

ORIGINAL PAPER

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## Differential expression patterns of the tight junction-associated proteins occludin and claudins in secretory and mature ameloblasts in mouse incisor

Received: August 10, 2009 / Accepted: October 28, 2009

**Abstract** Tight junctions (TJs) function primarily as a barrier against paracellular transport between epithelial cells and are composed mainly of occludin (OLD) and claudins (CLDs). The CLD family consists of 24 members that show tissue- or cell-specific expression. Ameloblasts, which originate from the oral epithelium, form enamel, and enamel proteins and minerals are transported across the ameloblastic layer during amelogenesis. We immunohistochemically examined the distribution patterns of TJs in ameloblasts by observing the expression patterns of OLD and CLDs (CLD-1 to CLD-10). Secretory ameloblasts contained OLD and CLD-1, -8, and -9 at the distal end of the cell. In mature ameloblasts, OLD and CLD-1, -6, -7, -8, -9, and -10 were present mainly at both the distal and proximal ends of the cell, regardless of whether the ameloblasts were ruffle-ended or smooth-ended. Mature ameloblasts in which only the proximal ends were stained for OLD and CLDs were also found. These results indicate that the expression patterns of CLDs and the distribution patterns of TJs change drastically between the secretory and mature ameloblast stages, suggesting that these patterns reflect the different functions of these cells, specifically in the transport of proteins and ions for enamel formation.

**Key words** Tight junction · Occludin · Claudin · Ameloblast · Tooth

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A part of this study was reported at the 47th Annual Meeting of the Japanese Association for Oral Biology, held at Sendai, Japan, in 2005

### Introduction

Ameloblasts, which are differentiated from the inner enamel epithelium of the enamel organ, form tooth enamel and line the enamel surface as a simple columnar epithelium, through amelogenesis. Amelogenesis is divided into two distinct stages, the secretory stage and the maturation stage, and ameloblasts undergo morphological and functional changes during the different stages of amelogenesis. At the secretory stage, ameloblasts, called secretory ameloblasts, characteristically exhibit Tomes' processes and well-developed cell organelles, such as rough endoplasmic reticulum and Golgi apparatus. This cell type primarily functions to secrete abundant enamel proteins and participates in the partial calcification of the secreted enamel matrix. After the organic matrix formation is completed, ameloblasts drastically modulate their morphological characteristics to become maturation ameloblasts: the cell shortens, the cell organelles such as rough endoplasmic reticulum and Golgi apparatus decrease, and the Tomes' process disappears. At this stage, the organic matrix that was secreted at the secretory stage is degraded and resorbed, and calcium (Ca) influx across the ameloblastic layer into the enamel matrix increases. These qualitative changes in the enamel matrix are achieved by both ruffle-ended ameloblasts (R-As) and smooth-ended ameloblasts (S-As) at the maturation stage. These two types of mature ameloblasts periodically undergo dramatic modulations of their morphological characteristics, switching between the R-A and S-A phenotypes. The sequence of amelogenesis is described in several reviews.<sup>1–3</sup>

It is well accepted that the transportation of materials across the epithelial cell layer is carried out by paracellular and/or transcellular pathways. The tight junction (TJ) is an intercellular junction that acts as a seal between epithelial cells to prevent the diffusion of materials via the paracellular pathway. The molecular architecture of the TJ has recently been clarified: it is composed mainly of the integral transmembrane proteins occludin (OLD) and claudin family members (CLDs). OLD was the first identified component of TJs<sup>4</sup>; the CLDs were found later as TJ-associated

adhesion molecules.<sup>5,6</sup> The CLDs comprise a gene family with 24 members and are expressed in tissue-specific distribution patterns, where they presumably play specific roles in the paracellular transport between epithelial cells in each organ or tissue.<sup>7</sup> Freeze-fracture studies reveal TJs between ameloblasts,<sup>8-13</sup> and proteins, minerals, and water are transported across the ameloblastic layer in amelogenesis; thus, it has been suggested that the TJs between ameloblasts play a role for the transportation of solutes and Ca.<sup>1-3</sup> However, the distribution patterns of the TJs and the molecular architecture of the junction at each ameloblast stage have not been fully clarified. In the present study, we used the immunohistochemical analysis of ameloblasts from mouse incisors to identify the distribution pattern of TJs and their molecular architecture at each ameloblast stage, focusing on the contributions of the claudin family.

