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Original

Histological and Immunohistochemical Observation of the Furcation Area Formation with the Subpulpal Lobus of Rat Molar

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Abstract: In the human molar, multirooted tooth formation occurs through the formation of dentin islands, termed subpulpal lobus, in the dental papilla. However, the mechanisms underlying subpulpal lobus formation currently remain unclear. Thus, using the rat molar, in which multirooted teeth formed through subpulpal lobus formation, similar to that in humans, we observed the process of subpulpal lobus formation over multirooted tooth formation histologically and immunohistologically, and discussed accessory root canal formation in the furcation area. The upper second molar (M2) was examined from rats 8 to 18 days after birth, stained with hematoxylin, and observed under a stereoscopic microscope. The maxilla including M2 was dissected out *en bloc* and decalcified, and paraffin-embedded sections were prepared following the standard method. Sections were subjected to hematoxylin-eosin double staining and immunohistochemical staining with anti-pan-keratin (PK), anti-heat shock protein 27 (HSP27), and anti-sonic hedgehog (Shh) antibodies. After the completion of tooth crown formation, a part of Hertwig's epithelial root sheath (HERS) in the cervical region extended and formed an epithelial projection (EP) that was reactive with the anti-PK antibody. The EP induced the differentiation of dental papilla cells facing it into anti-Hsp27 antibody-reactive odontoblasts and formed the subpulpal lobus. EP and subpulpal lobus-forming odontoblasts reacted with the anti-Shh antibody, suggesting that cell differentiation in subpulpal lobus formation occurs through the epithelial-mesenchymal interaction. While forming subpulpal lobus, the EP extended without making contact with the opposing EP or HERS in the cervical region. The furcation area was formed by fusion between the dentin projection (DP) formed in the cervical region and subpulpal lobus and between subpulpal lobus. HERS in the cervical region and EP formed the furcation area without making contact with each other, suggesting that the accessory root canal is formed where DP and the subpulpal lobus do not fuse.

Key words: Furcation area, Subpulpal lobus, Multirooted tooth, Molar, Rat

Introduction

Tooth morphogenesis is regarded as a model system of morphogenesis through the epithelial-mesenchymal interaction. A large number of studies have investigated the epithelial-mesenchymal interaction in tooth morphogenesis[1)]. The findings obtained demonstrated that the primary enamel knot that appear in tooth bud in the bud stage and secondary enamel knot appearing in enamel organ in the cap stage function as a signal center[1-3)], i.e., many genes and signal molecules expressed by the epithelium and mesenchyme are involved while influencing each other in the differentiation of odontoblasts (OB) and ameloblasts (AM)[1,4)]. However, these studies focused on tooth crown morphogenesis; the presence of a signal center in tooth root morphogenesis similar to enamel knots has not yet been reported[5)]. On the other hand, the involvement of signals from the inner enamel epithelium of Hertwig's epithelial root sheath (HERS) in the differentiation of dental papilla cells facing the inner enamel epithelium of HERS into OB has been demonstrated[1)]. These reports suggested that the epithelial-mesenchymal interaction in tooth morphogenesis occurs in the morphogenesis of not only the tooth crown, but also the tooth root.

Signals involved in tooth formation include sonic hedgehog (Shh)[6,7)], msh homeobox 2[5,8)], fibroblast growth factors[9)], lymphoid enhancer-binding factor[10)], wingless int[9,11)], and bone morphogenetic protein (Bmp)[5-10)]. Shh is expressed from the early developmental stages in various organs, such as the four limbs[12)], and is regarded as a morphogenetic factor in many organs. Shh is also expressed in the inner enamel epithelium, mainly in enamel knots, during the tooth developmental process. Its receptor, patched-1, is expressed in the mesenchyme around the dental

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papilla and tooth germ[13,14], and was previously shown to influence many genes, such as smoothened and Bmp4[15]. Furthermore, Shh is expressed in OB forming dentin[6-7], indicating its important role in tooth root morphogenesis.

Most mammals including humans are heterodonts. The incisors cut off food, whereas the molars grind food in the oral cavity. Accordingly, the surface area of the molar tooth root is widened by multirooted tooth formation in order to tolerate strong occlusal forces.

