Three-Dimensional Observation of the Furcation Area during Multi-Rooted Tooth Formation in Rat

Nobue Kikuchi\textsuperscript{1,2}, Kei Kitamura\textsuperscript{1}, Norio Kasahara\textsuperscript{1}, Yudai Ogawa\textsuperscript{1}, Noboru Ishikawa\textsuperscript{2}, Masahito Yamamoto\textsuperscript{3} and Hitoshi Yamamoto\textsuperscript{1}

\textsuperscript{1} Department of Histology and Developmental Biology, Tokyo Dental College, Tokyo, Japan
\textsuperscript{2} Department of Forensic Odontology and Anthropology, Tokyo Dental College, Tokyo, Japan
\textsuperscript{3} Department of Anatomy, Tokyo Dental College, Tokyo, Japan

(Accepted for publication, June 3, 2022)

Abstract: Epithelial projections (EP), which form as Hertwig’s epithelial root sheath (HERS) extends, play an important role in the formation of multi-rooted teeth. However, three-dimensional (3D) observations of the process in which EP form the furcation area have not yet been conducted. To investigate the formation of the furcation area by EP, we used a 3D reconstruction model of the furcation area in rats, which form subpulpal lobes in the same manner as humans, and examined the extent of cell division around the epithelium. Wistar rats between postnatal days 3-18 (PN3-18) (n=3/age) were fixed by the perfusion of 4% paraformaldehyde solution, and the maxillae were dissected out and decalcified. Tissues were embedded in paraffin by the conventional protocol, and 5-µm-thick serial sections and some sections for immunohistochemistry using an anti-proliferating cell nuclear antigen antibody of the frontal plane were prepared. All serial sections were stained by hematoxylin and cosin, and the structures of HERS, EP, dentin projections, and subpulpal lobes were overlayed after delineation every 5 sections from the images obtained of the maxillary second molar for the 3D reconstruction using ITK-snap software. Reconstructed images showed that a portion of HERS in the cervical region had extended into the dental papilla on PN3 to form EP. Four EP formed and extended with age at different speeds. On PN11, EP fused with one another in the center of the dental papilla and started to form subpulpal lobes. During this process, apical elongation in the apical direction of EP was not observed. Significant cell proliferation was detected in the dental papilla around the epithelium of the cervical loop. Collectively, these results indicate that the subpulpal lobes form immediately after the fusion of EP, and HERS extend in the apical direction following the fusion of EP.

Key words: Three-dimension, Hertwig’s epithelial root sheath, Subpulpal lobus, Multirooted tooth, Rat

Introduction

Tooth morphogenesis occurs through epithelial-mesenchymal interactions\textsuperscript{1,2}. During the morphogenesis of the tooth crown, the primary and secondary enamel knots appear within the enamel organ and function as signaling centers to promote cell differentiation, resulting in epithelial-mesenchymal interactions within the dental papilla and enamel organ\textsuperscript{3-4}. Hertwig’s epithelial root sheath (HERS), which forms as a result of fusion between the inner and outer enamel epithelium in the enamel organ, plays an important role in tooth root morphogenesis\textsuperscript{5}. In tooth root morphogenesis, 1) dental papilla cells facing the inner enamel epithelium of HERS differentiate into odontoblasts and secrete the dentin matrix of the root, 2) calcification of the secreted dentin matrix leads to the fragmentation of HERS on the surface and the remaining cells are released from the calcified surface of dentin, 3) cells in the dental follicle that surround the dental papilla and enamel organ pass through the space created as a result of HERS fragmentation and migrate to the surface of calcified dentin. These cells then differentiate into cementoblasts to form cementum on the surface of calcified dentin. These processes occur in a continuum from the cervical region to the root apex and result in root formation. During these processes, a number of signaling molecules, such as bone morphogenetic proteins (BMPs)\textsuperscript{6-8}, sonic hedgehog (Shh)\textsuperscript{9-10}, msh homeobox 2 \textsuperscript{11}, and Smad-4\textsuperscript{12}, are expressed in HERS and dental papilla. However, the presence of signaling centers, which have a similar function as enamel knots during tooth crown formation, have not yet been identified in the process of root formation\textsuperscript{13,14}.

