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Hertwig's epithelial root sheath cell behavior during initial acellular cementogenesis in rat molars

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Abstract

This study was designed to examine developing acellular cementum in rat molars by immunohistochemistry, to elucidate (1) how Hertwig's epithelial root sheath disintegrates and (2) whether epithelial sheath cells transform into cementoblasts through epithelial–mesenchymal transition (EMT). Initial acellular cementogenesis was divided into three developmental stages, which can be seen in three different portions of the root: portion 1, where the epithelial sheath is intact; portion 2, where the epithelial sheath becomes fragmented; and portion 3, where acellular cementogenesis begins. Antibodies against three kinds of matrix proteinases, which degrade epithelial sheath-maintaining factors, including basement membrane and desmosomes, were used to investigate proteolytic activity of the epithelial sheath. Tissue non-specific alkaline phosphatase (TNALP) and keratin were used to investigate EMT. Epithelial sheath cells showed immunoreactivity for all three enzymes at fragmentation, which suggests that epithelial sheath disintegration is enzymatically mediated. Dental follicle cells and cementoblasts showed intense immunoreactivity for TNALP, and from portion 1 through to 3, the reaction extended from the alveolar bone-related zone to the root-related zone. Cells possessing keratin/TNALP double immunoreactivity were virtually absent. Keratin-positive epithelial sheath cells showed negligible immunoreactivity for TNALP, and epithelial cells did not appear to migrate to the dental follicle. Together, these findings suggest that a transition phenotype between epithelial cells and cementoblasts does not exist in the developing dental follicle, and hence that epithelial sheath cells do not undergo EMT during initial acellular cementogenesis. In brief, this study supports the notion that cementoblasts derive from the dental follicle.

246 words

Key words: Hertwig's epithelial root sheath, Cementoblasts, Epithelial–mesenchymal transition, Acellular cementum, Rat molars

Introduction

Cementum is a dental hard tissue that functions as a tooth-supporting structure, along with the periodontal ligament and the alveolar bone. Its formation is regulated by Hertwig's epithelial root sheath and cementoblasts. The prevailing notion on initial cementogenesis is that the epithelial root sheath covers the developing root edge and grows apically with cell proliferation. As root dentinogenesis advances, the epithelial sheath becomes fenestrated and fragmented. Dental follicle cells then approach the root surface through the gaps formed by sheath fragmentation, differentiate into cementoblasts, and secrete cementum matrices, including collagen fibrils and non-collagenous matrices. Epithelial cell clusters derived from the fragmented sheath survive in the periodontal ligament as the epithelial rests of Malassez.

However, questions remain in this theory, one of which concerns epithelial sheath fragmentation. It is known that the structural integrity of the epithelial sheath is maintained by the basement membrane and desmosomes. E-cadherins are also important for intercellular adhesion (Obara et al. 1999). During sheath fragmentation, however, these epithelial sheath-maintaining factors must suffer some sort of degradation. Two mechanisms have been proposed for the degradation. First, external mechanical damage or invasion of dental follicle cells into the epithelial sheath causes degradation (Cho and Garant 1988; Suzuki *et al*. 2002). Alternatively, epithelial sheath cells secrete matrix-degrading enzymes that disintegrate the factors (Hirata and Nakamura 2006). Which one of these phenomena is the actual degradation mechanism is presently unknown.

Another indistinct point in the theory, concerns the fate of the epithelial sheath cells. Many reports have emphasized that some epithelial sheath cells transform into cementoblasts through epithelial–mesenchymal transition, or EMT (Thomas 1995; Webb et al. 1996; Bosshardt et al. 1998; Bosshardt and Nanci 1998, 2004; Lésot et al. 2000; Zeichner-David et al. 2003; Bosshardt 2005; Huang et al. 2009; Akimoto et al. 2011). This novel idea can explain why the number of epithelial cells decreases immediately after epithelial sheath fragmentation, even though only a few epithelial cells die by apoptosis (Kaneko et al. 1999; Suzuki et al. 2002). Some investigators, however, have disputed EMT and support the original idea of cementogenesis (Diekwisch 2001; Suzuki et al. 2002; Yamamoto et al. 2007; Yamamoto and Takahashi 2009).

This study was designed to examine developing acellular cementum in rat molars by immunohistochemistry (IHC), to thereby elucidate (1) how the epithelial sheath disintegrates, or how the epithelial sheath-maintaining factors are degraded, and (2) whether the epithelial sheath cells undergo EMT. For point (1), antibodies against three kinds of matrix proteinases[kallikrein7(KLK7)], a disintegrin and metalloproteinase 10(ADAM10), and matrix metalloproteinase7(MMP7)), which are associated with the degradation of the epithelial sheath-maintaining factors, were used; KLK7 degrades desmosomal cadherins (Caubet et al. 2004), ADAM10 degrades desmosomal cadherins, type IV collagen and E-cadherins (White 2003; Edwards et al. 2008), and MMP7 degrades laminin and type IV collagen (Birkedal-Hansen et al. 1993). For point (2), antibodies against tissue non-specific alkaline phosphatase (TNALP) and keratin were used to verify whether epithelial sheath cells acquire mineralization-inducing activity. It has been established that cementoblasts, like other mineralization-inducing cells, such as odontoblasts and osteoblasts, show intense TNALP activity (Iwamatsu 1993; Yamamoto et al. 2007).