## Materials and methods

C57BL6/J mice at 5–6 weeks of age were used as experimental animals. A 0.04% 3,3-bis[*N,N*-di(carboxymethyl)aminomethyl] fluorescein (calcein) solution was administered to some mice via the left ventricle 10 min before death, to distinguish the R-As and S-As, as reported elsewhere.<sup>14,15</sup> The animal experiment in this study was approved by the Animal Research Control Committee of Okayama University.

### Immunohistochemistry

Freshly frozen undecalcified sections of mandibular incisors were used for the immunohistochemical detection of OLD and CLDs. The sections were prepared according to the method of Kawamoto.<sup>16</sup> Briefly, mice were decapitated after being anesthetized, and the heads were immediately frozen in liquid nitrogen-cooled isopentane and freeze-embedded in 5% carboxymethyl cellulose. Undecalcified fresh-frozen sections of entire incisors of the mandible approximately 5  $\mu$ m thick were cut with a cryostat (Leica CM3050S; Nussloch, Germany) at a temperature range of  $-23^{\circ}$  to  $-25^{\circ}\text{C}$  and mounted on adhesive film on glass slides. The sections were kept at  $-80^{\circ}\text{C}$  until use. Before immunohistochemical reactions, the sections were fixed in cooled 95% ethyl alcohol at  $-20^{\circ}\text{C}$  for 30 min and immersed in 100% acetone for 1 min at room temperature. The sections were then rinsed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) several times, and incubated with antibodies to OLD or the CLDs for 4 h at  $4^{\circ}\text{C}$ . The sections were rinsed briefly with PBS containing 1% BSA and then incubated with Cy3-conjugated secondary antibodies (diluted to 1:250 with PBS containing 1% BSA) for 1 h at room temperature. These antibodies were subjected to centrifugation at 15000 rpm before use to reduce nonspecific staining. The incubation was terminated by washing the sections several times with PBS. In some sections, the nuclei of ameloblasts were counterstained with Hoechst (bis-benzamide trihydrochloride). The slides were

then mounted with 95% glycerin containing 0.1% *p*-phenylenediamine. The sections were observed with a fluorescence microscope, and phase-contrast images were also obtained (Leica, New York).

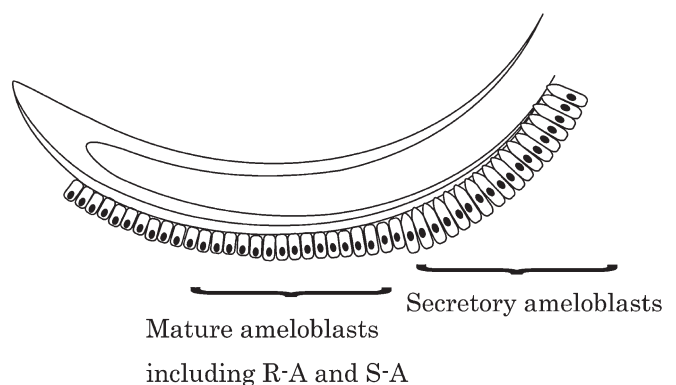
### Antibodies for occludin and claudins

Primary antibodies for detecting OLD and CLDs were an antimouse OLD monoclonal antibody and antimouse polyclonal antibodies for CLD-1, -2, -3, -4, -5, -6, -7, -8, -9, and -10, respectively. The anti-CLD-1, -3, and -5 polyclonal antibodies were purchased (Zymed Laboratories, San Francisco, CA, USA). The others were kindly provided by the late Prof. Shoichiro Tsukita (Kyoto University Graduate School of Medicine, Kyoto, Japan).

