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博 士 論 文

Immunolocalization of podoplanin, CD44, and endomucin in the odontoblastic cell layer of murine tooth germs

（マウス歯胚の象牙芽細胞層における podoplanin, CD44 および endomucin の局在）

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Immunolocalization of podoplanin, CD44, and endomucin in the odontoblastic cell layer of murine tooth germs

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Running title: *Podoplanin, CD44 and endomucin in odontoblast layers*

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Abstract

In this study, we attempted to localize the immunoreactivities of podoplanin and CD44, a counterpart possessing a high affinity to podoplanin, as well as endomucin-immunoreactive blood vessels in the regions of odontoblast layers and the underlying sub-odontoblastic layers in murine tooth germs. Endomucin-reactive small blood vessels were scattered throughout the dental papillae of the tooth germs at postnatal day 1 but came to be localized close to the odontoblast/sub-odontoblastic layers until day 3. After postnatal day 5, small blood vessels were seen forming in odontoblast cell layers, while blood vessels with relatively larger diameters were seen forming in sub-odontoblastic layers. Immunoreactivities of podoplanin and CD44 were not detectable in the cells of dental papillae facing the inner enamel epithelium at postnatal day 1. However, at around postnatal days 3–5, podoplanin was localized in the odontoblast layer but not in the sub-odontoblastic layer, whereas CD44 was observed in the sub-odontoblastic layer but not in the odontoblast layer. The exclusive immunolocalization of podoplanin and CD44 in the odontoblast layers and sub-odontoblastic layers was seen after postnatal day 3 of the tooth germs, which is when the mesenchymal cells of dental papillae have already differentiated into mature odontoblasts at the cusp tip. Taken together, it seems likely that endomucin-reactive small blood vessels extended to the podoplanin-positive odontoblast layers, whereas endomucin-reactive large blood vessels were already present in CD44-immmunopositive sub-odontoblastic layer, indicating the cellular regulation on the vascularization of endomucin-reactive endothelial cells during odontogenesis of the tooth germs.

245 words

Key words: *odontoblast, sub-odontoblastic layer, endomucin, podoplanin, CD44*

Introduction

Secretory odontoblasts are fully differentiated polarized cells containing numerous organelles, such as endoplasmic reticulum and Golgi complex. Odontoblasts synthesize abundant collagen fibrils and non-collagenous proteins, as well as matrix vesicles to form unmineralized predentin that is subsequently mineralized to become mature dentin[1)]. During odontogenesis in tooth germs, pre-odontoblasts differentiate from dental papillae beneath the inner enamel epithelium and increase the height of the cell bodies, thus extending their cytoplasmic processes toward the enamel organs. The differentiated odontoblasts move away toward the underlying dental pulp, formerly termed dental papillae, leaving their cytoplasmic processes within the dentinal tubules[2)]. Beneath the odontoblast layers, a cell-free zone, called the zone of Weil, is present specifically in the coronal pulp and overlays a cell-rich zone showing high cell population. The cell-free and cell-rich zones are collectively referred to as the sub-odontoblastic layer[3)]. Meanwhile, the distribution patterns of peripheral blood vessels seem to be associated with the odontoblast layer and sub-odontoblastic layers. Yoshida and Ohshima[4, 5)] have described that when dentin deposition begins, capillaries begin to invade into the odontoblast layer and finally settle close to the predentin while continuous capillaries form a coarse vascular network distant from the odontoblast layers, *e.g.,* sub-odontoblastic layers. Therefore, they have suggested that the changes in the peripheral capillaries are closely related to the secretory activity of odontoblasts[4-6)]. Thus, the distribution patterns of peripheral blood vessels appear to be related to the cellular activities of mature odontoblasts and cells in sub-odontoblastic layers. In addition, it must be significant to investigate if tooth germs include bone-specific blood vessels, which show CD31^{high}/endomucin^{high}[7–9)]. However, few studies have examined the presence of endomucin-immunoreactive blood vessels in tooth germs.

Odontoblasts have been reported to express podoplanin,[10, 11)] also called E11[12)], gp38[13)], OTS-8[14)], and T1a[15)]. Podoplanin is a 38-kDa type I transmembrane glycoprotein[16, 17)] and has been demonstrated to play pivotal roles in cell motility[18)], cell adhesion[19)], elongation of cytoplasmic processes[20)], and organogenesis[21–23)]. Most of these roles are presumably mediated

with the rearrangement of the actin cytoskeleton[24, 25]. When odontoblasts are differentiated enough to synthesize the dentin matrix, they may dynamically change the assembly of cytoskeletons that mediate podoplanin signaling. Meanwhile, CD44, a hyaluronan receptor of the cell membrane[26], is reportedly expressed in tooth germ, including secretory ameloblasts and the inner enamel epithelium, as well as stratum intermedium[27–29]. CD44 has been postulated to interact with hyaluronan and regulate the vascularization of endothelial cells by affecting the expression of the chemokines CXCL9 and CXCL12 and their receptors[30]. CD44 also reportedly interacts with podoplanin to promote directional cell migration, suggesting its co-operation with podoplanin[31]. Ohizumi *et al.* suggested that the interaction between CD44 and podoplanin regulates endothelial cell growth and/or migration in tumor[32].