Materials and Methods

Twenty 20-day-old male Wistar rats weighing about 50 g were used in this study. The animals and tissue specimens were treated in accordance with the guidelines of Hokkaido University's Experimental Animal Committee (No.10-0081).

After anesthesia with an intraperitoneal injection of sodium pentobarbital, animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. The maxillae were removed, freed of soft tissues and demineralized in 5% ethylene-diaminetetraacetic acid. Specimens were dehydrated in a graded series of ethanol and embedded in paraffin. Sagittal serial sections of the first maxillary molar were then cut at 5 μm thickness. Some sections were stained with hematoxylin and eosin (HE) for general histological examination and others were used for IHC as described below.

IHC for keratin

Deparaffinized sections were immersed in methanol containing 0.3% hydrogen peroxide to inhibit endogeneous peroxidase and treated with 0.5% trypsin in 0.01M Tris-HCl buffer (pH7.6) for 20 min at 37°C. Pre-treated sections were incubated with anti-pan keratin mouse monoclonal antibody (Abcam, Tokyo, Japan) and then with anti-mouse IgG goat polyclonal antibody conjugating horseradish peroxidase (HRP) (Histofine Simple Stain rat MAX-PO (M): Nichirei, Tokyo, Japan). The immunoreaction was visualized using 3, 3'-diaminobenzidine as a substrate. Immunostained sections were counter-stained with methyl green.

IHC for proteinases (MMP7, KLK7 and ADAM10) and double IHC for proteinase-keratin

Rabbit polyclonal antibodies against MMP7 (Bioss Inc., Woburn, MA, USA), KLK7 (Bioss Inc.) and ADAM10 (Sigma, St. Louis, MO, USA) were used. After inhibition of endogeneous peroxidase, sections were incubated with the antibodies and then with anti-rabbit IgG goat polyclonal antibody conjugating HRP (Histofine Simple Stain rat MAX-PO (R): Nichirei), and visualized by the 3, 3'-diaminobenzidine method. Sections were counter-stained with methyl green, mounted with glycerin and photographed. Sections were then processed for proteinase-keratin double staining.

After removal of glycerin, sections were treated with trypsin and incubated with anti-pan keratin antibody (Abcam), followed by incubation with anti-mouse IgG secondary antibody. Keratin immunoreaction was visualized with the Vector® VIP substrate Kit (Vector Laboratories, Burlingame, CA, USA). Double-immunostained sections were again counter-stained with methyl green.

IHC for TNALP staining and double IHC for TNALP-keratin

After inhibition of endogeneous peroxidase, sections were incubated with anti-TNALP rabbit polyclonal antibody (Oda et al. 1999), and then with anti-rabbit IgG secondary antibody, for posterior visualization by the 3, 3'-diaminobenzidine method. Sections were counter-stained with methyl green, mounted with

glycerin and photographed for successive TNALP-keratin double staining. After removal of glycerin, sections were treated for keratin staining as described in the proteinase-keratin double staining method.

For all sets of IHC experiments, controls were obtained by substitution of normal rabbit or mouse serum for the primary antibodies. These control sections did not show any specific immunoreactivity.

Results

The mesial side of the mesial root of the maxillary first molars displayed the full range of stages of acellular cementogenesis (Fig. 1a, b). Hence, the apical portion of this side was examined for investigation of initial acellular cementogenesis. For descriptive convenience, initial acellular cementogenesis was divided into three stages, which can be seen in three different portions: portion 1, where the Hertwig's epithelial root sheath is intact; portion 2, where the epithelial sheath becomes fragmented; and portion 3, where acellular cementogenesis begins.

Histology

In portion 1, Hertwig's epithelial root sheath consisted of two cell layers, namely the inner and the outer enamel epithelial cells (Fig. 1c). Dental follicle cells were small and slender, and arranged in parallel with the epithelial sheath. In portion 2, dental follicle cells became large and plump; in contrast, the epithelial sheath cells turned into small, cytoplasm-poor cells. Dental papilla cells differentiated into odontoblasts and started to form predentin (Fig. 1d). With the onset of predentin formation, the epithelial sheath began to fragment. At this point, cytoplasm-poor cells adhering on the root surface could be recognized as epithelial cells. However, it was difficult to make a strict distinction between dental follicle cells and epithelial cells in HE-stained sections in portion 2. In portion 3, with the onset of dentin mineralization, hematoxylin-stainable initial acellular cementum started to form on the mineralized dentin, and cytoplasm-rich large cells, generally referred to as cementoblasts, appeared on the root surface (Fig. 1e). The cementoblasts located about 15-20 μm apart from the root surface.