Sohn et al. observed dental papilla cells facing HERS using murine molars\textsuperscript{15}. They demonstrated that cells in the dental papilla facing HERS did not proliferate in the area of furcation formation where HERS extended toward the dental papilla, while cells actively proliferated where HERS extended in the perpendicular direction. These findings suggest that the proliferation of dental papilla cells facing HERS is involved in the formation of epithelial projections (EP).

Molars are multi-rooted in most mammals, including humans. The murine model of multi-rooted tooth formation reported by Orban and Mueller is widely accepted\textsuperscript{16,17}. In this model, 1) following crown formation, a portion of HERS proliferates towards the dental papilla to form EP, 2) dental papilla cells facing EP differentiate continuously from the cervical region to the dental papilla into odontoblasts, 3) the formation of dentin projections (DP), interradicular projections that consist of dentin formed by differentiated odontoblasts, occurs from the cervical region toward the dental papilla, and 4) DP fuse in the center of the dental papilla to form the furcation area, resulting in multiple roots. However,
a study by Ooë examined the process of root formation in human deciduous teeth and demonstrated that, in contrast to mice, a mass of dentin termed the subpulpal lobes appeared in the center of the dental papilla. They further demonstrated that the subpulpal lobes fused with one another as well as with DP to form the furcation area, resulting in the formation of multi-rooted teeth. Multi-rooted tooth formation involving the subpulpal lobes has also been reported in rats and pigs.

In this complex process of root formation involving the subpulpal lobes, the mechanisms by which HERS, HERS-derived EP, and DP in the three-dimensional space contribute to the formation of the furcation area remain unclear.

To clarify the dynamics of HERS, EP, and DP in the process of multi-rooted tooth formation, we used rat molars to create a 3D reconstruction model of the furcation area and examined histological changes over time. Rats were used as the model system since the involvement of the subpulpal lobes in multi-rooted tooth formation is similar to that in humans. We also performed immunohistochemistry using an anti-proliferating cell nuclear antigen (PCNA) antibody and compared the images obtained to 3D reconstructed images in order to assess the degree of mesenchymal cell division in HERS and around EP.

Materials and Methods
The present study was approved by the Animal Research Committee of Tokyo Dental College and was performed in accordance with the guidelines for experiments involving animals (No. 200202, 210202).

Animals
Forty-two Wistar rats (Sankyo Labo Service Corporation INC., Tokyo, Japan) aged 3-18 days (PN3-18, 5-28 g) were used in the present study; 3 animals at each age were used for 3D reconstruction and immunohistochemistry, respectively. The maxillary second molar tooth germ (M2) was used for observations due to its large furcation area.

Animals were anesthetized with sevoflurane (3 vol%) and perfused with phosphate-buffered saline (PBS) until blood had cleared. They were then fixed by the transcardial perfusion of 0.1 M phosphate-buffered 4% paraformaldehyde (pH 7.4). The maxilla was dissected out, fixed in the same solution at 4°C for 2 days, and then decalcified using K-CX solution (Falma Co., Ltd., Tokyo, Japan) at room temperature for 1-2 days (n=3/age). Tissues were washed with PBS, dehydrated with ethanol, and embedded in paraffin by the conventional protocol.

Histology
Using the rotary microtome HYRAX M25 (Zeiss, Oberkochen, Germany), 5-µm-thick serial sections of the frontal plane were prepared and stained with hematoxylin and eosin (H-E). Tissue sections were observed using an optical microscope (cellSens, Olympus, Tokyo, Japan).