Thus, the interplay of CD44 and podoplanin appears significant for cell activities of odontoblasts, pre-odontoblastic cells, and vascular endothelial cells during the development of tooth germs. Herein, we aimed to examine the immunolocalization of podoplanin and CD44, as well as endomucin-positive vascular endothelial cells in the regions of odontoblast layers and sub-odontoblastic layers in the coronal tooth germs, which may provide some clues for better understanding of the cellular interaction between odontoblasts/pre-odontoblasts and vascular endothelial cells.

Material s and Methods

Animals and tissue preparation

Postnatal mice at 1, 3, 5, and 7 days and 2 and 3 weeks after birth (CLEA Japan Co. Ltd, Tokyo) were used this study (n = 4–6 for each), and the animal experiments were conducted under the Hokkaido University guidelines for animal experimentation (approval No. 15-0041).

Mice were euthanized with an intraperitoneal injection of sodium pentobarbital and were immediately extracted heads. The samples were then immersed in 4% paraformaldehyde solution diluted in a 0.067M phosphate buffer (pH 7.4) for 24 h at 4°C[33, 34]. After fixation, the specimens were decalcified with 10% ethylenediamine tetraacetic disodium salt (EDTA-2Na) solution between 3 day and 3 weeks before paraffin-embedding. Five-micrometer-thick paraffin sections parallel to the longitudinal axis of the line of upper molars were made, and all the paraffin sections subjected to the following immmunohistochemistry were photographed under a Nikon Eclipse E800 microscope (Nikon Instruments Inc. Tokyo, Japan), and light microscopic images were acquired with a digital camera (Nikon DXM1200C, Nikon).

Immunolocalization of podoplanin, endomucin, and CD44 in the developing tooth germs

Dewaxed paraffin sections were subjected to immunodetection of podoplanin, endomucin, and CD44 as previously described[35]. The sections were immersed in PBS containing 0.3% H_2O_2 for 30 min to block endogenous peroxidase. To reduce non-specific binding, 1% bovine serum albumin (BSA, Seologicals Proteins Inc. Kankakee, IL) in PBS (1% BSA-PBS) was applied to the sections for 20 min. Then, they were incubated with polyclonal goat IgG against mouse podoplanin (R&D systems Inc., Minneapolis, MN) at a dilution of 1:100 with 1% BSA–PBS at room temperature (RT) for 2 h. Following several washings in PBS, they were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (American Qualex Scientific Products, Inc., San Clemente, CA) for 1 h. For the detection of endomucin and CD44, after treatment with 0.3% H_2O_2 and 1% BSA-PBS, the dewaxed sections were reacted with rat anti-CD44 monoclonal antibody (BD Biosciences, Systems & Reagents Inc., San Jose, CA) for 1 h at a dilution of 1:100 or with rat antibody against endomucin (Santa Cruz Biotechnology, Inc.,

Dallas, TX) at a dilution of 1:100 overnight at 4°C. After washing with PBS, these sections were incubated with HRP-conjugated anti-rat IgG (Zymed Laboratories Inc., South San Francisco, CA) at a dilution of 1:100. Immune complexes were visualized using 3, 3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan).

Results

The distribution of endomucin-immunoreactive blood vessels in odontoblasts/sub-odontoblastic layers of the coronal areas of developing tooth germs

Endomucin-immunoreactive blood vessels were uniformly scattered throughout dental papillae at postnatal day 1; at this time, tooth germs did not yet show abundant enamel and dentin **(Figs. 1A, 2A)**. Endomucin-reactive small blood vessels reached the region of mesenchymal cells adjacent to the inner enamel epithelium **(Fig. 2D)**. On postnatal day 3, when tooth germs exhibited apparent enamel and dentin **(Figs. 1B)**, the endomucin-reactive blood vessels tended to be localized in the peripheral end of dental papillae (dental pulp at this development stage), *i.e.*, close to or along odontoblasts/sub-odontoblastic layers **(Fig. 2B)**. At a higher magnification, small blood vessels were found to have extended to the odontoblast layers, while some larger blood vessels appeared, at least in part, to be present in sub-odontoblastic layers **(Fig. 2E)**. By postnatal days 5–7, abundant enamel and dentin had formed **(Figs. 1C, D)**, and enlarged endomucin-reactive blood vessels predominantly existing in sub-odontoblastic layers were localized slightly distant from the odontoblast layers, whereas the smaller blood vessels were evenly scattered in both the odontoblast layers **(Figs. 2C, F, G, J)**. At postnatal weeks 2 and 3, when tooth germs were about to erupt or had erupted **(Figs. 1E, F)**, a tendency of small endomucin-positive blood vessels forming in the odontoblast layers and reaching close to the predentin was observed, whereas the enlarged endomucin-reactive blood vessels were positioned in sub-odontoblastic layers **(Figs. 2H, I, K, L)**.

