



# Periodontal Ligament Cells Are Involved in the Formation of Intracanal Cementum-Like Tissue After Regenerative Endodontic Procedures: A Mouse *in situ* Model

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Regenerative endodontic cell-homing procedures are frequently performed on injured immature teeth diagnosed with pulp necrosis and/or apical periodontitis. The representative histological finding after those procedures is cementum-like tissues filling in the root canal but details of the healing process remain unknown. We investigated that healing process histologically using a mouse *in situ* model. Regenerative endodontic procedures were experimentally performed on noninfected maxillary first molars of 6-week-old male C57BL/6 mice, after which the healing process was investigated using histology and immunohistochemistry. Immediately after the regenerative endodontic cell-homing procedures, blood clots were seen in the root canals that disappeared over time. On day 7, the blood clot in the root canal was replaced by granulation tissue. From day 14 onward, cementum-like tissues were filled in the root canals, while the amount of fibrous tissue was reduced. Immunohistochemically, positive reactions for periostin were seen in the fibrous tissue in the root canal, the apex, and periodontal ligament cells. On the other hand, positive reactions for nestin were not detected in the root canal. CD31-positive cells with a luminal structure were also observed in the fibrous tissue around the apex and around the newly formed cementum-like tissues in the root canal. Thus, in this study, we have established an *in situ* mouse model of regenerative endodontic procedures. The results of this study suggest that periodontal ligament cells and vascular endothelial cells grow into the root canals from the apex, replace the blood clots, and participate in the formation of cementum-like tissues with angiogenesis during the healing process of regenerative endodontic procedures.

**Keywords:** regenerative endodontic procedures, pulp revascularization, cementum, cell-homing, mouse *in situ* model, periodontal ligament, periostin, angiogenesis

## INTRODUCTION

Endodontic treatment of immature permanent teeth due to dental caries or trauma is frequently encountered. Conventionally, apexification has been performed as a therapy for immature permanent teeth that has resulted in pulp necrosis or apical periodontitis (1, 2). That therapy promotes hard tissue formation at the dilated apical region of the root and closes the region (3), but

root development with the thickness of the root canal wall and apical formation does not occur. As a result, teeth show a poor prognosis such as root fracture (4–7) and new therapies that overcome that disadvantage were needed.

In recent years, regenerative endodontics (pulp revascularization) has been in the limelight as a new regenerative endodontic therapy that overcomes that shortcoming. Since the first description by Iwaya et al. (8), many successful cases of pulp revascularization for immature permanent teeth with infected root canals have been reported (8–13). Regenerative endodontics has been defined as “biologically-based procedures designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as cells of the pulp-dentin complex” (14, 15). The American Association of Endodontists has advocated the clinical use of this therapy, stating “After cleaning and disinfecting the root canal, the apical tissue is intentionally damaged mechanically and bleeding causes a blood clot in the root canal.” As a result, the thickness of the root canal wall and narrowing of the root canal space, and continuous root elongation and apical closure are observed radiographically (16, 17). Previous case reports showed histological findings of human immature permanent teeth after regenerative endodontics (18–21). The representative findings were fibrous connective tissue with bone-like or cementum-like tissue within the root canal, and no pulp-like tissue including odontoblasts or dentin-like hard tissue was observed. To investigate the wound healing process histologically after regenerative endodontics, animal models have been used. Earlier experimental models using large animals (22–28) are clinically similar to humans and can provide a large number of teeth for the experiment; however, small animals are easy to handle and more economical. However, one problem with experimental models using large animals is that it is difficult to perform detailed histological investigations during the healing process over time. Furthermore, the cells involved in the healing process, such as mesenchymal stem cells, vascular cells, and periodontal ligament (PDL) cells, were not well characterized. Moreover, the process of cell migration into the root canal and new hard tissue formation that was added to the root canal wall and the apical region has not been fully elucidated.

To overcome these problems, establishing a small animal model that can easily elucidate histological changes over time is necessary, and we, therefore, developed a mouse experimental model of regenerative endodontics. The purpose of this study was to elucidate the involvement of migrating cells into the root canal immunohistochemically and the formation of hard tissue added to the root canal wall and the apical region.

## MATERIALS AND METHODS

### Animals

All animal experiments were performed according to the Guidelines for the Treatment of Animals at the Tokyo Dental College (Approval No. 212301). Eighty-three 6-week-old C57BL/6 male mice, which still had thin root dentin with wide root apex, were used in these experiments. Seven mice were used at each time point for histological and immunohistochemical

analyses. In total, thirty-four mice that had their temporary seals removed during the postoperative course and had root canal infection or apical lesions were excluded.