3D reconstruction
Images of the serial sections obtained using the optical microscope (4× objective) were imported into a computer and co-registered using image processing software (ImageJ, National Institute of Health). Re-
Figure 2. 3D reconstruction. (A) A CT image of a 9-week-old rat maxilla with fully mature roots. Second molars are highlighted in yellow squares. The X-axis (B-H) corresponds to a 3D image of the region of interest highlighted in yellow observed from the side of the crown. The Z-axis (I-O) corresponds to 3D images of the roots (PN3-18) highlighted in yellow observed from the distal direction. (B) Indications of the directions (mesial, distal, buccal, and palatal). In I, the distal side is shown towards the front and the mesial side towards the back. B-H and I-O were each obtained from the same directions. E’ is a magnified image of the yellow boxes in E. On PN3, a portion of HERS extended into the dental papilla to form EP (B). On PN5, EP extended from the 4 directions (C). EP further extended into the dental papilla on PN8 (D). In a side view, we observed that EP faced the crown rather than the cervical region on PN8 (K). On PN11, EP fused in the center of the dental papilla and the subpulpal lobes formed (E’, arrowhead). DP (F, arrowhead) and enlarged subpulpal lobes (F, arrow) were observed on PN13. On PN15, the root indicated with the arrow appeared to be the largest and subpulpal lobes fused with DP (G). At the same time, HERS started extending towards the apex (N, arrow). The epithelium in all areas other than the furcation area further extended towards the apex (O). All scale bars: 200 µm. Red: HERS and EP, Blue: dentin and subpulpal lobes.
Figure 3. Immunohistochemistry. A-G) The PCNA-stained maxillary second molar tooth germ on PN3-18. H-N) Magnified images of the regions of interest highlighted in the black square in A-G. O-U) Magnified images of H-N. Squares in the upper right are higher magnified images of boxes in the cervical region (O-U). A small number of PCNA-positive cells were homogeneously distributed in the dental papilla of the cervical region on PN3 and 5 (O, P, squares). On PN8, 11, and 13, significant cell proliferation was observed in the cervical region (Q-S, squares). By PN15 and 18, the number of PCNA-positive dividing cells decreased in the cervical region (T, U, squares). Scale bars: 200 µm (A-G), 100 µm (H-N), and 50 µm (O-U).
regions of interest at which the furcation area was observed were selected from the processed images and converted to a gray scale. Images were saved in the NIfTI format. Regarding image reconstruction, ITK-SNAP software (www.itksnap.org) was downloaded and used to manually trace structures that corresponded to HERS and EP (red) and dentin and subpulpal lobes (blue) every 5 images. Traced images were interpolated at a 25-µm interval and overlaid automatically to create a 3D reconstruction. Three-dimensional images of the tooth root were collected from the frontal and transverse planes.

**Immunohistochemistry**

Five-micrometer-thick paraffin-embedded sections of the frontal plane (n=3/age) were prepared and stained by the avidin-biotin complex (ABC) method using the VECTASTAIN Elite ABC Kit (Vector Laboratories Inc, California, USA). Tissue sections were de-paraffinized using xylene and immersed in 0.3% H2O2-containing methanol at room temperature for 30 minutes to remove endogenous hydrogen peroxide. Sections were then washed with PBS, immersed in 0.01% citrate buffer, and heated using a microwave for antigen retrieval at 60°C for 15 minutes. Following a cooling period, sections were washed with PBS and blocked with 2.5% normal goat serum. A rabbit anti-PCNA antibody (1/150, Abcam, Cambridge, UK) was employed to examine the status of cell division, while a normal goat anti-rabbit IgG antibody was used as the biotinylated secondary antibody. A positive reaction was stained brown using 3,3'-diaminobenzidine tetrahydrochloride, and hematoxylin was used as a counterstain. Normal goat serum was used as the negative control instead of the primary antibody.