Immunolocalization of podoplanin and CD44 in the coronal odontoblasts/sub-odontoblastic layers of developing tooth germs

Faint podoplanin immunoreactivity was observed mainly in the inner enamel epithelium at postnatal day 1 **(Fig. 3A)**. Cervical loops showed podoplanin in both outer and inner enamel epithelia. In contrast, CD44 was prominently present in both outer and inner enamel epithelia but not in dental papillae **(Fig. 4A)**. On postnatal day 3, podoplanin was intensely in stellate reticulum and in odontoblasts facing dentin but was only sparsely present in inner enamel

epithelium/ameloblasts **(Fig. 3B)**. Conversely, CD44 was intensely present in inner enamel epithelium/ameloblasts and the surrounding stratum intermedium and papillary layers but not in mature odontoblasts **(Fig. 4B)**. On postnatal days 5–7, podoplanin immunoreactivity became apparent in odontoblast layers and stellate reticulum but not in ameloblasts **(Figs. 3C, D)**. CD44 showed intense localization in ameloblasts and the surrounding stellate reticulum but only faint localization in the dental pulp **(Figs. 4C, D)**. After 2 weeks, podoplanin was seen in odontoblast and papillary layers **(Fig. 3E)** and was still observable in the odontoblast layers after tooth eruption at 3 weeks **(Fig. 3F)**. In contrast, CD44 was seen faintly in dental pulp and intensely in papillary layers at 2 weeks **(Fig. 4E)** but was observed mainly in periodontal ligaments after eruption **(Fig. 4F)**.

Comparative localization of podoplanin and CD44 in odontoblasts/sub-odontoblastic layers during tooth development

Next, we compared the immunolocalization of podoplanin and CD44 in the regions of odontoblasts and the underlying sub-odontoblastic layers at a higher magnification **(Fig. 5)**. At postnatal day 1, the inner enamel epithelium showed very weak podoplanin immunolocalization and relatively intense CD44 immunolocalization, whereas the adjacent mesenchymal cells of dental papillae showed weak immunolocalization of both podoplanin and CD44 **(Figs. 5A, B)**. At postnatal day 3, however, mature odontoblasts facing dentin showed intense podoplanin but not CD44 **(Figs. 5C, D)**. In contrast, sub-odontoblastic layers showed weak immunolocalization of CD44 but not of podoplanin. At postnatal day 5, mature odontoblasts featuring a typical cylindrical cell shape demonstrated strong immunoreactivity of podoplanin but not of CD44, whereas sub-odontoblastic layers displayed CD44 but not podoplanin. At this developmental stage, enlarged blood vessels were seen in the CD44-immunoreactive sub-odontoblastic layers **(See Figs. 5E and F)**, which is consistent with the observation of endomucin-reactive blood vessels **(Compare with Fig. 2F)**. At postnatal day 7, podoplanin reactivity was prominently observed in odontoblasts and weakly in sub-odontoblastic layers, whereas CD44 was predominantly detectable in sub-odontoblastic layers **(Figs. 5G, H)**. Large blood vessels were still present in the region of sub-odontoblastic layers.

Discussion

To our knowledge, this is the first report that blood vessels in developing tooth germs exhibit intense endomucin immunoreactivity[7], which has been documented to a hallmark of bone-specific blood vessels, being co-expressed with intense CD31[8]. In addition, the distribution patterns of small and large blood vessels were restrictedly related to the odontoblast layers and sub-odontoblastic layers, respectively. In this study, our findings suggest that the size and distribution patterns of endomucin-positive blood vessels are regulated with cell-surfaced molecules expressed in odontoblasts and the cells of sub-odontoblastic layers. Therefore, we believe that this study provides a new insight on the spatial relation between the endomucin-reactive blood vessels and podoplanin/CD44-positive odontoblast/sub-odontoblastic layers.

Consistent with the previous reports[9, 36–38], we have successfully localized podoplanin immunoreactivity in odontoblasts as shown in **Figs. 3** and **5**. The previous reports have demonstrated the presence of CD44 and not podoplanin in human mature odontoblasts,[27, 28] whereas our immunohistochemistry results showed no CD44 in odontoblast layers but intense presence in the inner enamel epithelium/ameloblasts, stratum intermedium, and outer enamel epithelium, which is in agreement with the report by Nakamura and Ozawa[29]. This discrepancy may be due to interspecies differences between human and mouse. Our observation suggests that podoplanin and CD44 interact reciprocally at the interface between odontoblasts and cells of sub-odontoblastic layers. When mesenchymal cells of the coronal dental papillae differentiated into podoplanin-reactive odontoblasts at postnatal day 3, sub-odontoblastic layers expressed CD44. The extracellular domain of podoplanin can reportedly bind to CD44[31], and thus, it may, at least in part, influence odontoblastic activities, such as cell polarity, adhesion, and elongation of cytoplasmic processes[18–22]. Among a variety of interactions between odontoblasts and the surrounding sub-odontoblastic layers, podoplanin/CD44 may be the candidates that regulate the cellular activities of odontoblasts and sub-odontoblastic layers.