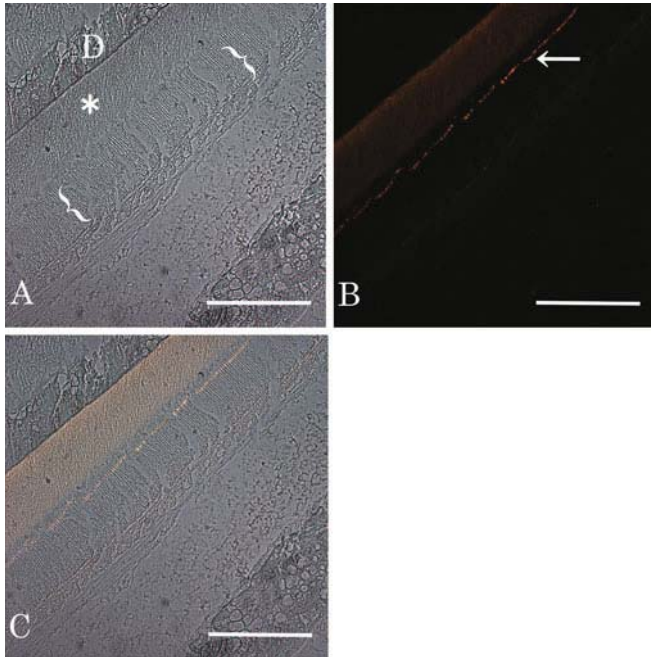
## Results

In the present study, three types of ameloblasts were observed: secretory ameloblasts, mature R-As and mature S-As. The observed regions of each incisor are illustrated in Fig. 1.

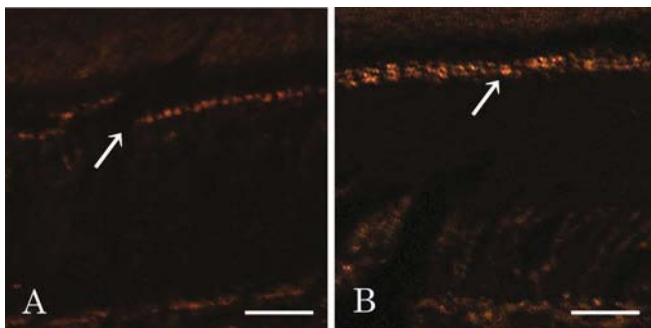
In the secretory ameloblasts, OLD staining was observed at the proximal end of the cells, as were CLD-1, -8, and -9 (Figs. 2, 3). In contrast, CLD-2, -3, -4, -5, -6, -7, and -10, were not detected in these cells. OLD and CLD-1, -6, -7, -8, -9, and -10 were detected, however, in maturation-stage cells; i.e., CLD-6, -7, and -10 were newly expressed by the cells at this stage. OLD and these CLDs also showed interesting localization patterns in the mature ameloblasts. OLD fluorescence was mainly localized to both the proximal and distal ends of the cells, forming a railroad-like pattern in the aligned cells (Fig. 4). The CLDs showed the same distribution pattern as OLD. For example, as shown in Fig. 5, CLD-1 was detected at both ends of the mature ameloblastic layer, and this pattern was not associated specifically with the R-A or S-A region, as judged by calcein fluorescence. In addition to this distribution pattern, some groups of mature ameloblasts showed labeling only at their proximal ends (Fig. 6). In these cells, CLD-6, -7, -8, -9, and -10 showed the same



**Fig. 1.** Diagram of the regions observed for the three types of ameloblasts: secretory ameloblasts, ruffle-ended ameloblasts (R-As), and smooth-ended ameloblasts (S-As)



**Fig. 2.** Phase-contrast image and claudin-1 localization of secretory ameloblasts in the same section. **A** Secretory ameloblasts are identified by their tall and columnar cell profiles (*brackets*). *Asterisk* indicates the enamel of the secretory stage. **B** Claudin-1 was detected at the distal end of each secretory ameloblast, and the positive fluorescence ran as a single line along the enamel (*arrow*). The fluorescence of the enamel matrix was from the nonspecific adsorption of antibodies. **C** Merged image composed of phase-contrast and fluorescence images. *D*, dentin. *Bars* 100  $\mu\text{m}$