**Results**

**H-E staining**

Enamel and dentin started forming in the crown on PN3. HERS began extending from the cervical loop into the dental papilla and formed a long and thin EP structure (Fig. 1O, arrow). On PN5, HERS started forming from both of the cervical regions and EP was observed at the base of the dental papilla (Fig. 1P, arrow). EP extended from the cervical region into the crown on PN8 (Fig. 1Q, arrow). Similar to PN8, an epithelium was observed in the dental papilla towards the crown rather than the cervical region on PN11 and 13. However, the apical elongation of a portion of HERS was noted on PN15 (Fig. 1T, arrow). On PN18, the outermost epithelium and the epithelium around the furcation area further extended towards the apex, while the epithelium in the center of the dental papilla did not (Fig. 1N, arrow head).

**3D reconstruction**

Fig. 2A shows a computed tomography (CT) image of a 9-week-old rat with a fully matured root. The CT image was used as a guide for 3D reconstruction. The X-axis corresponds to the 3D image of the root, which is the region of interest highlighted in yellow (A) observed from the crown side. The Z-axis corresponds to 3D images of the region of interest highlighted in yellow (A) observed from the distal side.

On PN3, a portion of HERS extended into the dental papilla to form EP (Fig. 2B). In the lateral view, EP extended horizontally towards the dental papilla (Fig. 2I). The circular formation of HERS from the cervical region was observed on PN5, and 4 EP extended into the dental papilla. Each EP varied in length, but formed at almost even intervals (Fig. 2C). Similar to PN3, EP formed towards the center of the dental papilla (Fig. 2J). On PN8, EP extended further into the dental papilla, but had not yet fused (Fig. 2D). EP faced towards the crown rather than the cervical region (Fig. 2K). EP had grown by PN11 and fused with one another in the center of the dental papilla. Several subpulpal lobes were observed facing the area at which the epithelium fused (Fig. 2E’, arrow head). It was unclear which epithelium fused first. HERS did not extend towards the apex until EP extended to the dental papilla and fused with one another (Fig. 2L). The width of all EP increased with age (Fig. 2F) and DP started to form (Fig. 2F, arrow head) on PN13. Several subpulpal lobes were observed in the center of the dental papilla, and some had enlarged (Fig. 2F, arrow). Similar to PN11, EP invaded from the cervical region into the crown (Fig. 2M). On PN15, each EP fused to form the furcation area and the mesiopalatal root appeared to be the largest (Fig. 2G, arrow). The fusion of DP and the subpulpal lobes was also observed (Fig. 2G). As the furcation area formed, HERS on the outer side of the distal buccal root started extending towards the apex (Fig. 2N, arrow). The fusion of DP and subpulpal lobes progressed further to form the floor of the pulp chamber on PN18 (Fig. 2H). HERS in areas around the furcation area further extended towards the apex (Fig. 2O).
**Immunohistochemistry**

Fig. 3A-U show the PCNA-stained maxillary second molar tooth germ collected on PN3-18.

On PN3, PCNA-positive cells homogeneously distributed in the dental papilla (Fig. 3O). The extent of cell division in the dental papilla was similar on PN5 (Fig. 3P). However, PCNA-positive cells aggregated in the cervical region on PN8 (Fig. 3Q). The number of PCNA-positive cells in the cervical region was higher on PN11 and 13 than on PN3 and 5 (Fig. 3R, S). Although cell division was also observed in the cervical region, the number of PCNA-positive cells decreased on PN15 and 18 (Fig. 3T, U).

**Discussion**

In the present study, we used 2D H-E staining and 3D reconstruction methods to assess the dynamics of EP, DP, and subpulpal lobes in the process of multi-rooted tooth formation involving the subpulpal lobes.