It would be interesting to know if the interplay between podoplanin/CD44 is associated with the distribution and size of endomucin-immunoreactive blood vessels in odontoblasts/sub-odontoblastic layers. Nandi *et al.* reported that CD44 anchors hyaluronan to endothelial cell surfaces and that the activation of CD44 is a major regulator of hyaluronan expression on the endothelial surface. Further, they reported that the non-covalent interaction between CD44 and hyaluronan is sufficient to provide resistance to shear under physiologic conditions[39]. Endomucin is a mucin-like sialoglycoprotein that interferes with the assembly of focal adhesion complexes and inhibits the interaction between cells and the extracellular matrix[40]. Therefore, it is assumed that endomucin interacts with CD44 or some growth factors/local factors trapped within the complex of CD44/hyaluronan. However, unlike CD44, podoplanin-Fc, a fusion protein comprising the extracellular portion of human podoplanin linked to the Fc region of human IgG1, reduced lymphatic vessel formation *in vitro* and *in vivo*[41]. Therefore, the podoplanin expressed in odontoblasts possibly inhibits the vascularization of endomucin-reactive blood vessels. In addition, podoplanin-mediated platelet activation is critically involved in the separation of blood and lymphatic vessels[42], and therefore, it is possible that when large blood vessels in the sub-odontoblastic layer extend to the podoplanin-reactive odontoblast layers, the large blood vessels branch off into smaller vessels to invade into the odontoblast layers.

However, further examination is necessary to elucidate the cellular mechanism involved in the distribution of endomucin-reactive blood vessels and the interaction between podoplanin and CD44 during tooth germ development.

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Disclosures

The authors have no financial conflicts of interest to declare.

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Figure legends

Figure 1

Histological aspects of the developing tooth germs

At postnatal day 1, the tooth germ did not show enamel and dentin (Fig. 1A). During postnatal days 3–7, the tooth germs continuously showed progressive development of enamel and dentin (Fig. 1B–D). The tooth root began to form at 2 and 3 weeks when the tooth germs were about to erupt or had erupted on the oral cavity (Fig. 1E, F).

E: enamel, D: dentin, DP: dental papilla, EP: enamel pulp

Bars: A-F: 200 μm

Figure 2

The distribution of endomucin in developing tooth germs

The endomucin-positive small blood vessels were scattered in the region of dental papilla surrounded by the inner enamel epithelium at postnatal day 1 (Fig. 2A). After postnatal day 3, the endomucin-positive blood vessels tended to be localized to the dental papilla close to odontoblasts/sub-odontoblastic layers (Fig. 2B, C, G–I). At this stage, the blood vessels located in the odontoblast layers showed a small vascular diameter, whereas the enlarged endomucin-positive blood vessels existed in sub-odontoblastic layers (asterisks, Fig. 2F, J–L).

DP: dental papilla

Bars: A-C, G-I: 200 μm; D-F, J-L: 30 μm

Figure 3

The distribution of podoplanin in the developing tooth germs

The immunoreactivity of podoplanin was displayed on the inner enamel epithelium. The outer enamel epithelium also expressed podoplanin immunoreactivity (See the inset; Fig. 3A). On the specimens of postnatal days 3–7 and postnatal week 2, podoplanin was intensely observed in odontoblasts facing on dentin and enamel pulp including stellate reticulum and papillary layers (Fig. 3B–E). The ameloblasts did not show podoplanin immunoreactivity at postnatal days 5 and 7 (Fig. 3C, D); however, the inner enamel epithelium/ameloblasts weakly expressed podoplanin at postnatal day 3 (See the inset; Fig. 3B).

DP: dental papilla, EP: enamel pulp

Bars: A-F: 200 μm

Figure 4

The distribution of CD44 in the developing tooth germs

At postnatal day 1, there is strong immunoreactivity of CD44 in both outer and inner enamel epithelia (Fig. 4A). The inner enamel epithelium still displayed CD44 immunoreactivity at postnatal day 3 (Fig. 4B). Thereafter, CD44 continuously appeared on the ameloblasts and stellate reticulum until tooth eruption at postnatal week 3 (Fig. 4C–F).

DP: dental papilla, EP: enamel pulp

Bars: A-F: 200 μm

Figure 5

The comparison of podoplanin- and CD44-immunoreactivite cells in odontoblasts/sub-odontoblastic layers at postnatal days 1, 3, 5, and 7

At a higher magnification as seen in Fig. 4, in the region of odontoblasts and the underlying sub-odontoblastic layers, the inner enamel epithelium showed very faint podoplanin and comparatively intense CD44 activity at postnatal day 1 (Fig. 5A, B). At this stage, the mesenchymal cells of dental papillae close to the inner enamel epithelium did not show either podoplanin or CD44. At postnatal days 3 and 5, mature odontoblasts revealed intense podoplanin but no CD44, whereas sub-odontoblastic layers showed weak CD44 but no podoplanin (asterisks, Fig. 5C – F). At postnatal day 7, podoplanin was seen in both odontoblasts and sub-odontoblastic layers (asterisks); however, the distribution of CD44 did not change in former specimens (Fig. 5G, H). Note that large blood vessels are located on the region of sub-odontoblastic layers at postnatal days 5 and 7 (Fig. 5E–H).

