interacts with ZO-1 and occludin (Haskins et al., 1998). To date, occludin/ZO-1, ZO-1/ZO-2, ZO-1/ZO-3 and ZO-1/actin interactions have been characterized based on a biochemistry approach (Wittchen et al., 1999). It has also been demonstrated that ZO-1, ZO-2 and ZO-3 bind to the COOH-terminal YV sequence of claudin 1 to 8 through their PDZ1 domains in vitro (Itoh et al., 1999).

Tight junction formation during development is critical for embryonic patterning and organization. However, information on molecular assembly and functional regulation of TJ proteins during organogenesis is scarce. Data concerning formation, distribution and function of TJ proteins during tooth development is lacking. In order to study the developmental dynamics of TJ-associated proteins and the possible role in morphogenesis and cell differentiation, we have analyzed the temporal and spatial expression of ZO-1, ZO-2, ZO-3 and occludin during tooth development.

Materials and methods

Antibodies

Rabbit anti-ZO-1, -ZO-2 (Zymed Laboratories, Inc., CA, USA), and -ZO-3 (Chemicon International, Inc., CA, USA) polyclonal antibodies were raised against aminoacids 463-1109 of human ZO-1 protein, a peptide from the central portion of the ZO-2 protein or canine ZO-3 (aminoacids 754-898). The polyclonal rabbit anti-occludin (Zymed Laboratories, Inc., CA, USA) was raised against the C-terminus of human occludin. All antibodies were used at 1 μg/ml for immunoblotting and 10 μg/ml for immunofluorescence.

MDCK cell culture

Madin-Darby canine kidney (MDCK) cells, donated by Dr. JS Gutkind, were cultivated in culture flasks in DMEM medium, supplemented with 5% foetal calf serum, 50 i.u./ml penicillin, 50 mg/ml streptomycin, at

37 ºC in a humidified atmosphere containing 5% CO2. MDCK cells, growing on sterilized glass slides were fixed and immunofluorescence for ZO-1, -2, -3 and occludin was performed as described for tissue cryosections.

Tissue preparation, cryosections and histology

On embryonic day (ED) 13.5, 14.5, 17.5 and 19.5 pregnant Swiss mice were killed by cervical dislocation. Embryo heads were frozen in dry ice and stored at –80 °C. Coronal or sagittal sections at a thickness of 10 μm containing first molar or incisor were cut on a cryostat at –20 °C. Sections were mounted on glass slides and stored at –80 °C until use.

Some embryo heads were fixed in Bouin, dehydrated in ethanol and embedded in paraplast. Sections (5 μm) were stained with hematoxilin-eosin.

Immunoblot assay

E14.5, E17.5 and E19.5 mouse embryo first lower molars were dissected away from mandibles under a stereomicroscope. Molars were suspended in a lysis buffer containing 10 mM Tris-HCl, 1% TritonX-100, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 1mmol/L phenylmethylsulphonyl fluoride, 0.28 U/ml aprotinin, 50mg/ml leupeptin and 7 mg/ml pepstatin. Samples were sonicated with a microtip sonicator until reaching clarity and the protein content determined by the bicinchoninic acid method with a kit from Sigma. Alliqots of 25-50mg of total protein were electrophoresed on 12% SDS polyacrylamide gels and transferred to nitrocellulose filters. Membranes were washed with 10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20 (TBS, pH 7.4), blocked with 5% non-fat dry milk in TBS, and then incubated with rabbit anti-ZO-1, anti-ZO-2, anti-ZO-3 or anti-occludin antibodies for 2 h at room temperature. After washings in TBS, blots were incubated for 1h with peroxidase-conjugated antibodies. The membranes were developed with ECL (Amershan Pharmacia Biotech) according to the manufacturers' instructions.

Immunofluorescence

Frozen sections were pre-warmed at room temperature and air-dried for 30 minutes. Sections were rinsed in PBS and fixed with methanol (for ZO-1, ZO-2 and ZO-3 detection) or acetone at –20 ºC for 10 minutes (for occludin detection). The slides were rinsed in PBS, blocked with 1% BSA in PBS for 30 min and incubated with primary antibody (or PBS for control sections) for 1 hour at room temperature. After rinsing in PBS, tissue sections were incubated for 1 hour with a 1:200 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, USA) in PBS. After washing in PBS, the slides were mounted with Fluoromount-G (Electron Microscopy Sciences, PA, USA) and examined under an epifluorescence microscope.

Actin detection

Frozen sections were air-dried as previously, and incubated with FITC-phalloidin (Sigma Chemical Co., St. Louis, USA), diluted in PBS (2 mg/ml) for 1 h at room temperature. Slides were rinsed in PBS, mounted and photographed with an epifluorescence microscope.