The mouse model of multirooted tooth formation reported by Orban and Mueller in 1929 is widely accepted to show the mechanisms underlying this process[16] in which multirooted teeth are formed as follows: (1) The part of HERS surrounding the tooth crown grows toward the dental papilla and forms an epithelial projection (EP), (2) The EP makes contact with the opposing EP, (3) Dental papilla cells facing the EP differentiate into OB, and a dentin projection (DP) is formed from the cervical side, and (4) It fuses with the opposing DP. However, Ooë showed that when human deciduous teeth were examined, unlike the multirooted tooth formation mechanism in mice, a mass of dentin, termed the subpulpal lobus, appeared in the center of the dental papilla regardless of the state of dentin formation around it[17]. The subpulpal lobus became a furcation area, inducing multirooted tooth formation[18]. Multirooted tooth formation accompanied by the appearance of subpulpal lobus has been reported in pigs[19] and rats [20]; however, the mechanisms underlying subpulpal lobus formation currently remain unclear.

A tubular structure termed the accessory root canal is present in furcation areas in multirooted teeth[21]. Since dental pulp and periodontal tissue communicate through the accessory root canal, which is involved in the spread of dental pulp lesions to periodontal tissue and vice versa, the accessory root canal is regarded as a clinically important structure; however, the mechanisms responsible for its formation have not yet been elucidated.

Previous studies on tooth germ formation have used anti-pan keratin (PK) for the observation of the epithelium, which is known to stain HERS and Malassez epithelial rests[22]. In addition, anti-heat shock protein 27 (HSP 27) is used for observation of odontoblast differentiation[23].

In the present study, using rat molars in which multirooted teeth form through subpulpal lobus formation, similar to that in humans, the process from subpulpal lobus formation to multirooted tooth formation was investigated histologically, and an important factor involved in tooth formation, Shh, was immunohistochemically examined in order to clarify the mechanisms underlying the formation of the subpulpal lobus and furcation area. The mechanisms responsible for accessory root canal formation were also discussed.

Materials and Methods

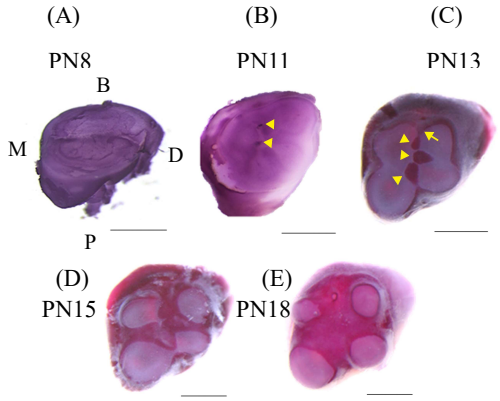

Figure 1. Furcation area formation process observed under a stereoscopic microscope
When the tooth germ being formed was observed through the bottom surface, subpulpal lobus (A,B) appeared independently of the surrounding dentin (cervical region dentin and DP) at PN11 and grew with the progression of tooth formation (C). The furcation area was formed by the fusion of a subpulpal lobus with another subpulpal lobus and also with a DP (C-E).
A M: Mesial, D: Distal , B: Buccal, P: Palatal
B, C arrowheads: subpulpal lobus, C arrow: dentin projection
Bars: 500 μm

This study was approved by the Experimental Animal Committee of Tokyo Dental College and performed in conformity with the specified guidelines for animal experiments (No.250206).

Animals

Twenty-five male Wistar rats (5 per stage) at 8, 11, 13, 15, and 18 days after birth (PN8, PN11, PN13, PN15, and PN18) were used as experimental animals in the present study. The upper second molar tooth germ (M2) with a large furcation area was examined.

Animals were fixed by the perfusion of 0.1 M PBS-buffered 4% paraformaldehyde solution (pH7.4) under deep anesthesia with an intraperitoneal injection of pentobarbital (200 mg/kg), followed by immersion fixation at 4 ℃. The maxilla including the teeth was then dissected out *en bloc*.

Stereoscopic microscopy

M2 was examined from the maxillae of 10 rats (2 per stage) and entirely stained with hematoxylin solution. The tooth germ was observed through the bottom side under a stereoscopic microscope (LEICA MZ75, Leica, Germany).

Histology and immunohistochemistry

The maxillae of 15 rats (3 per stage) were decalcified with 10% EDTA at 4 ℃ for 3 weeks. After washing with PBS, speciments were dehydrated with ethanol series, paraffin blocks were prepared following the standard method, and 4-μm-thick serial sagittal sections were prepared.