IHC for keratin

In portion 1, the intact epithelial sheath was consistently immunoreactive for keratin (Fig. 2a). In portions 2 and 3, this consistency was lost with fragmentation of the epithelial sheath and epithelial rests of Malassez appeared. The epithelial rests were located in close proximity to the root surface. Keratin-positive epithelial cells decreased in number after fragmentation (Fig. 2b). Cementoblasts were not immunoreactive for keratin (see Fig. 1e).

IHC for proteinases (MMP7, KLK7 and ADAM10) and double IHC for proteinase-keratin

From portion 1 through to 3, the dental follicle was positive for MMP7 staining. More cervically, the periodontal ligament showed more intense immunoreactivity (Fig. 3a). This trend was consistent for KLK7 and ADAM10 (data not shown). Magnified views of portion 1 showed that the epithelial sheath was immunoreactive for all three enzymes and immunoreactivity was most intense at the apical tip (Fig. 3b, c, d). By comparing single- and double-stained sections, keratin-positive epithelial cells were also found immunoreactive for MMP7 (Fig. 4a, b), KLK7 (Fig. 4c, d) and ADAM10 (data not shown) in portions 2 and 3.

IHC for TNALP staining and double IHC for TNALP-keratin

It has been established that cellular TNALP exists on cell membrane of mineralization-inducing cells, and that extracellular matrices as well as cementoblasts show TNALP activity in the periodontal ligament (Iwamatsu 1993). Hence linear TNALP immunolabelling along cell outlines was regarded as cellular immunoreactivity.

In portion 1, the epithelial sheath stained only weakly for TNALP. The dental follicle cells, particularly in the alveolar bone-related zone, showed more intense immunoreactivity than the epithelial sheath (Fig. 5a). Near portion 2, intense cellular TNALP immunoreactivity appeared in the epithelial sheath- or root-related zone, and in portion 3, the entire dental follicle showed intense cellular immunoreactivity. The immunoreactivity decreased more cervically. By comparing single- and double-stained sections (Fig. 5b, c), it was found that: 1) keratin-positive epithelial cells showed negligible immunoreactivity for TNALP; 2) epithelial cells did not appear to migrate to the developing dental follicle; and 3) intense TNALP-positive cells, including cementoblasts, did not show any immunoreactivity for keratin in the dental follicle.

Discussion

Fragmentation of Hertwig's epithelial root sheath

In this study, the epithelial sheath cells showed immunoreactivity for all three matrix proteinases throughout the three developmental stages. These findings suggest that the epithelial sheath secretes these enzymes to disintegrate itself. Cho and Garant (1988) present transmission electron micrographs in which dental follicle cells appeared to invade the epithelial sheath with their cytoplasmic projections, suggesting that dental follicle cells force their projections into the epithelial sheath to disintegrate it. An electron microscopy study by Suzuki et al. (2002) also suggests that invasion of dental follicle cells causes epithelial sheath fragmentation. Contrary to these opinions, we believe that dental follicle cells approach and extend their projections to the root surface only after enzymatic disintegration of the epithelial sheath.

The intact epithelial sheath shows its most intense proteolytic activity at the apical tip, where epithelial cells proliferate actively for apical elongation (Kaneko et al. 1999). Accordingly, the apical tip may be remodeled continuously, which may explain why the apical tip has a pronounced proteolytic activity.

Dental follicle cells also display proteolytic activity, and even though they may aid epithelial sheath disintegration, we believe that these proteolytic enzymes function mainly for the organization and/or remodeling of the developing periodontal ligament.

Fate of epithelial sheath cells

The epithelial rests of Malassez die gradually by apoptosis as individuals age (Cerri and Katchburian 2005; Luan et al. 2006; Wang et al.2013). However, apoptotic cell death does not occur or occurs only rarely during epithelial sheath fragmentation (Kaneko et al. 1999; Suzuki et al. 2002). Nevertheless, the number of epithelial cells decreases immediately after epithelial sheath fragmentation (see Fig. 2a, b). In addition, cultured epithelial sheath cells show mesenchymal characteristics under given conditions (Thomas 1995; Zeichner-David et al. 2003; Sonoyama et al. 2007; Akimoto *et al.* 2011). These findings have lead many investigators to assume that epithelial sheath cells transdifferentiate into cementoblasts through EMT during *in vivo* regular cementogenesis (Thomas 1995; Webb et al. 1996; Bosshardt et al. 1998; Bosshardt and Nanci 1998, 2004; Lésot et al. 2000; Zeichner-David et al. 2003; Bosshardt 2005; Sonoyama et al. 2007; Akimoto *et al.* 2011). Some of these investigators emphasize that cementoblasts of epithelial origin are associated with only acellular cementogenesis, but not with cellular cementogenesis (Webb et al. 1996; Zeichner-David et al. 2003), and the others mention that cementoblasts of epithelial origin are associated with both acellular and cellular cementogenesis, or do not distinguish between two types of cementogenesis. We suspect that some of these reports are based on misidentification of epithelial cells as cementoblasts or cementocytes, and that this misidentification arises from the misunderstanding that epithelial sheath cells behave similarly in rodent and human cementogenesis. Briefly, in human acellular and cellular cementogenesis, epithelial sheath cells leave the root surface before cementum formation. Consequently, they locate considerably away (about 50 μm or more) from the root as epithelial rests of Malassez and are