Results

Odontogenesis in vivo

At a histological level odontogenesis begins during the early stages of cranio-facial development. The progressive development of teeth, involving oral epithelium and mesenchyme has been subdivided in

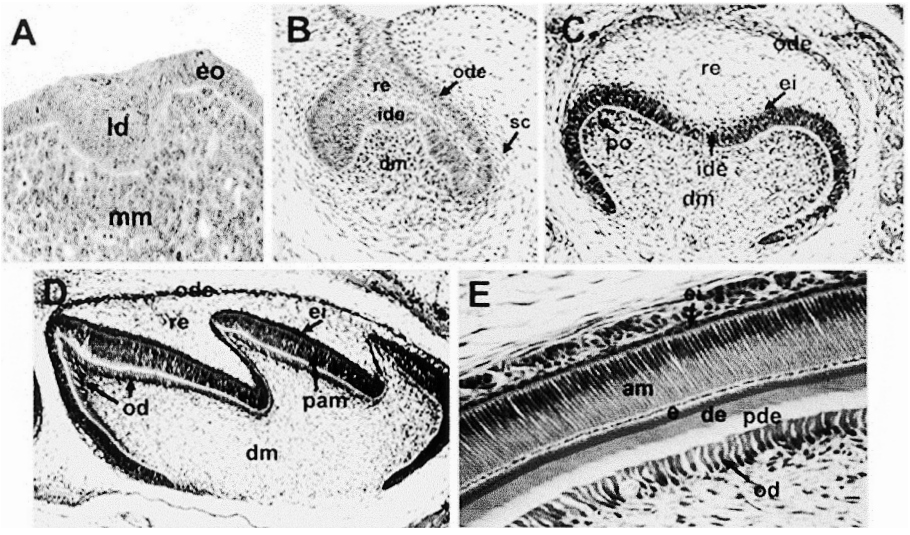

Fig. 1. Histological aspects of mouse first molar odontogenesis. **A.** Dental bud: initiation stage. **B.** Cap stage: the morphogenesis is initiated. **C.** Early bell stage: pre-differentiation stage. Pre-odontoblasts (po) are aligned facing inner dental epithelium (ide). **D.** Late bell stage: odontoblasts (od) differentiate and secrete pre-dentin. **E.** High magnification of functional odontoblasts (od) and ameloblasts (am) which secrete predentin-dentin (pde, de) and enamel (e), respectively. ld: dental lamina; mm: mandibular mesenchyme; eo: oral epithelium; re: stellate reticulum; ode: outer dental epithelium; ei: intermediate stratum; sd: dental sac; dm: dental mesenchyme; pam: preameloblasts.

several successive stages: bud, cap and bell stages. Figure 1 summarizes some of the morphological aspects of odontogenesis in the mouse embryo.

Immunoblotting analysis

Lysates prepared from E14.5 (lanes 1), E17.5 (lanes 2) and E19.5 (lanes 3) first lower molars were immunoblotted to nitrocellulose membrane (Figure 2). ZO-1 and ZO-2 TJ-associated proteins were detected in all samples as two bands each of around 220 kD and 160 kD, respectively. Some cross-reactivity with proteins of unknown identity in the 55 kD region for anti-ZO-2 antibody was found. On the contrary, ZO-3 protein was negative for the three embryo molar samples. However, occludin was seen as a single band of 65 kD in E17.5 (lane 2) and E19.5 (lane 3), but in E14.5 molars (lane 1) this transmembrane protein was not detected.

Distribution of ZO-1, ZO-2, ZO-3 and occludin in MDCK cells

To examine the tight junction-associated proteins in MDCK cells we carried out immunofluorescence experiments. ZO-1, -2, -3 and occludin were found to be located at the cellular periphery, giving rise to a strong and uniform staining along the whole circumference of the cells (Fig. 3), as described by other authors (Gottardi et al., 1996; Haskins et al., 1998; Mitic and Anderson, 1998).

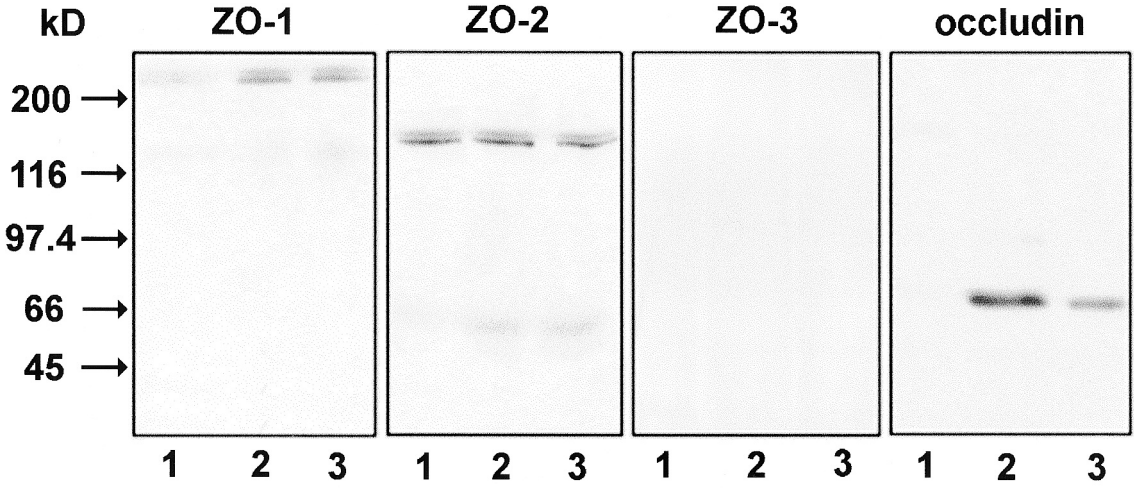

Fig. 2. Immunoblotting of TJ-associated proteins, ZO-1, ZO-2, ZO-3 and occludin in total proteins obtained from E14.5 (lanes 1), E17.5 (lanes 2) and E19.5 (lanes 3) mouse embryo first lower molars. Samples are electrophoresed (25-50 μg per lane), transferred onto nitrocellulose membranes, and incubated with rabbit anti ZO-1, ZO-2, ZO-3 or occludin antibodies. These antibodies are detected with peroxidase anti-rabbit followed by ECL.