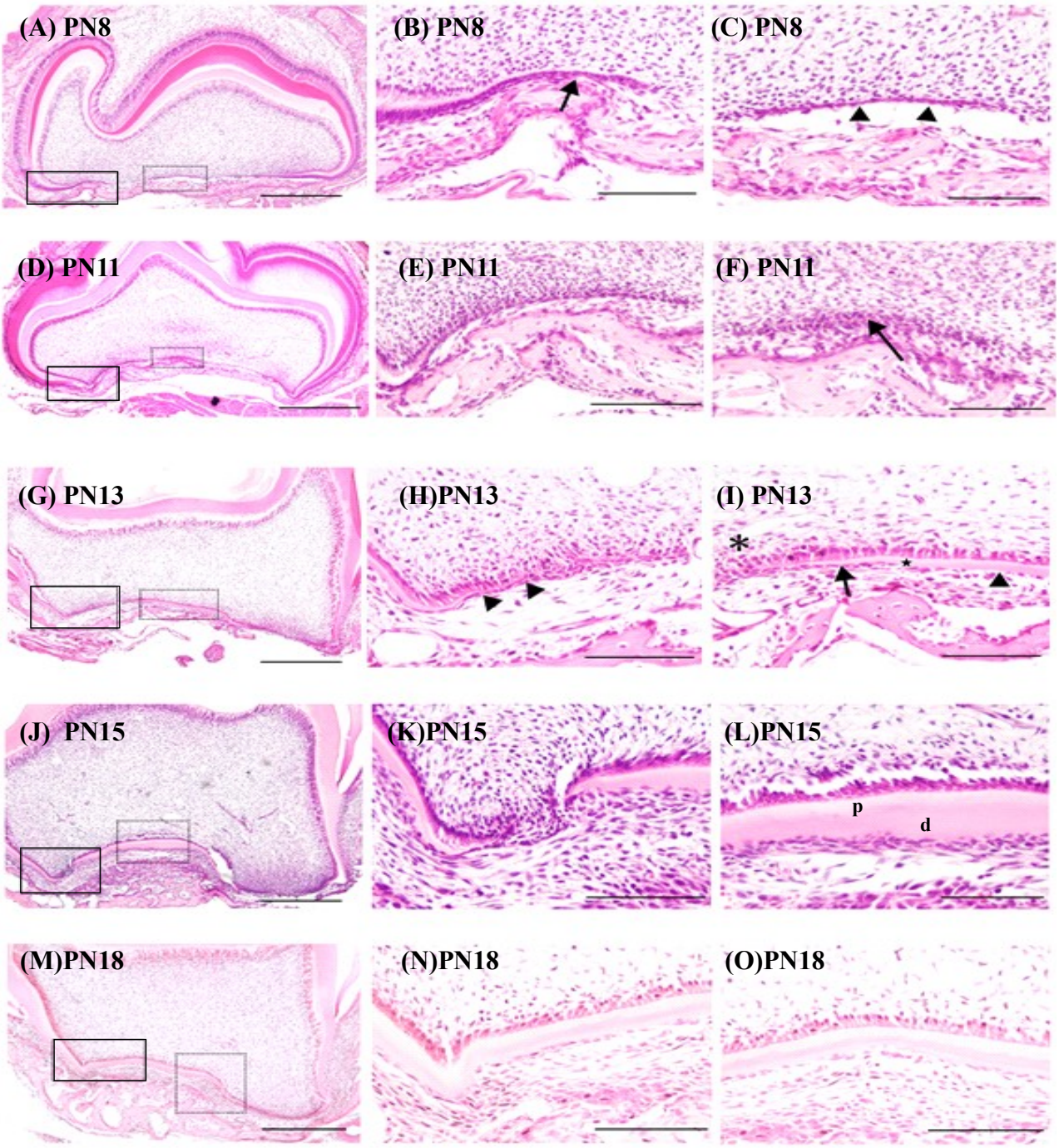

Figure 2. H-E staining of the furcation area formation process.
Dental papilla cells facing the epithelial cord-like structure (C arrowheads) observed at PN8 differentiated into columnar cells, and subpulpal lobus formation had started at PN11 (F arrow). Dental papilla cells facing the EP (B arrow) extending from the cervical region also differentiated into columnar OB with development, and the formation of DP was observed (B, E, H arrowheads). Subpulpal lobus thickened (I arrowhead, L) and, at the same time, cell differentiation occurred at the side of the subpulpal lobus (I arrow, I*), and the lateral progression of subpulpal lobus formation was noted (I). Subpulpal lobus formed in the lateral direction and DP that formed continuously from dentin in the cervical region were fused and formed dentin in the furcation area (K, N).
B, E, H, K, and N are magnified views of the black-framed regions in A, D, G, J, and M, respectively. C, F, I, L, and O are magnified views of the dotted boxes in A, D, G, J, and M, respectively.
B. arrow: epithelial projection; C. arrowheads: epithelial cord-like structure; H. arrowheads: a dentin projection being formed; I. arrowhead: thickened subpulpal lobus, arrow: subpulpal lobus comprised of predentin, ⁎ : region of cell differentiation, ★ : predentin; L. p: predentin, d: dentin
Bars: A, D, G, J, M. 500 μm, and B, C, E, F, H, I, K, L, N.100 μm