The experimental group was a group in which blood clots were formed on the mesial root of the right maxillary first molar (M1) and then covered and filled. The untreated left maxillary first molars on the opposite side were used as a control.

### Experimental Procedure of Regenerative Endodontics

The procedure was performed according to the method of Xu et al. (29). General anesthesia was administered intraperitoneally using a mixed anesthetic of medetomidine hydrochloride (0.3 mg/kg, Nippon Zenyaku Kogyo, Koriyama, Japan), midazolam (4 mg/kg, Astellas Pharma, Tokyo, Japan), and butorphanol tartrate (5 mg/kg, Meiji Seika Pharma, Tokyo, Japan). After the operative field was disinfected with ethanol, endodontic access opening into the pulp chamber was performed on the mesial occlusal surface of M1 using a round-type carbide bur (Zipperler; VDW GmbH, München, Germany) and the coronal pulp was removed. The dental pulp of the mesial root canal was mechanically removed with a #6 K-file (MANI, Utsunomiya, Japan) under a surgical microscope (Stemi 508; Zeiss, Oberkochen, Germany), and was chemically cleaned with 10% sodium hypochlorite solution (Neo Cleaner; Neo Dental Chemical Products Co., Tokyo, Japan) and 3% ethylenediaminetetraacetic acid (EDTA) solution (Smear Clean; Nishika, Shimonoseki, Japan). The #6 K-file was then approached *via* the mesial root canal and bleeding from the outside of the apical foramen was initiated. Hemostasis was done using a sterile article point to form a blood clot. The upper part of the blood clot (just below the root canal orifice) was covered with mineral trioxide aggregate (MTA) cement (Bio MTA cement, Morita, Tokyo, Japan), and the cavity was sealed with adhesive resin cement (Super-Bond, Sun Medical, Moriyama, Japan).

### Tissue Preparation and Histological Observation

Mice were sacrificed at 1 h, 1 day, 3 days, 7 days, 14 days, 21 days, and 28 days after the operation. At each time point, perfusion fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (PFA; pH 7.4) was performed transcardially, and the dissected upper jaws were immersed for 24 h in the same fixative. After decalcification with 10% EDTA for 1 week at 4°C, the tissues were trimmed at the midline of the palate with a razor and embedded into the paraffin with the midline facing the bottom. The paraffin block was cut from the palatal side and the 4- $\mu$ m-thick serial sagittal sections were prepared. The sections were stained with Hematoxylin and eosin (H&E) and were observed and photographed using a universal photographic microscope (UPM Axiophoto2, Zeiss).

### Immunohistochemistry (IHC)

After deparaffinization, antigen activation treatment was performed using the microwave method. Briefly, the slides were microwaved at 70°C continuously for 20 min using a

microwave processor (Microwave Processor MI-77, Azumaya, Tokyo, Japan) and then were reacted at room temperature for 30 min. For the anti-nestin antibody, a 10 mM sodium citrate buffer (pH 6.0) was used, and for the anti-periostin and anti-CD31 antibodies, a Tris-EDTA buffer (pH 9.0) was used. The sections were immersed in 3% hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidase. After blocking with 3% bovine serum albumin (BSA; Sigma-Aldrich, Darmstadt, Germany) at room temperature for 1 h, they were incubated with an anti-nestin antibody (dilution 1:300; anti-nestin monoclonal antibody, clone rat-401, Merck Millipore, Darmstadt, Germany), an anti-periostin antibody (dilution 1:500; anti-periostin antibody, Abcam, Cambridge, UK), and an anti-CD31 antibody (dilution 1:500; anti-CD31 antibody, Abcam) at 4°C overnight. After washing in phosphate buffered saline (PBS) three times, the sections were incubated with horseradish peroxidase (HRP) conjugated secondary antibody (EnVision TM<sup>+</sup> Dual Link System-HRP, Agilent) at room temperature for 1 h. After washing three times in PBS, a 3,3'-diaminobenzidine-tetrahydrochloride (DAB) solution (ImmPACT<sup>®</sup> DAB, Vector Laboratories, Burlingame, CA, USA) was used to visualize reactivity. The sections were counterstained with Mayer's hematoxylin solution for 60 s and then were observed and photographed using a photographic microscope (UPM Axiophoto2, Zeiss). As a negative control, the sections were incubated with 3% BSA instead of the primary antibodies.

### Root Length and Width Measurements

We measured the root length and width of the mesial roots of the maxillary M1 in each group postoperatively. The root length was set as the distance from the mesial cement-enamel junction to the apical foramen and was measured at the longest distance. Measurement of root width was carried out according to the method of Lee et al. (30). The root width included the root dentin and cementum in the mesial and distal walls was measured at the widest site, and the average width was calculated. If the apex foramen or root canal was obliterated, the full width of the root from the mesial wall to the distal wall was measured, then half of the value was designated as the root width. As a control, the length and the width of untreated roots were measured. The time course for measuring those parameters was selected from 7 to 28 days postoperatively.