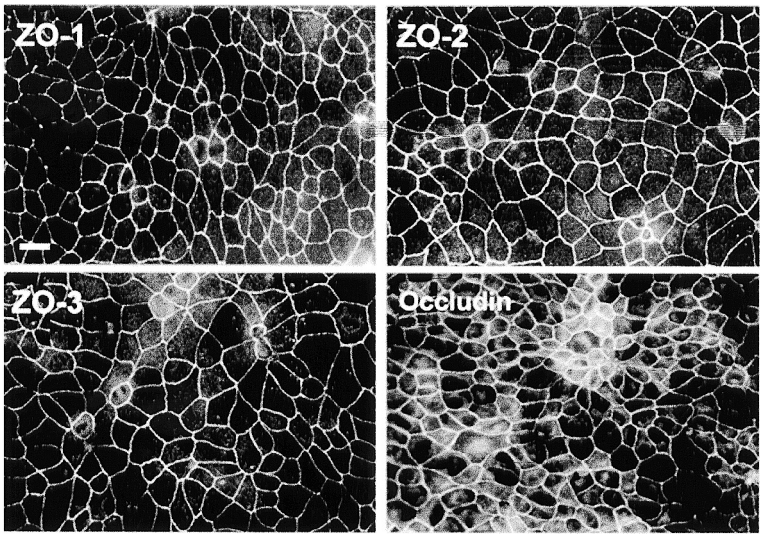

Fig. 3. Immunoflurescence for ZO-1, ZO-2, ZO-3 and occludin in MDCK cells. Cells plated on glass slides were incubated with antibodies against tight junction-associated proteins. All antibodies were strongly detected at cell-cell contacts continuously. Scale bar: 10 μm.

Localization of ZO-1, ZO-2 and ZO-3 in molar development

In the ED 14.5 first lower molar (bud/cap stage) the expression of ZO-1 was irregularly distributed in the dental epithelium and mesenchyme. A discontinuous fluorescent signal was observed at the cell membrane of dental lamina and dental papilla cells. However, the signal in the inner and outer dental epithelium was continuously located at the epithelia-mesenchymal transition. In addition, the anti-ZO-1 antibody labeled the endothelial cells of the developing blood capillaries in a continuous fashion, both in dental papilla and dental follicle (Fig. 4A,B). In sections of the ED 17.5 first molar (early bell stage) stratum intermedium cells showed a punctuated fluorescent labeling for ZO-1. Likewise, the inner dental epithelia cells showed a weak fluorescence. Dental mesenchyme cells accumulated ZO-1 in specific areas of the cell membrane. The endothelial cells of blood capillaries of various types and caliber were strongly stained on the cell membrane in a continuous fashion (Fig. 4C-D). In the ED 19.5 molars (late bell stage) the expression pattern for ZO-1 protein was modified in the enamel organ cells when compared to the early stages of tooth development. In this context, preameloblast/ameloblast cells were positive and the fluorescence accumulated at the basal and apical pole of the cell membrane. The intensity of this signal decreased at the tip of the dental cusps and in cervical loop direction. Punctuated fluorescence remained in the stratum intermedium and stellate reticulum. In dental papilla, endothelial cells of blood capillaries subjacent to the odontoblasts, and microvessels inside the dental mesenchyme were strongly positive (Fig. 4E-G).

The immunoreactivity for ZO-2 protein was different to that observed for ZO-1. Thus, ZO-2 was restricted to the enamel organ cells. At bud (ED 12.5-13.5) and cap (ED 14.5) stage the signal for anti-ZO-2 antibody was homogeneously distributed in the cell membrane of the dental lamina cells (Fig. 5A). In ED 14.5 coronal molar sections, the fluorescence was distributed at cell-cell contact of the inner and outer dental epithelium of the lingual side, whereas in the labial side the labeling accumulated at the basal pole of the cells (Fig. 5B,C). At early bell stage (ED 17.5), immunoreactivity was regularly distributed on the cell membrane of the dental lamina cells, stellate reticulum, stratum intermedium, and outer and inner dental epithelium (Fig. 5D-E). At late bell stage (ED 19.5), the fluorescence remained in the enamel organ and was specifically found at the lateral cell membrane of secretory and non-secretory poles of pre-ameloblasts/ameloblasts (Fig. 5F, G). However, the dental mesenchyme was negative for ZO-2 during all the tooth development.

Additionally, we performed immunohistochemistry against ZO-3 protein in mouse embryo molars. The results showed that no signal was exhibited in tooth tissues from early to late stages of tooth development. In ED 13.5 and 14.5 sections, we did not observe any signal for ZO-3 protein in the oral epithelium (data not shown), but a certain immunoreactivity was detected in the oral epithelium of the E17.5 and E19.5 molars (Fig. 6A-D).