not incorporated in the cementum. In contrast, as shown in the present study (Fig.2), the epithelial rests stay close to the root surface and coexist with cementoblasts during rodent acellular cementogenesis (Schroeder 1986; Bosshardt et al. 1998; Diekwisch 2001; Luan et al. 2006; Yamamoto and Takahashi 2009). Some investigators supporting EMT may misinterpret epithelial cells remaining on the root as cementoblasts (Webb et al. 1996; Lésot et al. 2000). In rodent cellular cementogenesis many epithelial sheath cells as well as true cementoblasts are incorporated in the cementum (Lester 1969a, b; Yamamoto and Hinrichsen 1993; Bosshardt et al. 1998; Yamamoto and Takahashi 2009). These incorporated epithelial cells may be misinterpreted as cementocytes (Lésot et al. 2000). Details of EMT in cellular cementogenesis will be discussed later.

It is well known that EMT occurs during a variety of normal developmental events. For example, when the opposing palate shelves adhere to each other in palatogenesis, epithelial cells undergo EMT at the midline seam. Palatogenesis is of particular interest in discussing EMT of epithelial sheath, because palatal epithelial cells belong to oral epithelium and the underlying mesenchymal cells, like dental follicle cells, are believed to be neural crest in origin (Bosshardt and Selvig 1997). Fitchett and Hay (1988) use keratin and vimentin as markers of epithelial and mesenchymal cells, respectively, and find coexisting keratin-vimentin immunostaining in the seam epithelium. In addition, Gibbins et al. (1999) find coexpression of keratin and vimentin mRNA in the seam epithelium. These findings suggest that an intermediate phenotype which expresses both epithelial and mesenchymal characteristics appears transiently during EMT.

This study used keratin and TNALP as markers of epithelial cells and cementoblasts, respectively. It is known that cementoblasts show intense TNALP activity, whereas epithelial sheath cells show negligible activity (Iwamatsu 1993; Yamamoto et al. 2007). If epithelial sheath cells became cementoblasts through EMT, an intermediate phenotype would appear in portions 2 and 3, in the following sequence of events. 1) Epithelial sheath cells would lessen their keratin immunoreactivity and leave the root surface. 2) Epithelial sheath cells would become large, cytoplasm-rich cells and express both keratin and TNALP immunoreactivity. 3) The enlarged cells would settle about 15-20 μm apart from the root surface as TNALP-positive cementoblasts, which have lost keratin immunoreactivity. However, our experiments were not able to observe the findings that support this hypothesis.

The present study dealt with EMT in only acellular cementogenesis. Previous IHC studies may be informative for discussion about EMT in cellular cementogenesis. Yamamoto and Takahashi (2009) do not observe colocalization of keratin-vimentin or keratin-runt-related transcription factor 2(used as a marker for mineralization-inducing cells) in any cells involved in rat cellular cementogenesis, such as epithelial sheath cells, epithelial rests of Malassez, dental follicle cells, cementoblasts and cementocytes. Hirata and Nakamura (2006) do not observe colocalization of keratin-osteopontin or keratin-bone sialoprotein in any cells involved in mouse cellular cementogenesis. These findings do not support EMT in *in vivo* cellular cementogenesis. Taken all together, there has been no concrete evidence supporting EMT during *in vivo* acellular or cellular cementogenesis. In conclusion, we support the original idea that the cementoblasts derive from the dental follicle in *in vivo* acellular and cellular cementogenesis. Recently, however, Huang et al. (2009) suggest that some incorporated epithelial cells become cementocytes through EMT in transgenic mice. On the contrary, Suzuki et al. (2006) suggest that incorporated epithelial cells gradually die by

apoptosis. We will investigate EMT in cellular cementogenesis further.