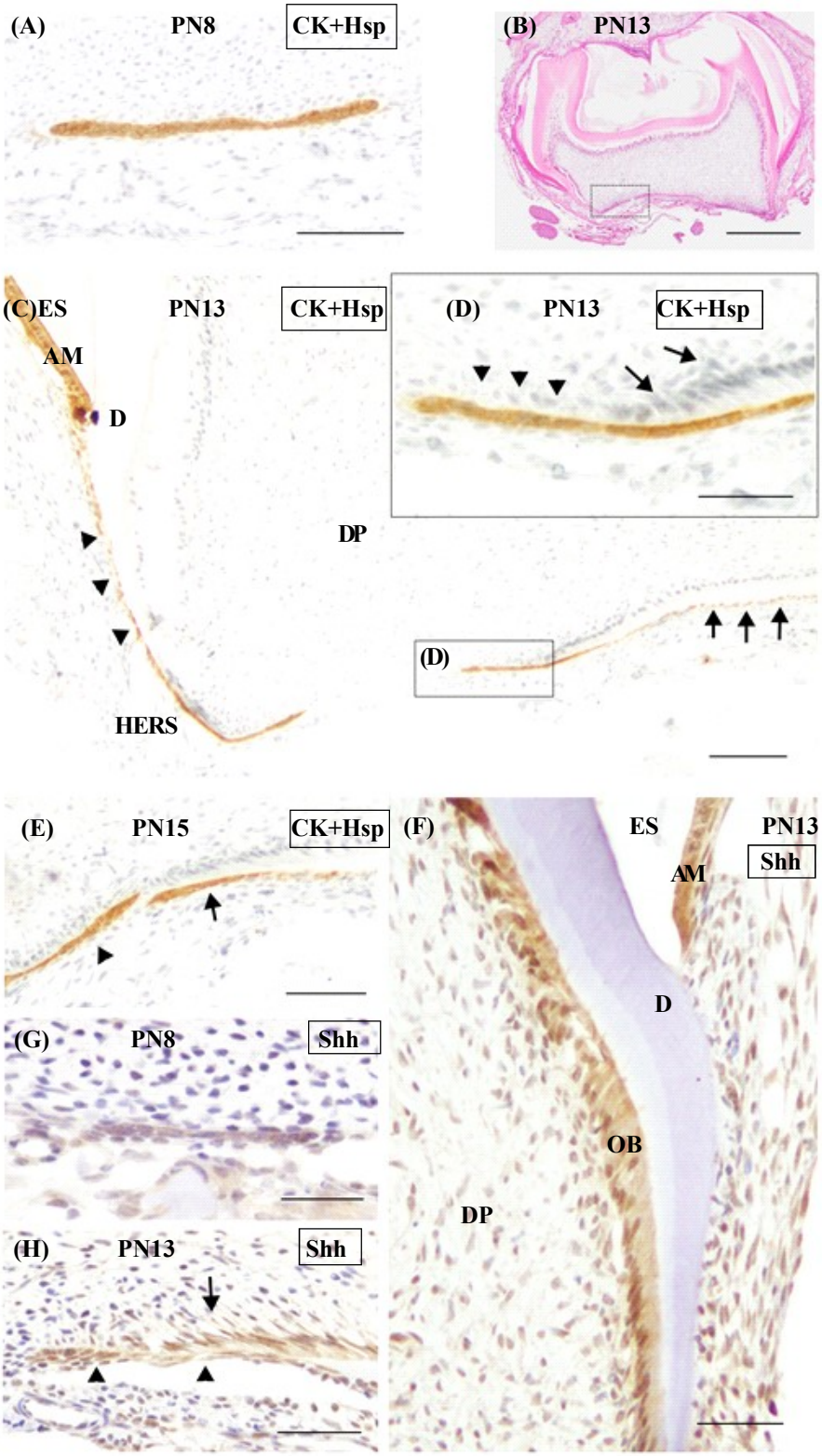

Figure 3. Immunohistochemical staining with anti-PK, anti-Hsp27 and anti-Shh antibodies.

On double staining with anti-PK (brown) and anti-Hsp27 (blue-gray) antibodies, an EP reacted with the anti-PK antibody at PN8 (A). C showed higher magnification of dotted square in B. Dental papilla cells facing the EP became high columnar odontoblast-like cells and reacted with the anti-Hsp27 antibody (D arrows). A reaction with the anti-PK antibody was noted in Hertwig's epithelial root sheath (C HERS) and Malassez epithelial rests (C arrowheads) formed by its disintegration. In the subpulpal lobus-formed region, the stainability of the tip of the EP was similar to that of HERS, and anti-PK antibody-positive cell masses disintegrated in the central region of the EP (C arrows). Anti-PK antibody-reactive HERS and the EP grew horizontally to the same position, but did not make contact with each other at PN15 (E). A reaction with the anti-Shh antibody was observed in AM and OB (F). A reaction with the anti-Shh antibody was only observed in the EP, even at the subpulpal lobus-formed region, at PN8 (G), but was noted in subpulpal lobus-forming odontoblasts in addition to the EP (H arrowheads) at PN13 (G).

A, C, D, and E: Immuno-double staining with anti-PK and anti-Hsp27 antibodies; B: H-E staining section at PN13; F-H: Immunostaining with the anti-Shh antibody; AM: ameloblast; S: enamel space; D: dentin; DP: dental papilla; OB: odontoblast

Bars: A, E, F. 100 μm, B. 500 μm, C. 200 μm, and D, E, H. 50 μm

Sections were subjected to hematoxylin-eosin double staining (H-E) following the standard method. Some sections were subjected to immunohistochemical staining using the method as described below.

After deparaffinization with xylene and alcohol series, sections were immersed in 0.3% H_2O_2-containing methanol at room temperature for 30 minutes to remove endogenous hydrogen peroxide. Sections were then immersed in 0.01% citrate buffer (pH 6.0) and treated with heat to activate antigens. After blocking with 2.5% normal goat serum, sections were subjected to double