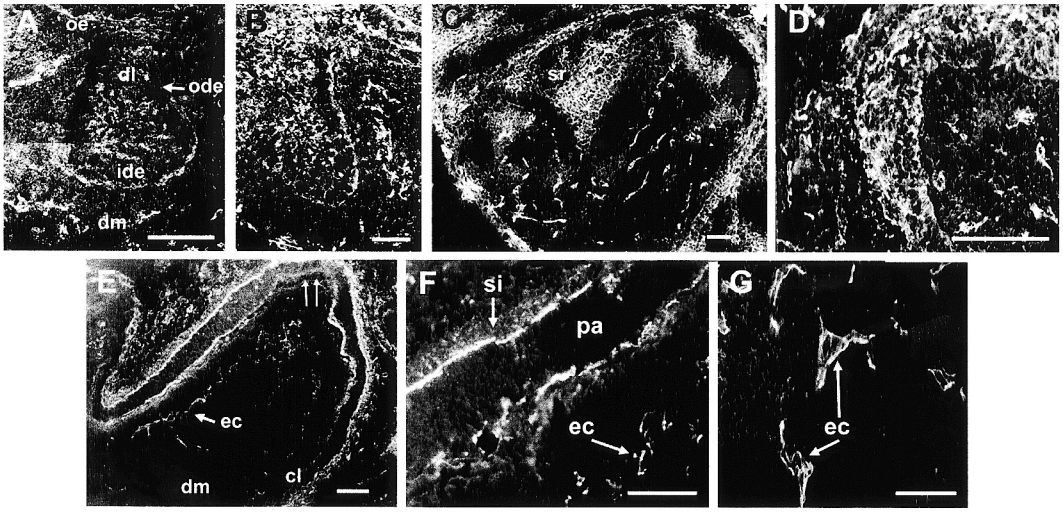

Fig. 4. Immunolocalization of ZO-1 in mouse molar development: **A-B** (ED 14.5) bud/cap stage. **C-D** (ED 17.5) early bell stage. **E-G** (ED 19.5) late bell stage. dl: dental lamina; dm: dental mesenchyme; ec: endothelial cells; ide: inner dental epithelium; ode: outer dental epithelium; oe: oral epithelium; pa: preameloblast/ameloblasts; si: stratum intermedium; sr: stellate reticulum. Note the discontinuous staining in the cell membrane of the dental lamina-derived cells **(A-D)** and in the dental mesenchyme cells **(A-D)**. On the contrary, the capillary endothelium showed a continuous staining **(G)**. The inner dental epithelium is positive in apical and basal pole **(E)** but the fluorescence decreased at the tip of the cusp (double arrow) to the cervical loop (cl) direction. Scale bar: A-F, 50 μm; G: 20 μm.

Localization of occludin during molar development

In contrast to ZO-1 and ZO-2 immunostaining, ED 14.5 molar sections exhibited fluorescence for occludin in the cell membrane of oral epithelium, but this protein was hardly detectable in dental tissues (Fig. 7A). In ED 17.5 samples, occludin remained in the cell membrane of oral epithelium but was weakly located in the stellate reticulum and stratum intermedium cells of enamel organ (Fig. 7B). At late bell stage, occludin was clearly observed in the cell membrane of endothelia cells of dental papilla blood capillaries similar to ZO-1 signal

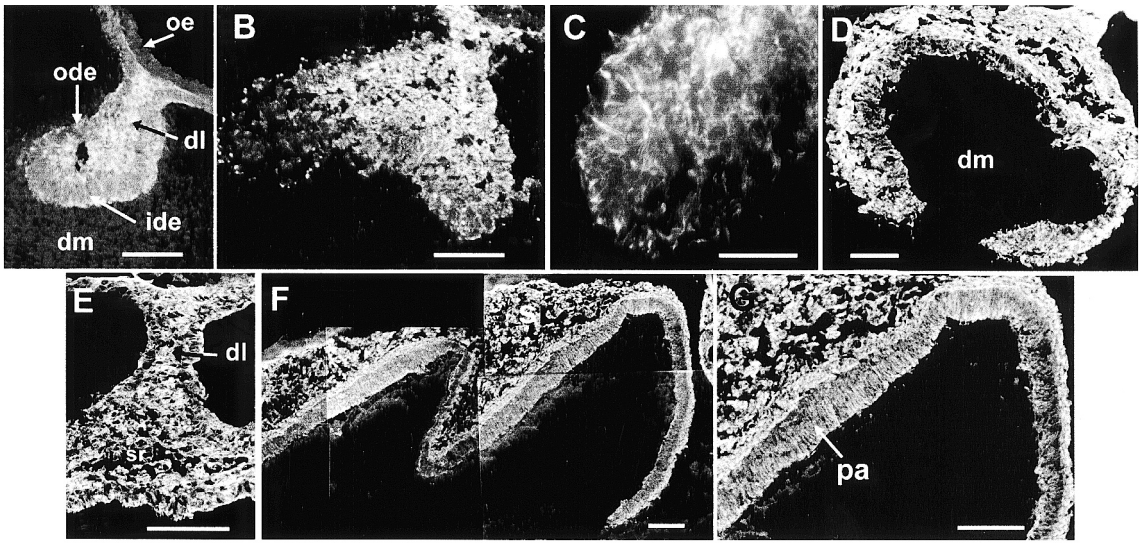

Fig. 5. Immunolocalization of ZO-2 in mouse molar development: **A** (ED13.5) and **B-C** (ED14.5) bud/cap stage; **D-E** (ED 17.5) early bell stage; **F-G** (ED 19.5) late bell stage. Expression of ZO-2 is detected in enamel organ cells, and dental mesenchyme is negative. Note the accumulation of ZO-2 in the inner dental epithelium and the fluorescence in lateral cell membrane of preameloblast/ameloblasts **(F-G)**. dl: dental lamina; dm: dental mesenchyme; ide: inner dental epithelium; ode: outer dental epithelium; oe: oral epithelium; pa: preameloblast/ameloblasts; sr: stellate reticulum. Scale bar: A,B, D-G, 50 μm; C, 25 μm.