### Statistical Analysis

All quantitative data in the experimental group and the untreated control group are expressed as the mean and SD of each group. Analysis software (IBM SPSS 27.0J for Windows; SPSS Japan, Tokyo, Japan) was used for statistical processing and a  $p < 0.05$  is considered significant. Statistical analysis of each measurement item was performed as follows: At each postoperative time, 2 groups were compared between the experimental group with the control group. After confirming the normality of the data and the uniformity of the homogeneity of variance, Student's *t*-test was performed on the comparison between the two homoscedastic groups.

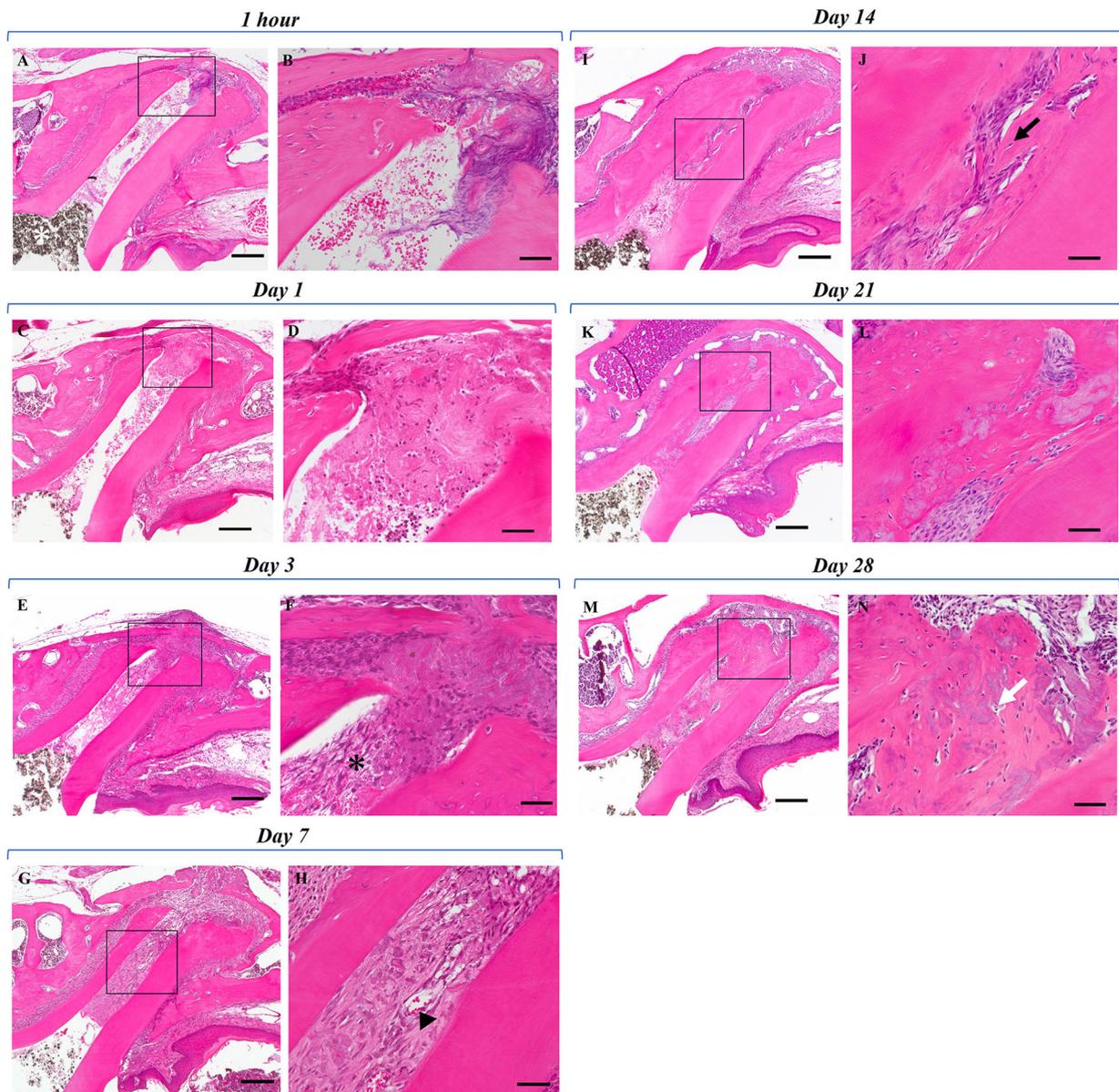