But still, why do the epithelial cells decrease in number immediately after epithelial sheath fragmentation? Regarding this question, Diekwisch (2001) has an interesting insight: “The epithelial root sheath cells proliferate only at the apical end of the sheath while the entire root surface grows considerably. As a result, the epithelial sheath only covers small portions of the developmentally advanced tooth root. The disproportionate growth rate may explain why the root surface is only covered by very few epithelial cells.” We agree with this comment, and consider that the shape of the intact epithelial sheath is also associated with the disproportionate growth rate. The intact epithelial sheath bends toward the dental papilla and forms a tapering cylinder (Fig. 6). The root grows somewhat straight, whereas the epithelial sheath maintains the tapering shape during root elongation (Kaneko et al. 1999; Luan et al. 2006). In this situation, the epithelial sheath could not cover the entire surface of the developing root owing to the discrepancy of surface area, even though epithelial cells proliferate actively at the apical tip. Therefore, the epithelial sheath may be enzymatically disintegrated and dispersed over the root surface. This may help cementoblasts approach the root surface and explain the decreased epithelial cell number in histological sections.

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Figure Legends

Figure 1. HE-stained sections. **a** Whole view of 20-day-old rat maxillary first molar. Developing mesial root is observed. Bar 200 μm. **b** Magnification of boxed area in **a.** Black and white arrows indicate Hertwig's epithelial root sheath and hematoxylin-stained acellular cementum, respectively. PL periodontal ligament, AB alveolar bone, DP dental pulp. Bar 50 μm. **c** Portion 1. Intact epithelial root sheath (between arrows) demarcates dental follicle (DF) and dental papilla (DP). Bar 10 μm (common in **c**-**e**). **d** Portion 2 and 3 partitioned by line. In portion 2 the epithelial sheath (arrow) is fragmented with predentin formation (asterisk). In portion 3, dentin mineralization (double asterisks) starts. OB odontoblasts. **e** Portion 3. With the onset of dentin mineralization (double asterisks), the initial acellular cementum (black arrow) appears on the dentin surface. Large and cytoplasm-rich cells suggestive of cementoblasts (white arrows) are observed.

Figure 2. Sections stained for keratin. **a** Apical half of the root. Black and white arrows indicate epithelial root sheath and epithelial rests of Malassez, respectively. Note that the epithelial rests lie close to the root surface. Bar 50 μm. **b** Magnification of boxed area in **a**. With epithelial sheath fragmentation, keratin-positive epithelial cells decrease in number. Bar 10 μm.

Figure 3. Sections stained for MMP7 (**a**, **b**), KLK7 (**c**), and ADAM10 (**d**). **a** Apical half of the root. Dental follicle (asterisk) stains moderately. Developing periodontal ligament stains more intensely (double asterisks). Bar 50 μm. **b**, **c**, **d** In portion 1, the intact epithelial root sheath (between arrows) shows immunoreactivity for all three enzymes. The apical tip (enclosed area) is most immunoreactive. Bar 10 μm.

Figure 4. Sections of portions 2 and 3, stained for MMP7 (**a**), double-stained for MMP7 and keratin (**b**), stained for KLK7 (**c**), and double-stained for KLK7 and keratin (**d**). **a** and **b**, and **c** and **d** pairs are the same section. Epithelial cells (arrows) are positive for proteinases (brown) and keratin (purple). Bars 10 μm.

Figure 5. Sections stained for TNALP (**a**, **b**) and double-stained for TNALP and keratin (**c**). TNALP and keratin stain brown and purple, respectively. **b** and **c** Figures were taken from the same section**. a** Apical half of the root. The intact epithelial sheath (arrow) stains weakly for TNALP. The dental follicle cells, particularly in the alveolar bone-related zone (asterisk), is more intensely immunoreactive than the epithelial sheath cells. Near portion 2, intense immunoreactivity encompasses the root-related zone. In the boxed area, which corresponds to portion 3, the entire dental follicle shows intense immunoreactivity. Bar 50 μm. **b** and **c** Magnification of portion 2 and 3. Linear immunolabelling corresponds to cellular TNALP immunoreactivity. In portion 2, epithelial cells (black arrows) are keratin-positive and show no or only negligible immunoreactivity for TNALP. Keratin-positive epithelial cells do not appear to move away from

the root surface. Dotted line indicates the extension of intense cellular TNALP immunoreactivity from the alveolar bone-related zone (asterisk) to the root surface. In portion 3, cementoblasts (white arrows) show intense cellular TNALP immunoreactivity drawing cell outlines and do not show any immunoreactivity for keratin. Note that there are no cells showing double immunostaining of TNALP and keratin in portion 2 or 3. Bars 10 μm.

Figure 6. Schematic diagram explaining the disproportionate growth rate between the epithelial sheath and the forming root. **a** Tapering epithelial sheath covers the root tip. **b** During root elongation the root grows straight (arrow) and the epithelial sheath maintains the tapering shape. Consequently, the surface area of newly formed root exceeds that of the epithelial sheath.