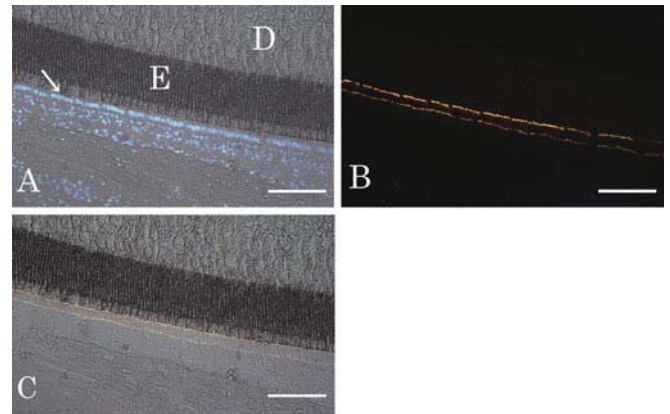


**Fig. 3.** Claudin-8 and -9 in secretory ameloblasts. Both Claudin-8 (**A**) and claudin-9 (**B**) were detected at the distal end of secretory ameloblasts (*arrows*). *Bars* 20  $\mu\text{m}$

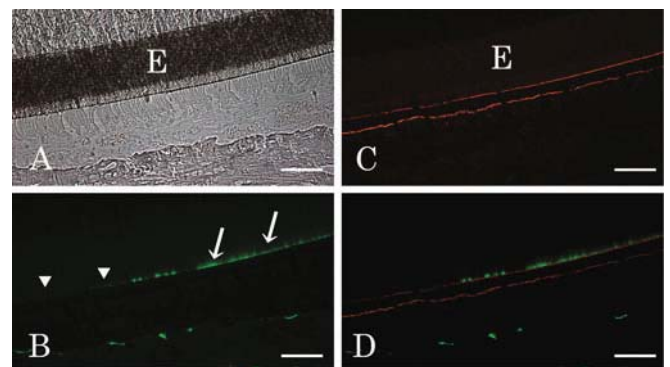
distribution patterns as OLD and CLD-1 (Fig. 7). As a summary, the localization of positively detected CLDs in ameloblasts is shown in Table 1. CLD-2, -3, -4, and -5 were not detected in mature ameloblasts in the present study.

## Discussion

Previous immunohistochemical studies have shown that ameloblasts begin to express OLD at the distal cell membrane as their differentiation progresses,<sup>17</sup> and that OLD and CLD-1 are detected at the proximal end of ameloblasts



**Fig. 4.** Occludin detection in mature ameloblasts. **A** Phase-contrast photograph showing the occludin-positive area. Nuclear staining for ameloblasts by the fluorescent dye Hoechst (*arrow*) is overlaid. **B** Occludin fluorescence appeared as *two parallel lines* in mature ameloblasts. **C** Phase-contrast image and occludin fluorescence image are overlaid. Occludin was detected at both the distal and proximal ends of the cells. *E*, enamel; *D*, dentin. *Bar* 100  $\mu\text{m}$



**Fig. 5.** Claudin-1 detection in mature ameloblasts. Calcein was administered before death to distinguish the S-A region from the R-A region. **A** Phase-contrast image of an observed area. **B** Same area showing the S-A region labeled with green fluorescence (*arrows*) and the R-A area as negative for fluorescence (*arrowheads*). **C** Orange fluorescence indicates claudin-1 localized to both the distal and proximal ends of the cells. **D** Overlaid image composed of **B** and **C**. The localization pattern of claudin-1 showed no difference between S-As and R-As. *E*, enamel. *Bar* 50  $\mu\text{m}$

at the late stage of differentiation.<sup>18</sup> In addition, a recent *in situ* hybridization study of claudin mRNAs in murine embryonic tooth germ cells showed that CLD-1, -3, -4, -6, -7, and -10 have restricted expression patterns in the tooth epithelium at the bud stage that correlate with the patterns of epithelial cell differentiation; only CLD-2 mRNA expression is observed in preameloblasts at the early bell stage.<sup>19</sup> These reports mainly examined the expression patterns of OLD and CLDs in differentiating ameloblasts. In the present study, OLD and CLDs (CLD-1 to -10) were immunohistochemically examined in functional ameloblasts, *i.e.*, secretory ameloblasts and mature ameloblasts in mouse incisors during amelogenesis. In the secretory ameloblasts, OLD and CLD-1, -8, and -9 were localized to the distal end of the cells. In mature ameloblasts, CLD-6, -7, and 10 were expressed in addition to OLD and the CLDs observed at