immunostaining with the following primary antibodies using ImmPRESS Reagent (Vector Laboratories Inc., California, USA): a rabbit anti-rat heat shock protein 27 polyclonal antibody (1/500) (Abcam, Cambridge, UK) to observe differentiated OB and a mouse anti-PK monoclonal antibody (1/100) (Abcam, Cambridge, UK) to stain epithelial cells. A positive reaction with the anti-Hsp27 antibody was visualized by the development of a blue-gray color using the Vector SG Substrate Kit for peroxidase (Vector Laboratories Inc., California, USA), followed by that of a brown color for a positive reaction with the anti-PK antibody using 3,3'-diaminobenzidine tetrahydrochloride (DAB). Control sections were reacted with normal horse serum instead of the primary antibodies. Some sections were blocked with 2.5% normal horse serum and then reacted with a rabbit anti-human Shh polyclonal antibody (1/250) (Santa Cruz, Texas, USA) as the primary antibody and the VECTASTAIN Elite ABC Kit (Vector Laboratories Inc., California, USA), and a dark brown color was developed using DAB. Sections were subsequently counterstained with hematoxylin. Control sections were reacted with normal goat serum instead of the primary antibody.

Results

Stereoscopic microscopy

No structure assumed to be hard tissue was observed in the dental papilla at PN8 (Fig. 1A), whereas hard tissue (the subpulpal lobus) intensely stained with hematoxylin was present in the central region of the dental papilla at PN11 independently of dentin and DP in the cervical region (Fig. 1B). These subpulpal lobus islands grew with tooth development and fused with the adjacent subpulpal lobus and DP extending from the cervical region, thereby forming the furcation area (Fig. 1C, D). At PN18, furcation area formation by subpulpal lobus and DP was complete, and the division of the tooth root into 5 parts was observed (Fig. 1E). The region in which subpulpal lobus appeared varied among the teeth examined (data not shown); however, the mechanisms underlying multirooted tooth formation were similar in all observed tooth germs.