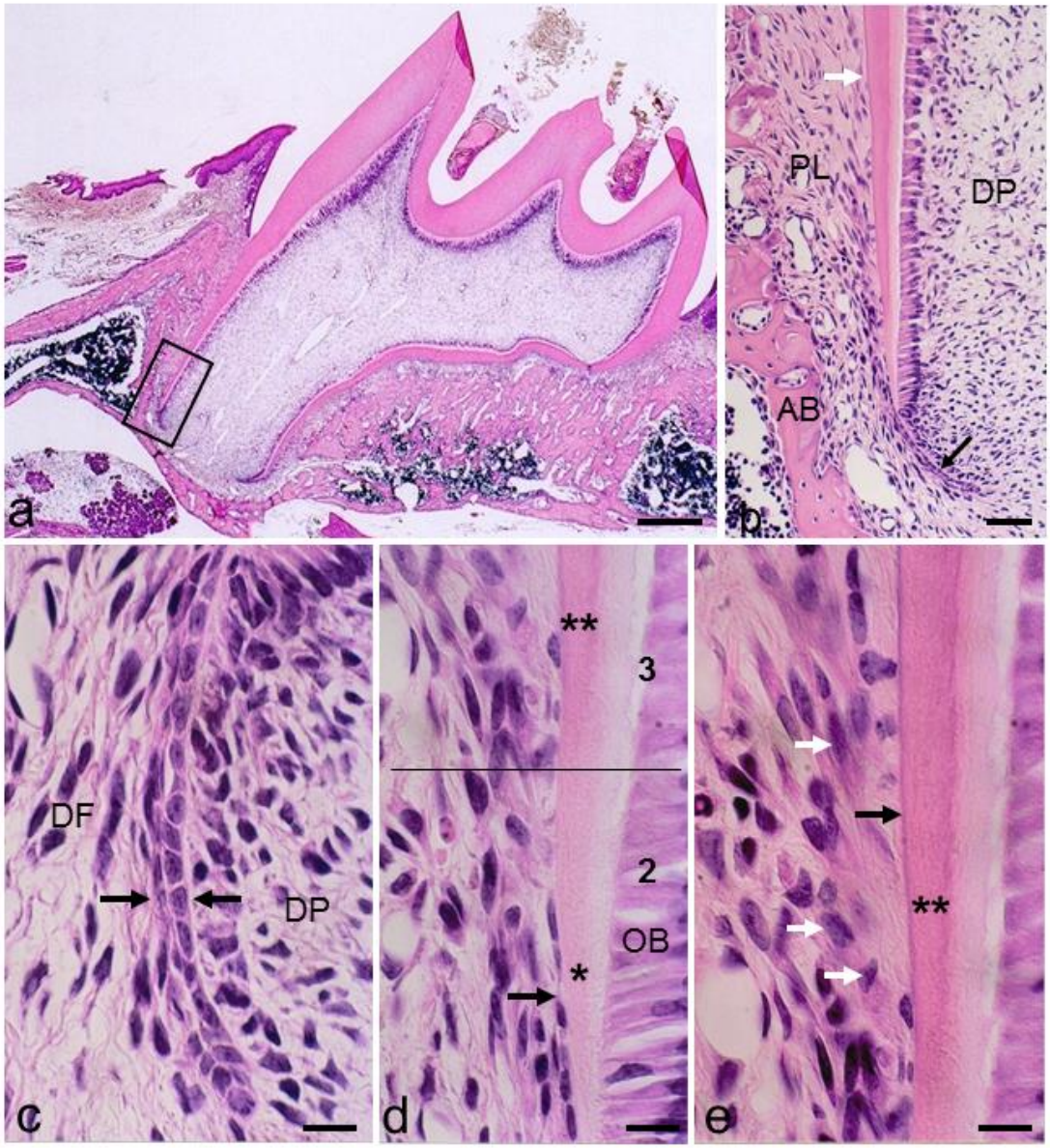

Fig.1

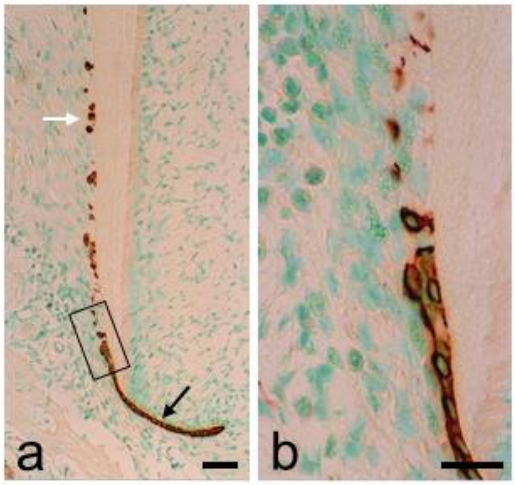

Fig.2

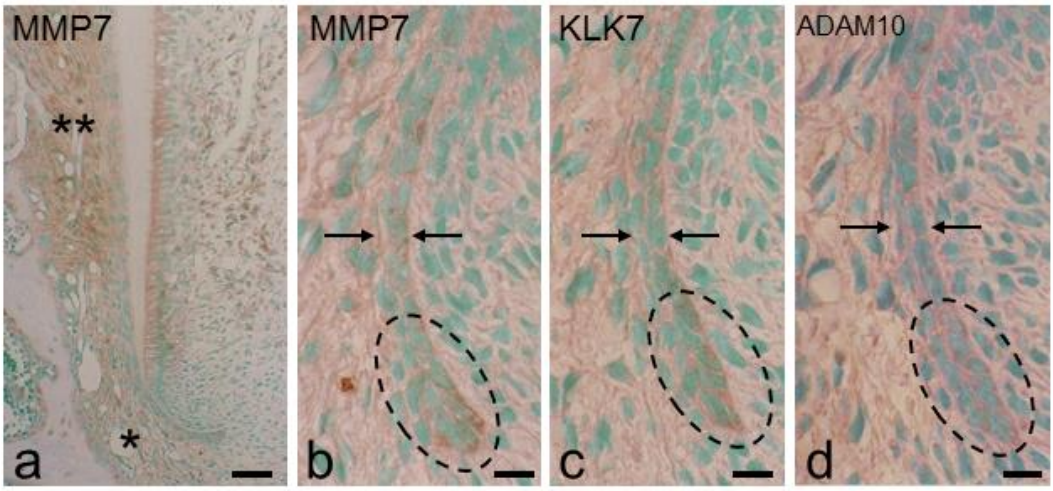