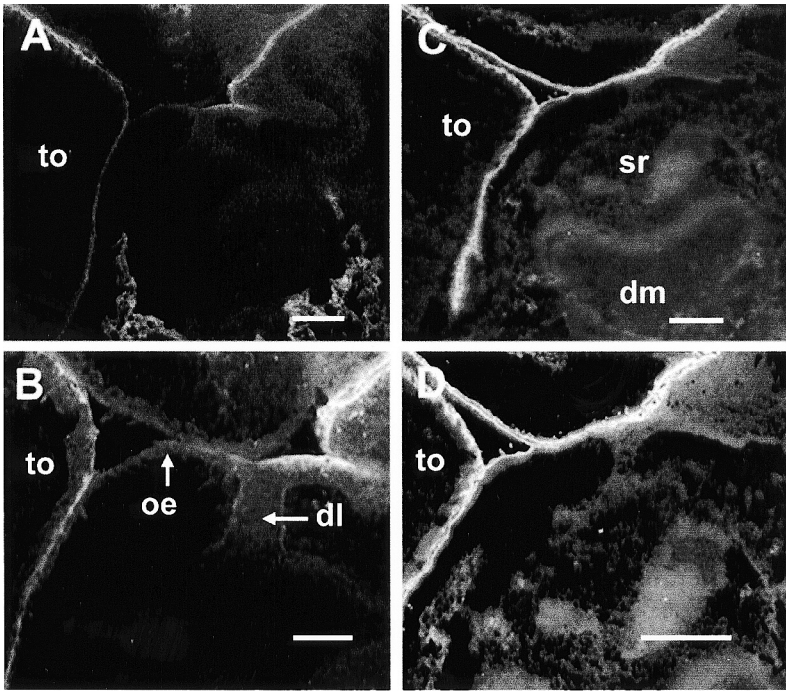

Fig. 6. Immunolocalization of ZO-3 in mouse molar development. **A-B** (ED17.5) early bell stage; **C-D** (ED19.5) late bell stage. dl: dental lamina; dm: dental mesenchyme; oe: oral epithelium; sr: stellate reticulum; to: tongue. Scale bar:A, C, D, 200 μm; B, 100 μm.

(Fig. 7C).

Localization of ZO-1, ZO-2, ZO-3 and occludin during incisor development

Rodent incisors are continuously growing teeth and exhibit gradients of odontoblast and ameloblast cytodifferentiation from distal to mesial direction on the labial side. However, the lingual side lacks enamel, since inner dental epithelia cells do not differentiate into ameloblasts. The location of ZO-1, ZO-2 and ZO-3 proteins in the mouse lower incisor (ED19.5) was similar to that described for molars. A gradient of fluorescence was observed for ZO-1 protein in the inner dental epithelia cells of the labial side. In the cell membrane of the non-secretory pole the fluorescent signal was continuous from undifferentiated cells to mature ameloblasts. However, in the secretory pole the signal was transiently reduced in polarized/differentiated ameloblasts and re-expressed in secretory and mature ameloblasts. In the dental mesenchyme, the cell membrane of endothelial capillaries displayed a strong fluorescence (Fig. 8A,B). In contrast, immunostaining for ZO-2 was found in the lateral membrane of the inner dental epithelial cells of the labial side, including preameloblast, secretory and mature ameloblasts. The stratum intermedium was also positive for ZO-2. In addition, the undifferentiated epithelial sheet of the inner dental epithelium of the lingual side expressed ZO-2 (Fig. 8C-D). Immunofluorescence for ZO-3 was

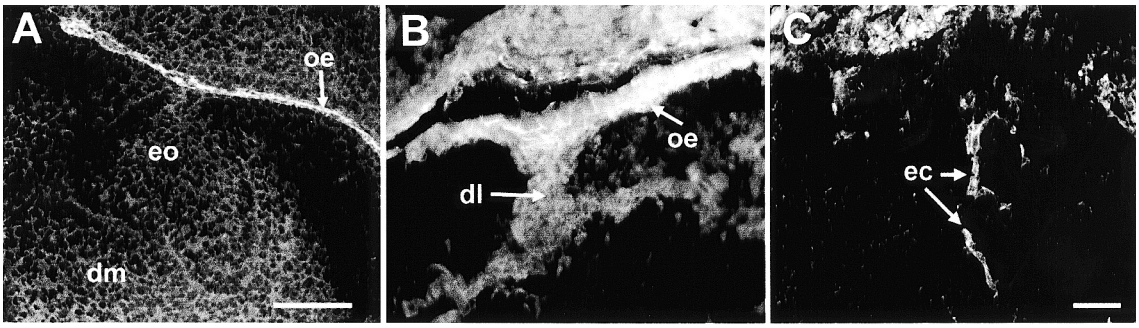

Fig. 7. Immunolocalization of occludin in mouse molar development. In the ED 14.5 bud/cap stage **(A)**, immunostaining is only exhibited in oral epithelium; in the ED 17.5 early bell stage **(B)**, the signal remains in oral epithelia cells; in the ED 19.5 late bell stage **(C)**, capillary endothelium expressed occludin in a similar manner to ZO-1. dm: dental mesenchyme; eo: enamel organ; oe: oral epithelium. Scale bar: A, 100 μm; 20 μm.