DP: dental papilla, IEE: inner enamel epithelium, OD: odontoblast, BV: blood vessel

Bars: A-H: 30 μm

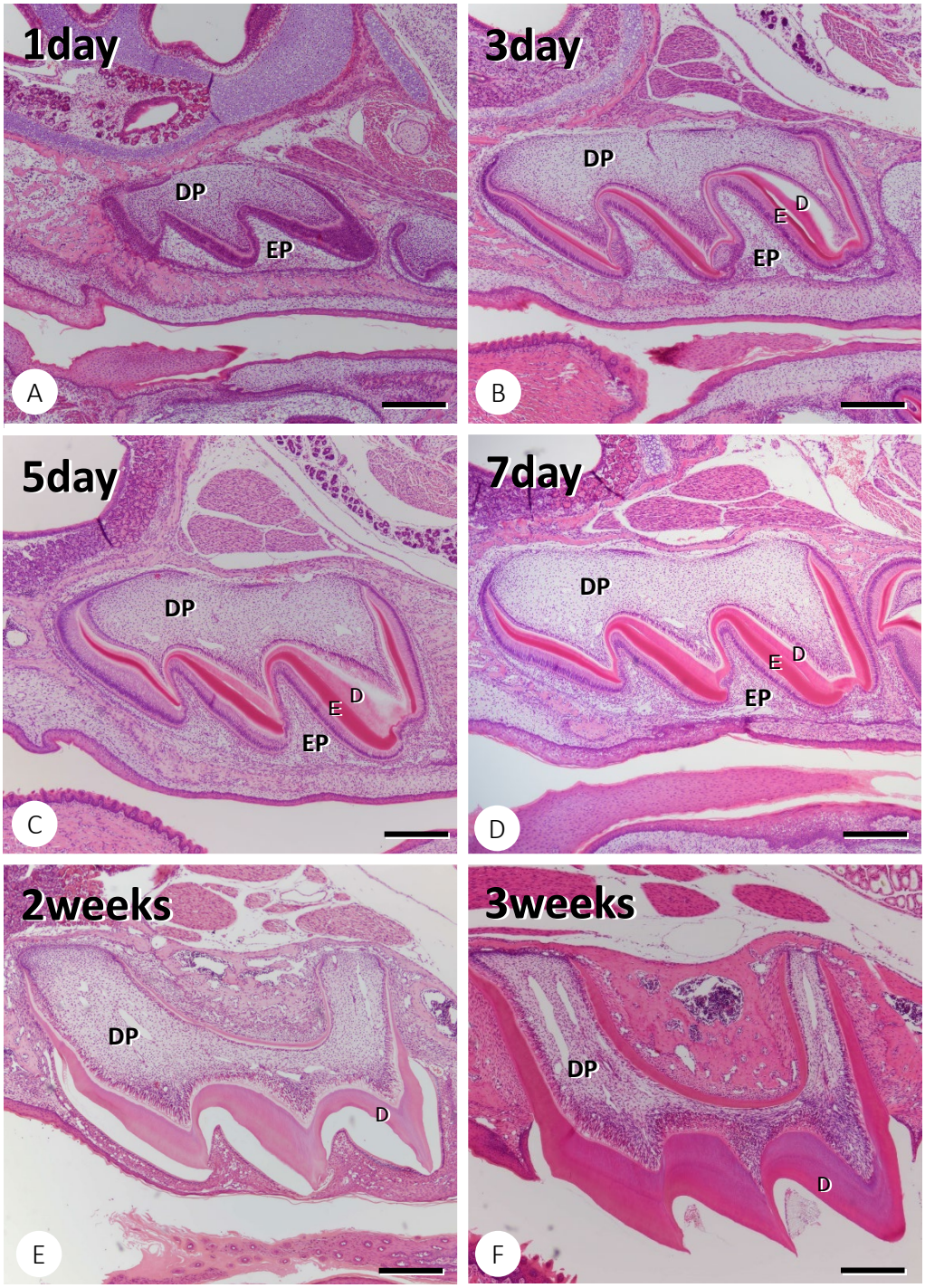
1day
DP
EP
A
3day
DP
E
D
EP
B
5day
DP
E
D
EP
C
7day
DP
E
D
EP
D
2weeks
DP
D
E
3weeks
DP
D
F