Fig.3

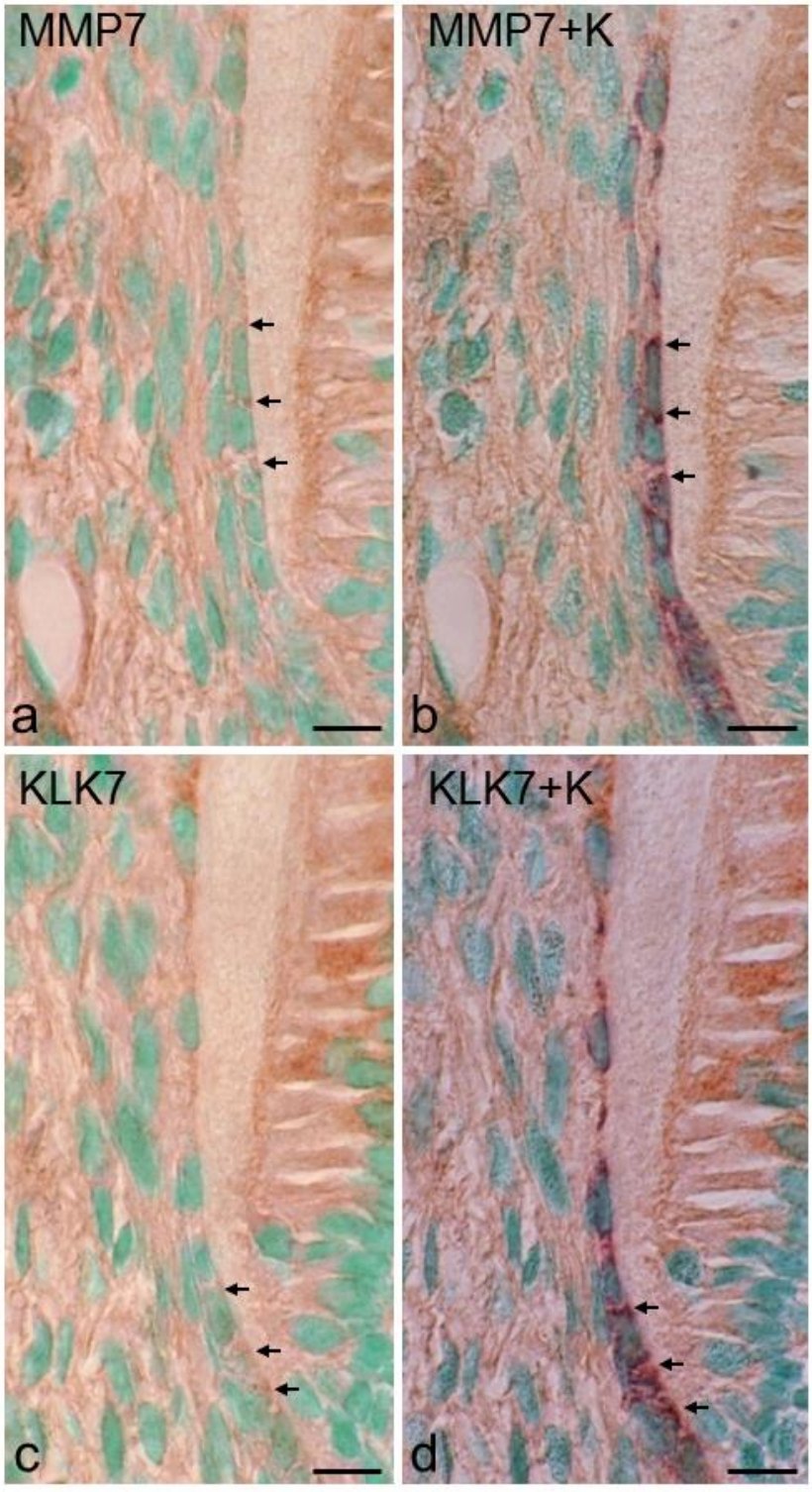

Fig.4

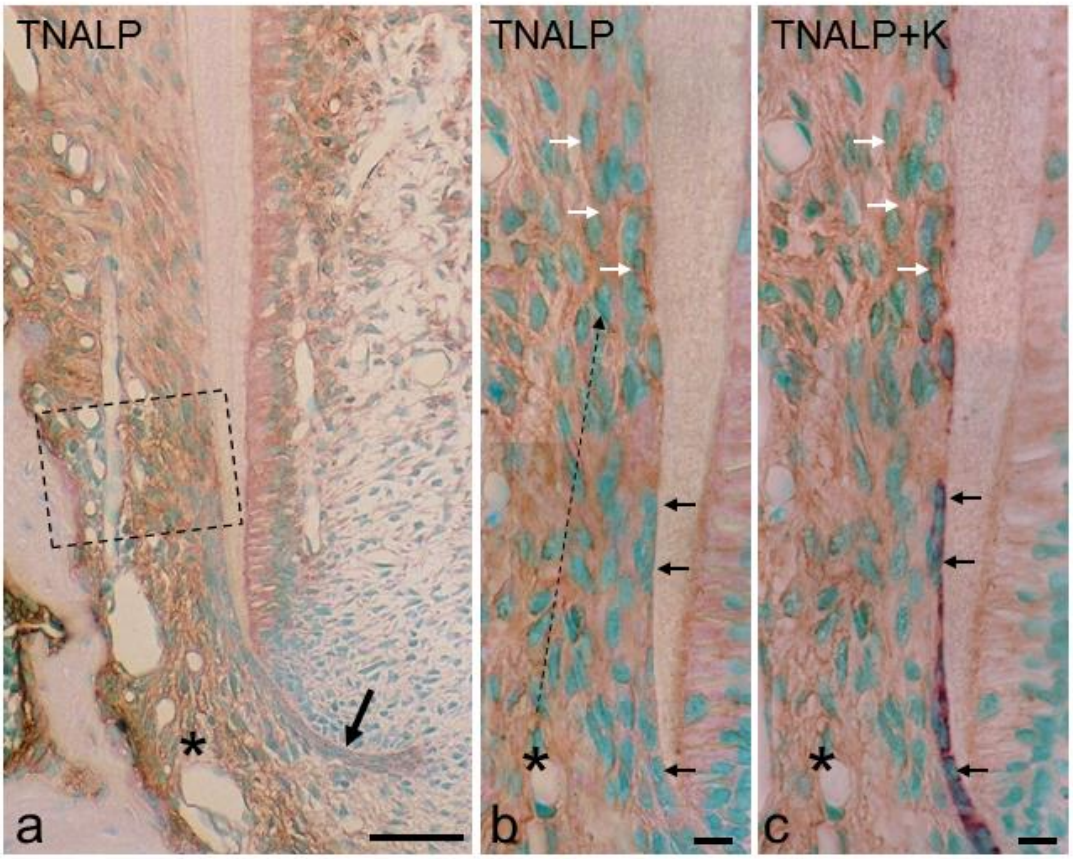
TNALP
TNALP
TNALP+K
a
b
c
*
*
*

Fig.5

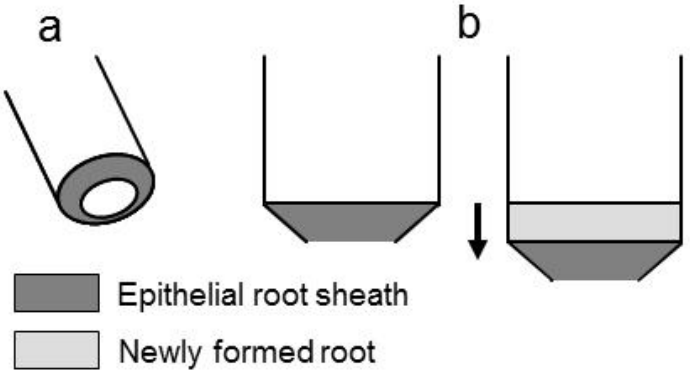
a
b
Epithelial root sheath
Newly formed root

Fig.6