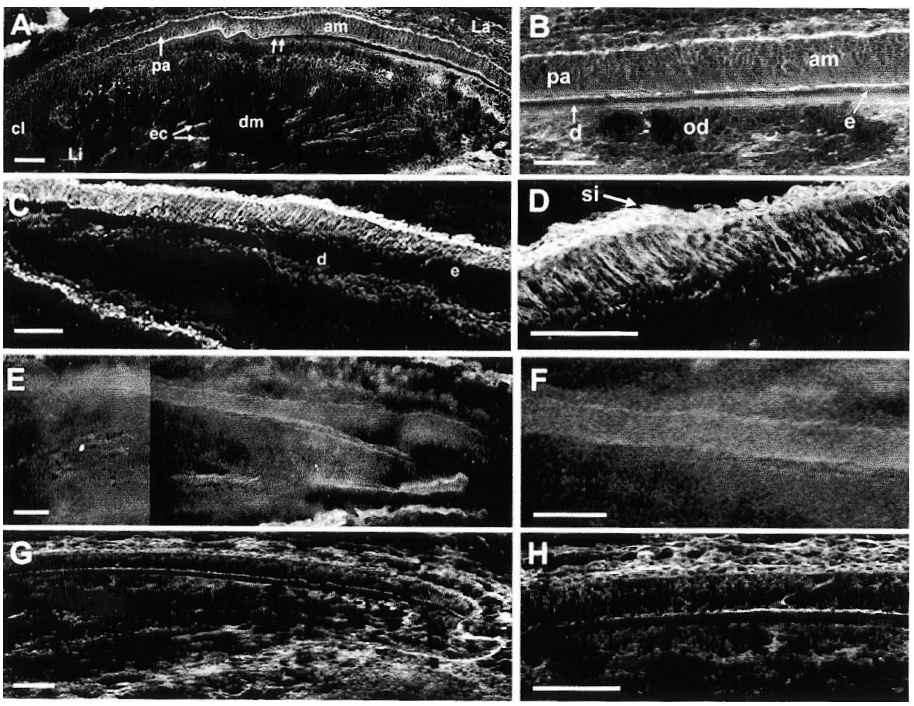

Fig. 8. Immunolocalization of ZO-1 **(A, B)**, ZO-2 **(C, D)**, ZO-3 **(E, F)** and occludin **(G, H)** in the ED 19.5 developing mouse incisor. Note the staining for ZO-1 along preameloblast and ameloblast cells in apical and basal pole **(A, B)**. The staining is reduced in preameloblasts and recently-differentiated ameloblasts (double arrows), and reexpressed in secretory and mature ameloblasts. Dental papillae capillaries are strongly stained. The fluorescence for ZO-2 is found in the inner dental epithelium of labial and lingual side **(C-D)**. In the labial side, the signal shows a continuous staining in sites of lateral cell-cell interaction. The immunofluorescence for ZO-3 is negative in incisor **(E-F)**. Occludin is found in the secretory pole of ameloblasts in a continuous fashion. The non-secretory pole of mature ameloblasts and the undifferentiated dental epithelium on the lingual side also express occludin. am: ameloblasts; cl: cervical loop; d: dentin; dm: dental mesenchyme; e: enamel; ec: endothelial capillaries; (La) labial side; (Li) lingual side; od: odontoblasts; pa: preameloblast/ameloblasts; si: stratum intermedium. Scale bar: 50 μm.

negative in the ED19.5 lower incisor (Fig. 8E,F). ED 19.5 incisor sagittal sections exhibited fluorescence for occludin in the cell membrane of the basal pole of differentiated and mature ameloblasts in a continuous fashion, similar to ZO-1 immunostaining (Fig. 8G,H).

Localization of actin in developing tooth

The ED14.5 molar sections stained with FITC-phalloidin exhibited an intense fluorescence. The signal was accumulated at the epithelial-mesenchymal junction of the inner and outer dental epithelial cells. Dental lamina cells also showed a strong fluorescence. In addition, actin was homogeneously distributed in dental mesenchyme cells (Fig. 9A). In ED17.5 sections, we observed a fluorescent signal in the inner dental epithelium at the basal and apical pole of the cells. Therefore, stellate reticulum and stratum intermedium were clearly stained (Fig. 9B). In ED19.5 molars, the distribution of actin was seen in the apical and basal pole of preameloblast/ameloblast cells. In the dental papilla, the signal was detected in the subodontoblastic cell layer (Fig. 9C).

In sagittal sections of the ED19.5 mouse incisor, we observed that actin was gradually expressed from distal to mesial side of the developing incisor. In the dental epithelium of the cervical loop the signal was weak but the fluorescence increased in preameloblast and functional ameloblasts. Intermediate stratum showed a strong fluorescent signal.

Discussion

In this work we have studied the temporal and spatial distribution of ZO-1, ZO-2, ZO-3 and occludin during tooth development. Our results indicate that the expression of TJ proteins is differentially regulated during tooth development. It suggests a dynamic and specific assembling of the TJ protein complex. For a better comprehension of results, Table 1 shows a summary of expression of TJ proteins in dental and oral tissues.