Histology

In the tooth germ at PN8, HERS extended from the cervical region toward the center of the dental papilla (Fig. 2A, B arrow). In addition, the EP with a cell cord-like structure was observed in the center of the bottom of the dental papilla (Fig. 2C, arrowheads). The morphology and arrangement of dental papilla cells facing HERS and the EP were similar to those of dental papilla cells in the other regions (Fig. 2B, C). No hard tissue was observed in the bottom of dental pulp at this time-point. In the tooth germ at PN11, no structure suggesting hard tissue was observed in the bottom of the dental papilla, which was similar to that in the tooth germ at PN8 (Fig. 2D); however, dental papilla cells facing HERS near the cervical region became taller than other dental papilla cells (Fig. 2E). Furthermore, dental papilla cells facing the EP in the center of the bottom of the dental papilla showed a columnar shape, and a region lightly stained with eosin, assumed to be the subpulpal lobus, was present between the EP and dental papilla cells (Fig. 2F arrow). At PN13, dentin projection (DP), continuous with that of the cervical region, formed in HERS near the cervical region (Fig. 2H arrowheads). Dentin assumed to be a subpulpal lobus was observed in the central region of the bottom of the dental papilla (Fig. 2G dotted square). When this region was magnified, dental papilla cells facing thin dentin had a high columnar shape and strong hematoxylin stainability, showing the characteristics of OB (Fig. 2I). Although the dentin that formed was thin, a region (Fig. 2I arrow) formed with predentin only, which was stained with eosin (Fig. 2I asterisk), was present at the side of calcified dentin stained with hematoxylin (Fig. 2I arrowhead), and the differentiation of dental papilla cells was observed at its side (Fig. 2I asterisk). At PN15, the subpulpal lobus observed in the central region of the bottom of the dental papilla were thicker than those observed at PN13, and the calcified dentin region stained with hematoxylin became thicker than predentin. In the cervical region, subpulpal lobus and DP were close to each other (Fig. 2K). At PN18, subpulpal lobus and DP had fused (Fig. 2M, N, O). Contact between EP or between the EP and HERS, bifurcation of the tip, or the disintegration of EP before dentinogenesis was not observed at any age in days (data not shown).

Immunohistochemistry

At PN8, a positive reaction of anti-PK antibody (brown) was observed on the EP, whereas dental papilla cells facing anti-PK antibody-reactive cells did not react with the anti-Hsp27 antibody (Fig. 3A). Figure 3C shows medium magnification of the tooth germ and cervical region (Fig. 3B dotted square). The left region was continuous with the cervical region of the tooth crown over the root, and AM, HERS, and Malassez epithelial rests (arrowheads) formed by the disintegration of HERS reacted with the anti-PK antibody. In the right region forming subpulpal lobus, the tip of the EP was continuous with anti-PK antibody-reactive cells, whereas anti-PK antibody-reactive cells disintegrated in the region indicated with arrows. Fig. 3D shows a magnified view of the black-framed region in Fig. 3C. Dental papilla cells facing the tip of the EP did not react with the anti-Hsp27 antibody (arrowheads), whereas those facing anti-PK antibody-reactive epithelial cells in the center of the EP reacted with the anti-Hsp27 antibody (blue-gray color). Fig. 3E shows the boundary between HERS (arrowhead) and the EP (arrow). Anti-PK antibody-reactive HERS and EP were present at the same horizontal position, but did not make contact with each other and a difference was observed between their vertical positions.

Fig. 3F shows immunostaining of the cervical region with the anti-Shh antibody at PN13. Both AM and OB reacted with the

anti-Shh antibody. In the subpulpal lobus-forming region, EP reacted with the anti-Shh antibody at PN8, whereas no immunological reaction was noted in the dental papilla region (Fig. 3G). At PN13, a reaction with the anti-Shh antibody was observed in HERS-like EP in the subpulpal lobus-forming region (Fig. 3H arrowheads), and subpulpal lobus-forming OB also reacted with the anti-Shh antibody (Fig. 3H arrow).