Figure 1

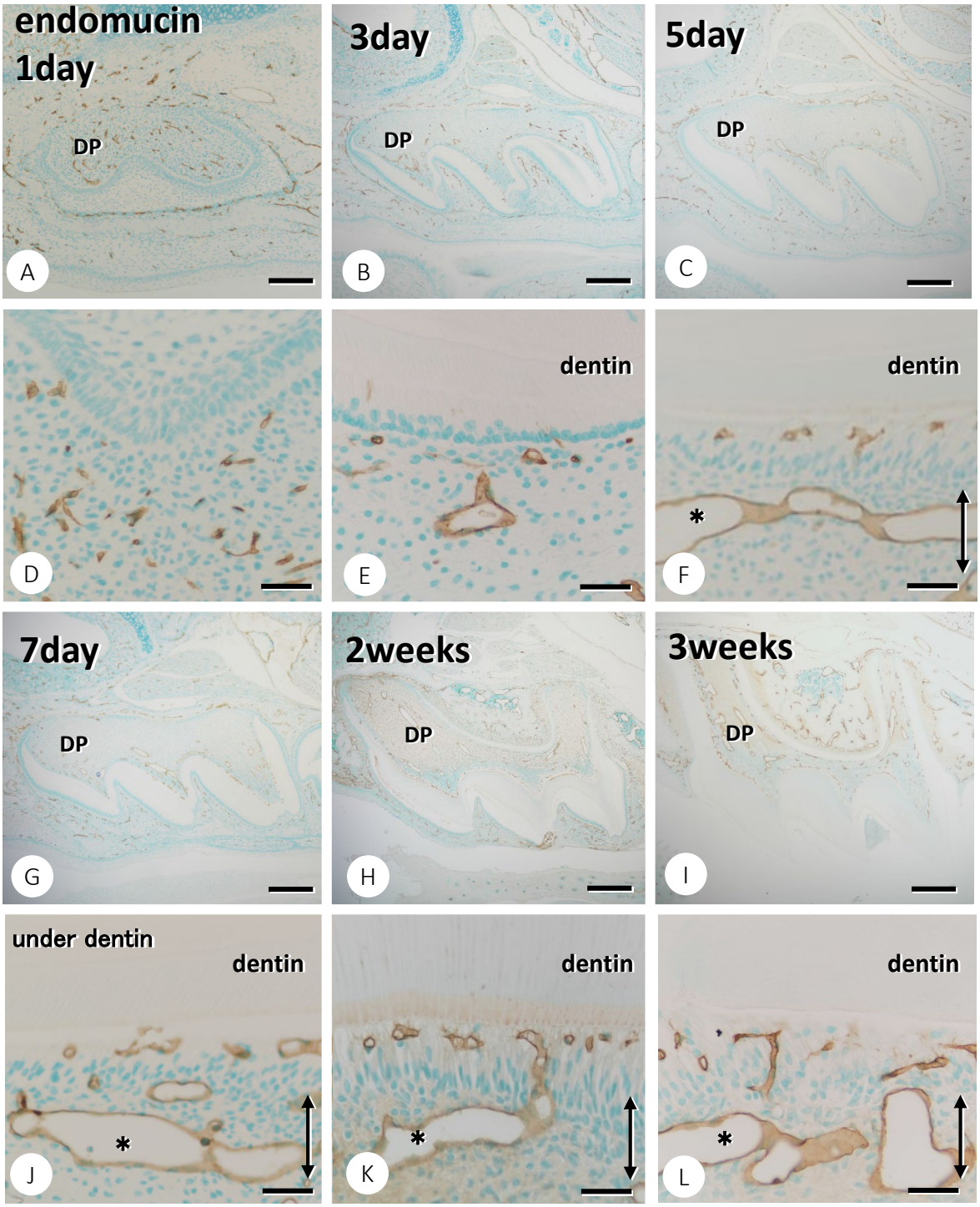
endomucin
1day
DP
A
3day
DP
B
5day
DP
C
D
dentin
E
dentin
*
F
7day
DP
G
2weeks
DP
H
3weeks
DP
I
under dentin
dentin
*
J
dentin
*
K
dentin
*
L

Figure 2

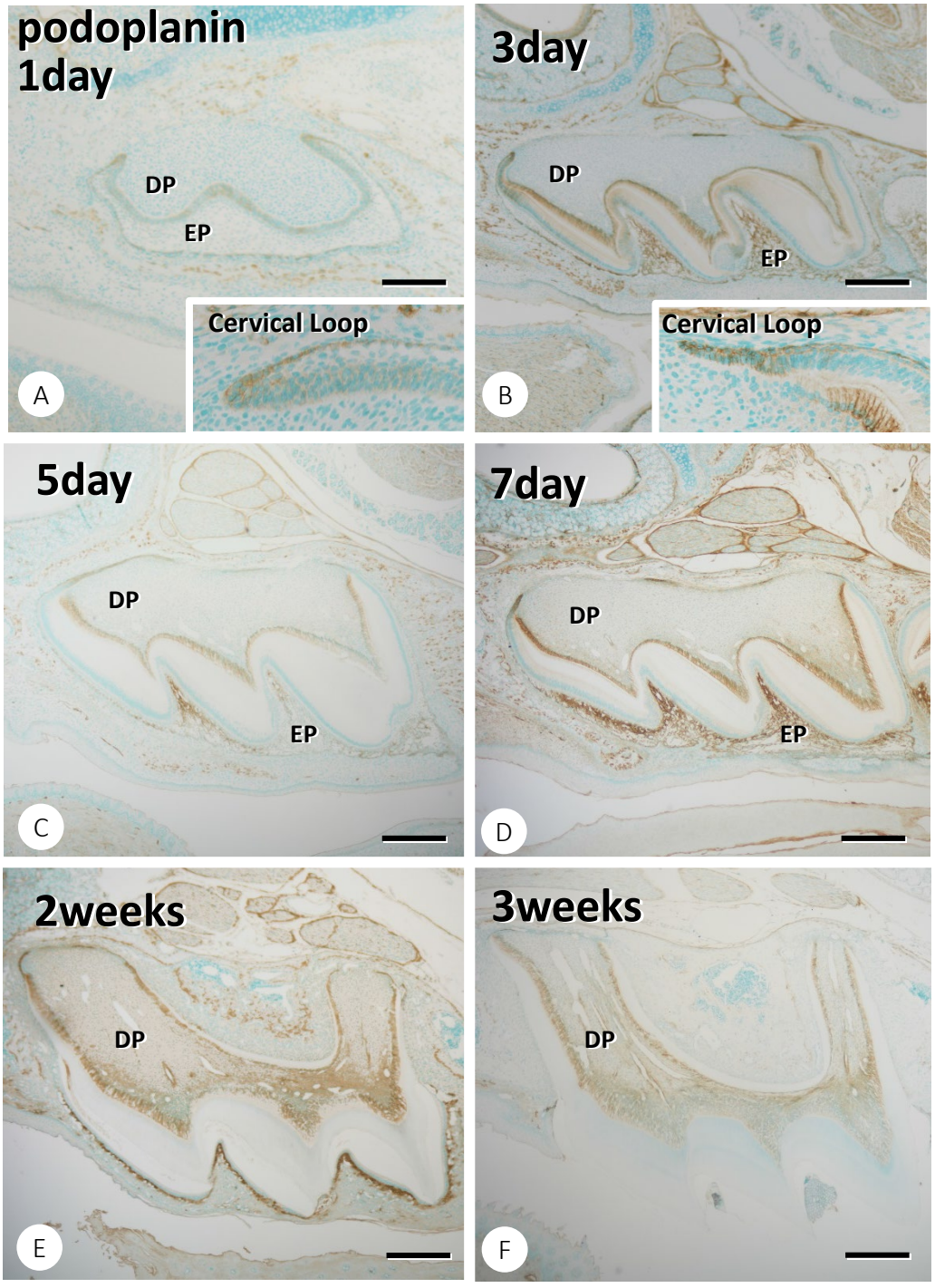
podoplanin
1day
DP
EP
Cervical Loop
A
3day
DP
EP
Cervical Loop
B
5day
DP
EP
C
7day
DP
EP
D
2weeks
DP
E
3weeks
DP
F