Dynamic assembly of ZO-1, ZO-2 and occludin

It has been considered that both ZO-1 and ZO-2 express and colocalize occludin in all cells that form TJ. For example, ZO-1 has been colocalized with ZO-2 at cell-cell borders in mouse epidermis (Morita et al., 1998). In our study, we have observed that the secretory and non-secretory cell membrane of preameloblast and ameloblasts of molar and incisor exhibited fluorescence for ZO-1 and ZO-2. However, occludin was hardly detected in preameloblasts, but was expressed in differentiated and secretory ameloblasts (Table 2, column B and F). It enables us to assure that TJ are completely formed after terminal differentiation of ameloblasts. These findings denote that TJ formation involves the expression of ZO-1 and ZO-2 at earlier stages of odontogenesis than occludin. The fact that ZO proteins were first present would suggest that ZO

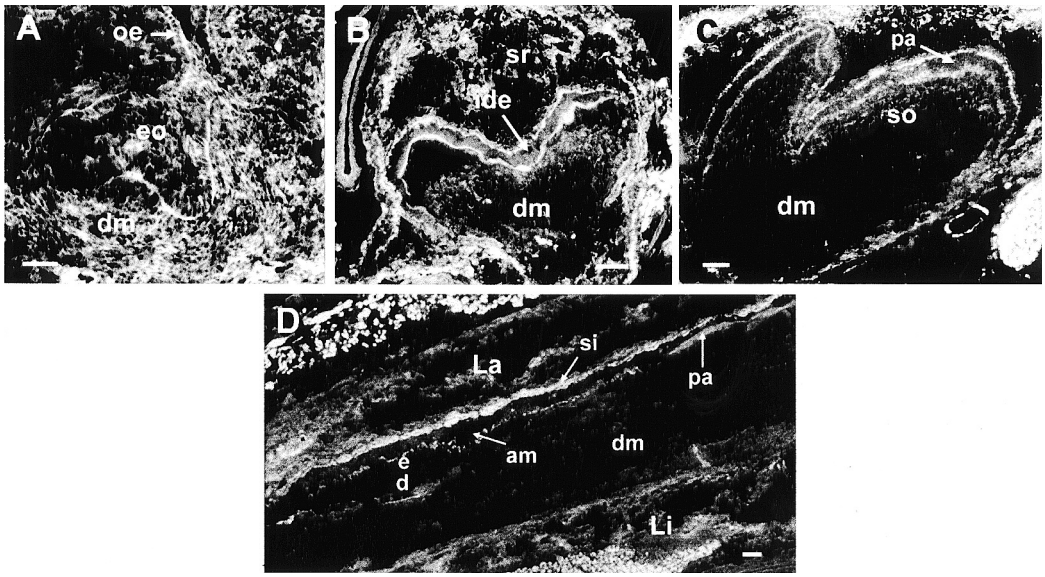

Fig. 9. Actin expression in mouse molar **(A-C)** and incisor **(D)** development. The ED 14.5 cap stage **(A)**. Actin is displayed in enamel organ and dental mesenchyme cells. ED17.5 early bell stage **(B)**. ED19.5 **(C)** late bell stage. Fluorescence is accumulated in apical and basal pole of preameloblast/ameloblast cells, stellate reticulum, and odontoblastic and subodontoblastic cell layer. ED19.5 lower incisor **(D)**. Actin is seen along the inner dental epithelium of labial side at secretoty and non-secretory cell membranes. am: ameloblasts; d: dentin; dm: dental mesenchyme; e: enamel; eo: enamel organ; ide: inner dental epithelium; La: labial side; Li: lingual side; oe: oral epithelium; pa: preameloblast/ameloblasts; si: stratum intermedium; so: subodontoblastic cell layer; sr: stellate reticulum. Scale bar: A, C-D, 50 μm; B, 100 μm.

proteins might direct TJ formation during embryonic development. Indeed, the appearance of AJ precedes that of TJ in the formation of epithelia (Collins and Fleming, 1995). Moreover, tooth morphogenesis implies the growing and remodeling of the enamel organ. TJs could prevent the cellular movements and the signal exchange between dental epithelium and mesenchyme for tooth morphogenesis and cell differentiation. This could explain, at least in part, the late expression of occludin, the only transmembrane protein specific for TJ, and the histomorphological remodeling taking place during enamel organ growing. In tall columnar ameloblasts the signal for ZO-1 in the secretory pole decreased and was re-expressed in secretory and mature ameloblasts. It suggests that ZO-1 could play a role in ameloblast differentiation.

Altogether, our data support that ZO-1, ZO-2 and occludin play a role in the establishment of a paracellular barrier in the inner dental epithelium only after tooth epithelium remodeling and exchanging of intracellular signaling between epithelium and mesenchyme. In this context, several signalling molecules, such as growth factors, are involved in tooth initiation, morphogenesis (Thesleff and Sharpe, 1997), odontoblast polarization, and odontoblast and ameloblast differentiation (Begue-Kirn et al., 1994; Martin et al., 1998; Unda et al., 2000).

Isolated expression of ZO-1 in undifferentiated dental mesenchymal cells

ZO-1 was expressed from early to late odontogenesis both in enamel organ and dental mesenchyme. The discontinuous distribution of ZO-1 in the cell membrane of the undifferentiated dental mesenchyme cells indicates that this protein is confined to specific areas of cell-cell contact to arrange macula junctions. Since only actin, but not occludin, ZO-2, ZO-3, E-cadherin and catenins, is also expressed in dental mesenchyme cells, ZO-1 could play a different TJ role or form cell-cell junctions by interaction with other cadherins or other TJ molecules (Table 2, column C).