Discussion

A previous study reported that multirooted teeth are formed through subpulpal lobus formation in humans and rats, unlike that in the mouse molar[16]. Subpulpal lobus are formed in the dental papilla independently of the surrounding dentin. Other than subpulpal lobus, denticles and dentin bridges are dentin or dentin-like structures that form in the dental papilla or dental pulp independently of the surrounding dentin. Denticles and dentin bridges are formed without epithelial tissue, and their formation is considered to start with the deposition of calcified bodies, followed by dentinogenesis by OB[24, 25]. Histological observations revealed that the morphology of dental papilla cells changed in the early phase of subpulpal lobus formation, predentin, which is un-calcified dentin, was observed before calcified dentinogenesis, and subpulpal lobus-forming cells reacted with the anti-Hsp antibody on immunohistochemical staining. These results confirmed that subpulpal lobus are formed by OB that differentiated from dental papilla cells from the beginning of their formation, unlike denticles and dentin bridges, suggesting that all dentin present as tooth structures in tooth development is formed by OB.

Many factors have been shown to play a role in the epithelial-mesenchymal interaction in tooth morphogenesis, and Shh is regarded as an important factor. It has been reported that in tooth crown formation, Shh is expressed in inner enamel epithelium, AM, which differentiated from this, and OB, which differentiated from dental papilla cells, although it is not expressed in dental papilla cells[6]. In the present study, EP and OB reacted with the anti-Shh antibody, as was also observed in tooth crown formation, suggesting that the epithelial-mesenchymal interaction is involved in subpulpal lobus formation, similar to that in tooth crown formation.

Although the morphology and size of subpulpal lobus varied among the teeth examined, the pattern of furcation area formation was similar: The furcation area formed by fusion between adjacent subpulpal lobus and between the subpulpal lobus and DP that was continuous with dentin in the cervical region. However, the site of the EP at which a subpulpal lobus was formed varied among the teeth and currently remains unclear, warranting further studies.

The EP formed by the extension of a part of HERS in the cervical region plays an important role in multirooted tooth formation[17,18]. Orban and Mueller[16] reported that EP extending from the cervical region made contact and fused with the opposing EP in mice, whereas Ooë[18] demonstrated that the EP did not make contact with the opposing EP in multirooted tooth formation by human molars, and lost continuity with HERS in the cervical region before dentinogenesis or was torn in the longitudinal direction, showing various shapes. In the present study, the EP of the rat molar was observed in serial sections using H-E staining. The EP of the rat molar did not make contact with the opposing EP, similar to that in humans; however, the tear observed in the human molar before dentinogenesis was not noted. Therefore, the morphology of the EP differs among species in animals in which multirooted teeth are formed through subpulpal lobus formation. However, the reason for these differences currently remains unclear. In addition, the EP in the rat molar disintegrated from the dentinogenesis-completed region, similar to HERS in tooth root formation, residual cell aggregates remained in the periodontal ligament, and the tip extended to the opposing cervical region. Although Malassez epithelial rests formed by the disintegration of HERS surrounding the tooth root like a net[26], it is unclear whether cell aggregates remaining after disintegration of the EP surround the furcation area, similar to Malassez epithelial rests in rats; therefore, further studies are needed in order to clarify this.

Accessory root canals are present in the furcation area in completed teeth. These canals have been investigated in human permanent[21,27-32] and deciduous teeth[27 33-36] and also in various animal species[37-40]. Regarding the accessory root canal formation mechanism, these canals are considered to form when subpulpal lobus do not fuse with dentin in the tooth crown, i.e., the DP[38-40], or in the disintegrated region of the EP[41]. We were unable to elucidate whether accessory root canals were formed due to disintegration of the EP because the EP did not disintegrate in the observed rat molar. However, HERS in the cervical region and the subpulpal lobus-forming EP never made contact, even though they were in close proximity to each other, suggesting that the accessory root canal was formed when dentin, formed by OB that differentiated from dental papilla cells facing each epithelium, did not fuse, and we strongly support the hypothesis related to accessory root canal formation described above. In addition, the presence of a suture line, termed the seam, in the furcation area has been reported in rats[43]. These findings suggest that the difference in the vertical level observed between the epithelia described above resulted in a difference in the level in dentin and became a structure termed the seam.

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Conflict of Interest

The authors have declared that no COI exists.

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