Figure 3

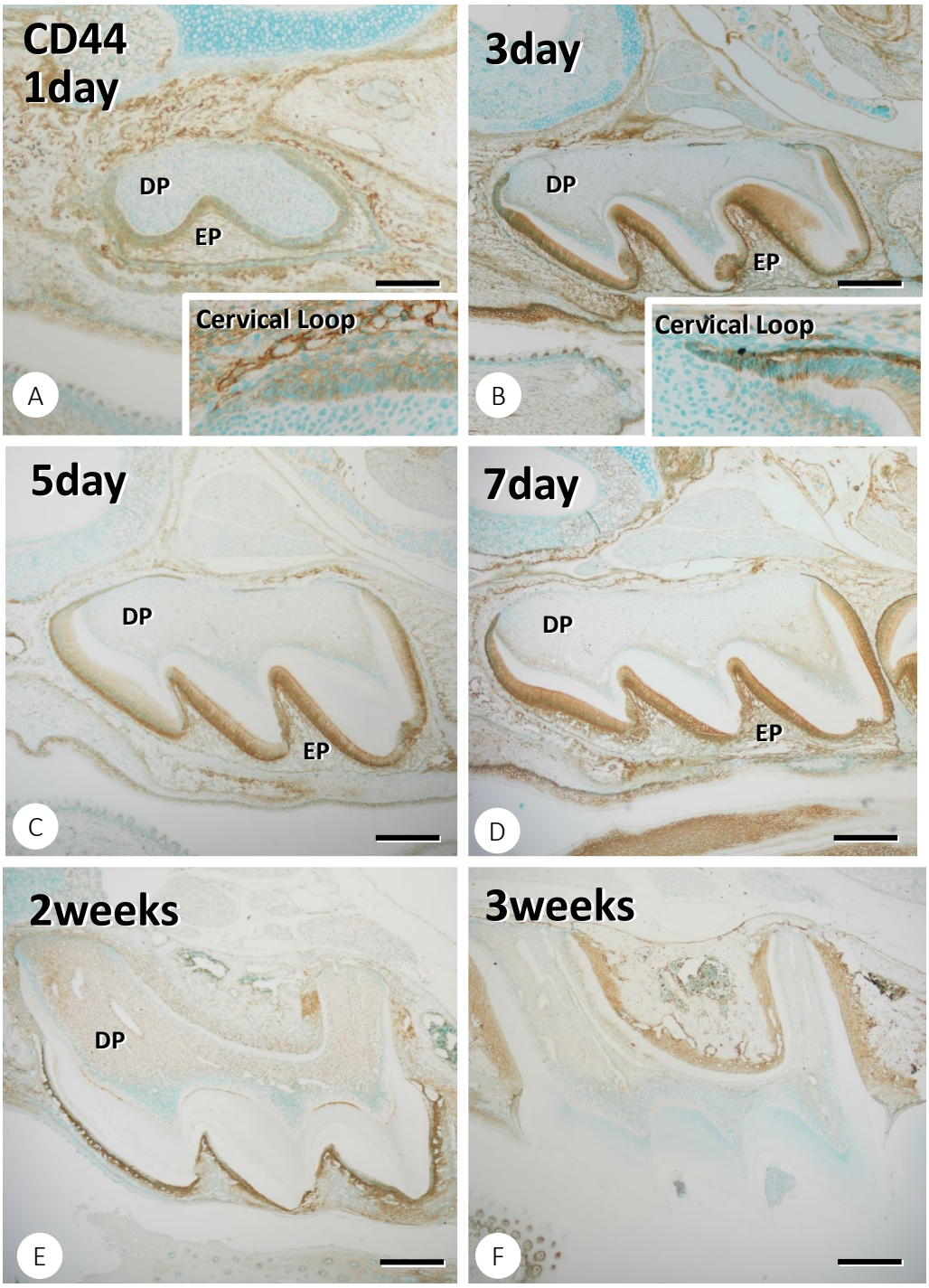
CD44
1day
DP
EP
Cervical Loop
A
3day
DP
EP
Cervical Loop
B
5day
DP
EP
C
7day
DP
EP
D
2weeks
DP
E
3weeks
F