Similarly, it has been reported that during neural tube closure occludin disappeared from all neuroepithelial cells but ZO-1 and N-cadherin expression increased from the neural plate to the neural tube stage (Aaku-Saraste et al., 1996). In dental epithelium, ZO-1 is concentrated in TJs through a direct

Table 1. Expression of tight junction-associated proteins in E13.5 to E19.5-day mouse embryo

SITE	OCCLUDIN	ZO-1	Z0-2	Z0-3	ACTIN
Oral epithelium	++	+	++	-/+	+
Dental lamina	+	+	++	-	+/-
Stellate reticulum	+/-	+	++	-	+/-
Intermediate stratum	-	+/-	++	-	+
Outer dental epithelium	-	+	++	-	+
Inner dental epithelium	-	+	++	-	+
Pre-ameloblasts	-	+	++	-	+
Ameloblasts	+	+	++	-	+
Odontoblasts	+/-	-	-	-	+/-
Dental mesenchyme	+/-	+	-	-	+/-
Dental sac	+/-	+	-	-	+/-
Endothelial cells	-	+	-	-	-

+/-: weak signal detected; -/+: no signal detected at early stage (bud/cap) of odontogenesis but fluorescence is displayed at bell stage.

Table 2. Adherens and tight junction associated protein combinatory.

	A	B	C	D	E	F
E-cadherin	+	nd	-	-	+	+
ß-catenin	+	+	-	-	+	+
Occludin	+	+	-	+	+/-	-
ZO-1	+	+	+	+	+	-
ZO-2	+	+	-	-	+	+
ZO-3	-	-	-	-	-	-
Actin	+	+	+	-	+	-
	E13.5M oe	E19.5I am	E13.5-17.5M dm	E19.5M ec	E13.5-19.5M dl, ide, ode	ED19.5M, I lm

am: ameloblast; dl: dental lamina; dm: dental mesenchyme; ec: endothelial cells; I: incisor; ide: inner dental epithelium; lm: lateral membrane of inner dental epithelium; M: molar; ode: outer dental epithelium; oe: oral epithelium.

binding to occludin, whereas in non-epithelial cells such as fibroblasts and cardiac muscle cells, ZO-1 is colocalized at AJ, where the cadherins are associated with actin-based cytoskeleton (Itoh et al., 1993; Jesaitis and Goodenough, 1994; Paria et al., 1999).

On the other hand, ZO-1 establishes a link between occludin and the actin cytoskeleton. Moreover, ZO-1 contains domains for binding both occludin and ZO-2 (Itoh et al., 1997; Fanning et al., 1998). In our work we observed a similar ZO-1 and actin expression pattern at early cap and late bell stage of odontogenesis in the inner dental epithelium. These results support that ZO-1 protein and actin are redistributed and linked during tooth morphogenesis. In addition, the colocalization of these proteins in predifferentiated ameloblasts suggests a role in cell polarization and terminal differentiation of ameloblasts. An active role for ZO-1 during epithelial-mesenchymal interactions has been suggested (Gottardi et al., 1996). However, we were not able to detect any nuclear ZO-1 labeling.

Occludin and ZO-1 are co-expressed in tooth blood capillaries

The continuous immunostaining in blood capillaries of dental papilla for ZO-1 from early bud/cap stage and occludin at late bell stage indicates that endothelial cells possess uninterrupted junction structures at cell-cell border, but TJ are finally constituted at late stage. Furthermore, endothelial cells were negative for ZO-2 and ZO-3, which is indicative of the non-association between ZO-1/occludin and ZO-2 or ZO-3 in the arrangements of TJ in endothelial cells of dental mesenchyme. Thus, the expression pattern of occludin does not merely reflect the distribution of the different ZO proteins (Table 2, column D).

Specific expression of ZO-2

We observed that ZO-2 expression pattern was restricted to enamel organ-derived cells. Lateral cell membrane of inner dental epithelium cells, including preameloblast/ameloblasts, was specifically labeled (Table 2, column F). In this context, E-cadherin and ß-catenin have also shown a lateral cell membrane location only in preameloblasts/ameloblasts of dental cusps (Fausser et al, 1998 and our own observations). These results indicate that ZO-2 could bind AJ proteins to form lateral cell-cell contacts and/or act in a different function to that of TJ. Therefore, the coincident expression of ß-catenin and ZO-2 indicates that these lateral cell-cell contacts in preameloblast/ameloblasts are formed during dental cusp growth. These results indicate that ZO-2 could bind adherent junction proteins and/or other TJ molecules to form lateral cell-cell contacts. Claudin multigene-family-encoding transmembrane protein components of TJ would be good candidates (Morita et al., 1999). It would suggest a more complex combinatory network of TJ proteins than anticipated.

Negative expression of ZO-3 during tooth morphogenesis

A third member of TJ has been identified: ZO-3. It has been shown that ZO-3 directly interacts with ZO-1 and occludin but not with ZO-2 (Haskins et al., 1998). Some evidence supports the existence of two independent TJ protein interactions: ZO-1/ZO-2 and ZO-1/ZO-3 complex, and both complexes bind occludin (Wittchen et al., 1999). However, in our study, we observed that ZO-3 is displayed in oral epithelium but is not expressed in the developing tooth tissues (Table 2, columns A, B, C, F). This indicates that TJ are formed in the absence of ZO-3 during tooth development. ZO-3 might fulfill a specific function during TJ assembly in the oral epithelium (Table 2, column A).

In summary, this work highlights a dynamic and specific assembling of TJ proteins and provides insight to TJ physiology in general. If the homology of the ZO proteins were indicative of a functional redundancy, the non-overlapping expression patterns would argue for unique functions. In addition, our data support that the interactions between the actin cytoskeleton and junction components are more complex than anticipated, consisting of multiple possible linkage mechanisms. Probably, combinatorial protein interactions are regulated with respect to morphogenetic movements of epithelia during development. In order to understand the functional basis of the interactions among TJ proteins, further investigations will be necessary to better comprehend the role of these proteins during vertebrate development and cell differentiation.

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