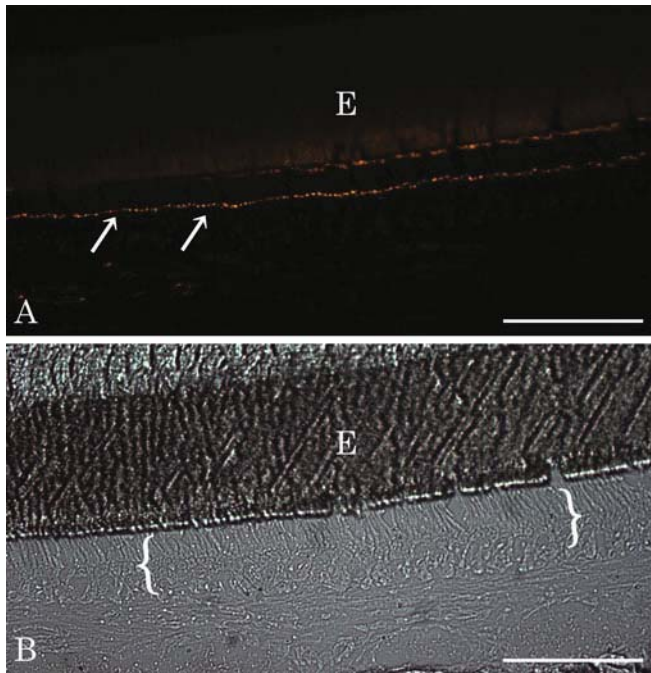
**Table 1.** Distribution patterns of claudins in ameloblast stages

Ameloblast stages	Localization	Claudin 1	Claudin 6	Claudin 7	Claudin 8	Claudin 9	Claudin 10
Secretory ameloblasts	Proximal end	–	–	–	–	–	–
	Distal end	+	–	–	+	+	–
Mature R-As <sup>a</sup>	Proximal end	+	+	+	+	+	+
	Distal end	+	+	+	+	+	+
Mature S-As <sup>a</sup>	Proximal end	+	+	+	+	+	+
	Distal end	+	+	+	+	+	+

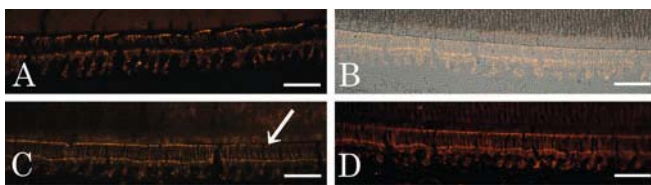
R-As, ruffle-ended ameloblasts; S-As, smooth-ended ameloblasts

Signals of immunohistochemistry: –, not detected; +, detected

<sup>a</sup>Mature ameloblasts in which only proximal ends were positive for occludin and claudins were also found in a few cases



**Fig. 6.** Claudin-1 localization in mature ameloblasts. **A** Mature ameloblasts showing claudin-1 at only the proximal end were sometimes observed (arrows). **B** Phase-contrast image of **A**. E, enamel; brackets, mature ameloblasts. Bar 50  $\mu$ m



**Fig. 7.** Claudin-6, -8, -9, and -10 were detected at both the distal and proximal ends of mature ameloblasts. **A** Claudin-6 localization. **B** Claudin-8 localization. The photograph is overlaid on the phase-contrast image. **C** Claudin-9 localization. Among the positive cells, a group showing positive reactivity at only the proximal end is indicated (arrow). **D** Claudin-10 localization. Bars 100  $\mu$ m

the secretory stage, and OLD and these CLDs were localized to both the distal and proximal ends of mature ameloblasts, although in some areas only the distal end of the cells was positively stained. Thus, this report is the first to identify the expressions of OLD and CLD-1, -8, and -9 in secretory

ameloblasts and those of OLD and CLD-1, -8, -9 plus -6, -7, and -10 in mature ameloblasts. With regard to OLD and CLDs expression in functional incisors, a recent immunohistochemical study reported that secretory ameloblasts were positive for CLD-4 but negative for OLD and CLD-1, whereas in mature ameloblasts OLD, CLD-1, and CLD-4 were positively detected using rat incisors.<sup>20</sup> In addition, the other CLDs examined in the study such as CLD-2, -3, -5, -6, -7, -8, -12, and -15 were negative in ameloblasts. The CLD-1 expression in mature ameloblasts is as in a previous report,<sup>20</sup> i.e., CLD-1 was positively stained in mature ameloblasts. However, the cause of the differences is still uncertain, although it may be pointed out that the animals used and the methods for specimen preparation are different in each study.