**Fusion of EP and the timing of subpulpal lobe formation**

In addition to humans, multi-rooted tooth formation that involves the subpulpal lobes occurs in rats[16] and pigs[17]. However, there is limited evidence for the relationship between the timing of subpulpal lobe formation and EP fusion. Osawa et al. recently examined the formation of the furcation area in rat maxillary second molars using an optical microscopy technique and demonstrated that subpulpal lobe formation occurred on PN11[15]. Consistent with these findings, we demonstrated that the fusion of the epithelium occurred in this time frame. We began observing subpulpal lobes on PN11, suggesting that subpulpal lobe formation occurs immediately after the fusion of EP in rat maxillary second molars.

**Process of subpulpal lobe formation**

A previous study demonstrated that subpulpal lobe formation in the dental papilla started from the buccal side, followed by the palatal, distal, and mesial sides[19]. However, we were unable to find a distinct order for subpulpal lobe formation. Another study reported that subpulpal lobes were formed by odontoblasts, which also form dentin in the crown and root[19]. Therefore, in the areas involved in subpulpal lobe formation, epithelial-mesenchymal interactions appeared to have occurred between the inner enamel epithelium and dental papilla cells that constitute EP. Additional studies are needed to identify the factors involved in the selection of sites for these interactions.

**Direction of EP extension and cell division in the furcation area**

In single-rooted teeth, HERS only extend toward the apex. In contrast, a portion of HERS extends towards the dental papilla to form EP during multi-rooted tooth formation. We demonstrated that HERS did not extend toward the apex when EP extended toward the pulp. It extended toward the apex only after EP fused with one another and stopped extending toward the dental papilla. This suggests that HERS only extended in a single direction during root formation.

Immunohistochemical staining for PCNA also revealed that the extent of cell division in the dental papilla changed as the epithelium matured. Specifically, while cells actively divided in the cervical region when EP extended into the center of the dental papilla, the number of dividing cells decreased when HERS started extending towards the apex. This suggests a relationship between the fusion of EP and the extension of cell division in the dental papilla.

**Speed of EP formation**

In the rat maxillary second molar, EP that emerged during multi-rooted tooth formation started extending from the mesiodistal and buccolingual centers. It currently remains unclear how, among all the HERS that surround the cervical region, only the epithelium in this particular region extended into the dental papilla.

We noted variations in the lengths of the 4 EP that formed during multi-rooted tooth formation of the maxillary second molar. In addition, EP extending mesiodistally before the fusion of EP formed closer to the center of the dental papilla than EP extending buccolingually. This result indicates that the speed of EP extension or the timing of EP formation differed. Since EP started forming by PN3, data need to be collected from an earlier time point to elucidate this mechanism.

**Assessing the size of the root during multi-rooted tooth formation**

The mesiopalatal root is the thickest of all 4 roots of the rat maxillary second molar. Alikhani et al. examined cross-sections of rat maxillary molar roots using micro-CT images[20]. The findings obtained showed that the diameter of the mesial root was greater than that of the distal root, and also that the palatal root was thicker than the buccal root in both the distal and mesial roots. In the present study, we created a 3D reconstruction model and showed that by PN11 when all EP fused, the area that corresponded to the mesiopalatal root was the thickest of all regions corresponding to the roots. This result indicates that the root size is already confirmed by the time EP fuse during the formation of the furcation area, and that the proportion of root thickness does not change just after EP fusion.

In conclusion, we herein demonstrated that the extension of EP temporarily terminated while they fused with one another around the center of the tooth germ, and HERS re-started extending toward the apex only once EP had fully fused. We also showed that the subpulpal lobes formed immediately after the fusion of EP.

**Acknowledgments**

The authors thank the members of the Department of Histology and Developmental Biology, the Department of Forensic Odontology and Anthropology, and the Department of Anatomy, Tokyo Dental College. This work was supported by JSPS KAKENHI Grant Numbers JP17K11629 to HY.

**Conflict of Interest**

The authors have declared that no COI exists.