Figure 4

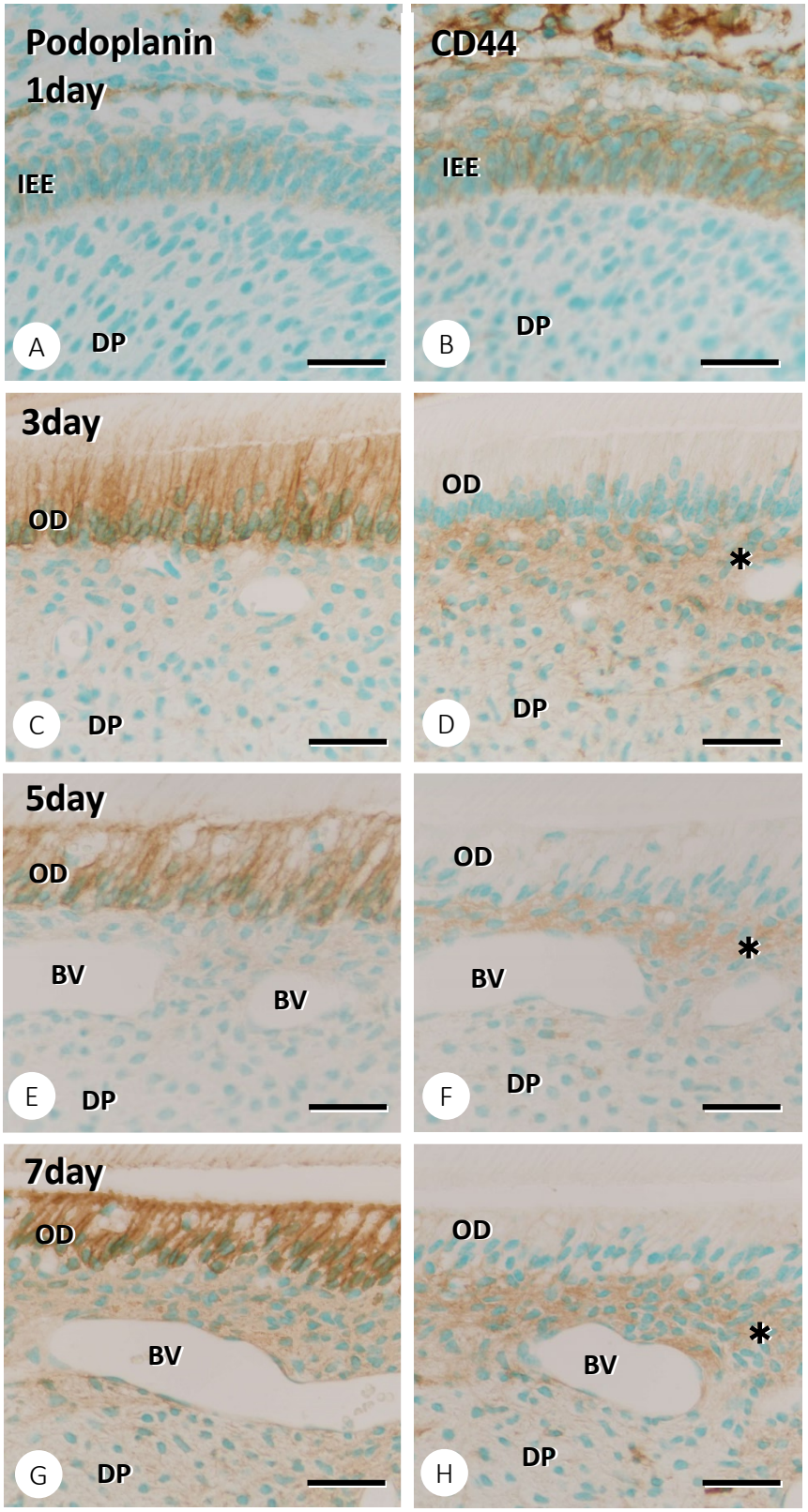
Podoplanin
1day
IEE
A
DP
CD44
IEE
B
DP
3day
OD
C
DP
OD
*
D
DP
5day
OD
BV
BV
E
DP
OD
*
BV
F
DP
7day
OD
BV
G
DP
OD
*
BV
H
DP

Figure 5