It is tempting to speculate that the expression of CLD-6, -7, and -10 in maturation ameloblasts reflects a functional difference between secretory ameloblasts and mature ameloblasts, which would suggest that the functions of the TJs shift in association with ameloblast differentiation. However, the functional significance of the newly expressed CLDs (CLD-6, -7, and -10) in maturation ameloblasts is not known. We point out only that mature ameloblasts primarily function in the absorption of degraded enamel proteins, dehydration, and the further mineral deposition of enamel.<sup>1-3</sup> Further examination will be necessary, for example, using CLD knockout mice, to determine the relationship between amelogenesis and the functions of each CLD. In addition, the expression of the other CLDs, CLD-11 to -24, in ameloblasts still remains to be studied.<sup>7</sup>

Mature ameloblasts consist of two cell types, R-A and S-A. The former mainly functions in the transport of Ca into enamel and the latter in the absorption of enamel matrix.<sup>1-3</sup> In the present study, we examined whether there were differences between R-As and S-As with regard to the expression patterns of CLDs. We used a proven method, administering calcein fluorescence, to identify regions containing the R-A and S-A ameloblasts in frozen sections.<sup>14,15</sup> Interestingly, the expression patterns of the CLDs examined showed no difference between the R-A and S-A cell types. Therefore, it appears that mature ameloblasts do not express different sets of CLDs (or at least of CLD-1 to -10), regardless of their functional state. It may be necessary that the TJs be maintained while the R-A and S-A cyclically change their functions and morphological characteristics, i.e., from R-A to S-A and from S-A to R-A.

Freeze-fracture studies have shown TJs to be located at the distal end of secretory ameloblasts and mature R-As but at the proximal end of mature S-As.<sup>8-13</sup> In the present study, TJs were localized to the distal end of secretory ameloblasts, in agreement with previous reports.<sup>8-13</sup> However, in mature ameloblasts, TJs were mostly localized to both the distal and proximal ends of the cells, regardless of whether they were R-As or S-As, although some mature ameloblasts showed TJs only at the proximal end of the cells. Regarding the localization of OLD and CLDs in secretory and mature ameloblasts, a previous immunohistochemical study showed the bipolar localization of OLD, CLD-1, and CLD-4 and stated that CLD-1 was detected at both ends of all ameloblasts except for the distal ends of S-As, and further that CLD-4 and OLD were detected at both ends of mature ameloblasts except for the distal ends of S-As.<sup>20</sup> In the present study it was shown that OLD and CLD-6, -7, -8, -9, and -10 also localized to both the distal and proximal ends of mature ameloblasts, in addition to previously reported OLD and CLDs localization.<sup>20</sup> Generally, TJs localize to the apical side of cells in epithelia, and no report is available that describes a bipolar localization of TJs, i.e., on both the apical and basal sides, of cells other than ameloblasts. Thus, the bipolar localization of TJs may be unique to ameloblasts. The reason for the discrepancy in the localization of TJs in mature ameloblasts between the present immunohistochemical study and freeze-fracture studies,<sup>8-13</sup> and/or a previous immunohistochemical study,<sup>20</sup> is unclear. However, to explain the complicated ion transport that is observed physiologically during amelogenesis, it is hypothesized that TJs modulate their localizations, i.e., at both the proximal and distal ends or solely at the proximal end.<sup>3</sup> Our immunohistochemical study may support this hypothesis.<sup>3</sup> Further examination is necessary to elucidate the functions of the CLDs and the distribution patterns of the TJs during amelogenesis.

**Acknowledgments** The authors thank Prof. Mikio Furuse, Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, for advice on occludin and claudin immunohistochemistry. This study was supported in part by a Grant-Aid for Scientific Research (C) from the Ministry of Education, Science, and Culture of Japan to T. Yamamoto (No. 17591909).

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