**References**

1. Jernvall J and Thesleff I. Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev 92: 19-29, 2000
2. Peter H and Balling R. Teeth: Where and how to make them. Trends Genet 15: 59-65, 1999
3. Jernvall J, Aberg T, Kettunen P, Keranen S and Thesleff I. The Life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot. Development 125: 161-169, 1998
4. Coboume MT, Miletich I and Sharpe PT. Restriction of sonic hedgehog signaling during early tooth development. Development 131: 2875-2885, 2004
5. Nadiri A, Kuchler-Bopp S, Haikel Y and Lesot H. Immunolocalization of BMP-2/-4, FGF-4, and WNT10b in the developing mouse first lower molar. J Histochem Cytochem 52: 103-112, 2004
6. Nakatomi M, Morita I, Eto K and Ota MS. Sonic hedgehog signal-
ing is important in tooth root development. J Dent Res 85: 427-431, 2006

7. Iseki S, Araga A, Ohuchi H, Nohno T, Yoshioka H, Hayashi F and Noji S. Sonic hedgehog is expressed in epithelial cells during development of whisker, hair, and tooth. Biochem Biophys Res Commun 218: 688-693, 1996

8. Hosoya A, Shalehin N, Takebe H, Shimo T and Irie K. Sonic hedgehog signaling and tooth development. Int J Mol Sci 21: 1587, 2020

9. Yamashiro T, Tummers M and Thesleff I. Expression of bone morphogenetic proteins and Msx genes during root formation. J Dent Res 82: 172-176, 2003

10. Xu X, Han J, Ito Y, Bringas P Jr, Deng C and Chai Y. Ectodermal Smad4 and p38 MAPK are functionally redundant in mediating TGF-β/BMP signaling during tooth and palate development. Dev Cell 15: 322-329, 2008

11. Huang X, Xu X, Bringas P Jr, Yee PH and Yang C. Smad4-Shh-Nfic signaling cascade-mediated epithelial-mesenchymal interaction is crucial in regulating tooth root development. J Bone Miner Res 25: 1167-1178, 2010

12. Yamamoto H, Cho SW, Kim EJ, Kim JY, Fujiwara N and Jung HS. Developmental properties of the Hertwig’s epithelial root sheath in mice. J Dent Res 83: 688-692, 2004

13. Sohn WJ, Choi MA, Yamamoto H, Lee S, Lee Y, Jung JK, Jin MU, An CH, Jung HS, Suh JY, Shin HI and Kim JY. Contribution of mesenchymal proliferation in tooth root morphogenesis. J Dent Res 93: 78-83, 2014

14. Orban B and Muller E. The development of the bifurcation of multirooted teeth. J Am Dent Assoc 16: 297-319, 1929

15. Ooë T. A propos de la formation de la bifurcation ou tripartition des racines dans les molaires humaines. Acta Anat 82: 512-524, 1972

16. Kodera H and Hashimoto I. On the subpulpal dentine islands and formation of the interradicular dentine in rat molar tooth germs. Tsurumi Univ Dent J 16: 455-462, 1990 (in Japanese)

17. Sawamura H. The mineralization patterns in the dentine of the floor of three pulp chamber of porcine molars. Tsurumi Univ Dent J 15: 487-513, 1989 (in Japanese)

18. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Jean-Yves T, White DJ, Hartenstein V, Eliceiri K, Tomancak P and Cardona A. Fiji: An open-source platform for biological-image analysis. Nature Methods 9: 676-682, 2012

19. Osawa E, Shintani S and Yamamoto H. Histological and immunohistochemical observation of the furcation area formation with the subpulpal lobus of rat molar. J Hard Tissue Biol 26: 149-156, 2017

20. Alikhani M, Alikhani M, Alansari S, Almansour A, Hamidaddin MA, Khoo E, Lopez JA, Nervina JM, Nho JY, Oliveira SM, Sangeswon C and Teixeira CC. Therapeutic effect of localized vibration on alveolar bone of osteoporotic rats. PLoS ONE 14(1): e021004, 2019