## RESULTS

### Histological Observations of the Healing Process After Regenerative Endodontics

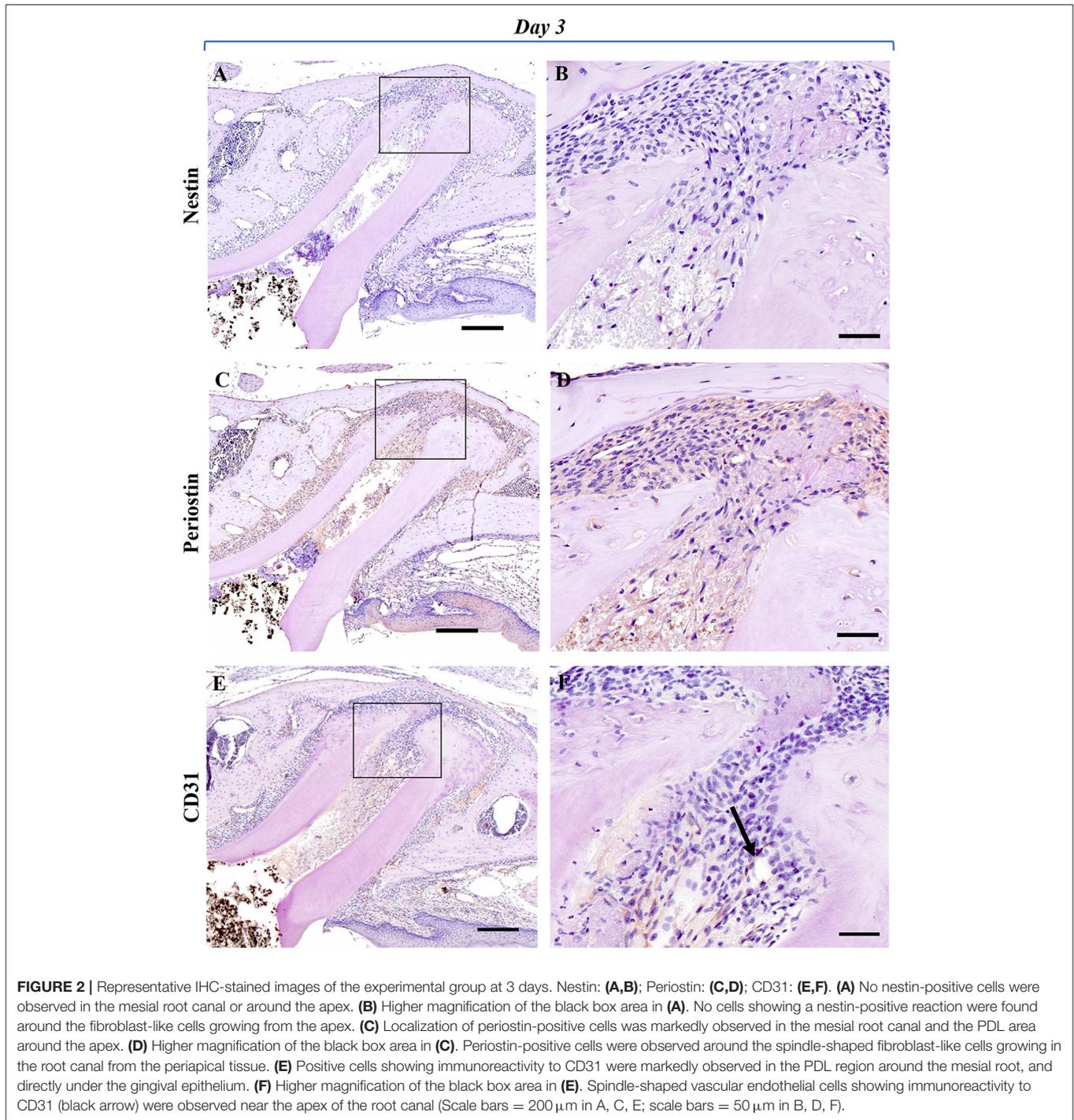
In H&E-stained sections of the experimental group 1 h and 1 day after the operation, blood clots containing blood components such as erythrocytes and fibrin were observed in the mesial root canal (Figures 1A–D). At 3 days, a decrease of blood clots in the root canal and an increase of spindle-shaped fibroblast-like cells at the apical portion of the root canal were observed (Figures 1E,F). The tissues were continuous to the apical PDL. No blood clots were observed in the root canals at 7 days (Figures 1G,H) and the root canals were filled with fibrous connective tissue containing numerous spindle-shaped fibroblasts and dilated capillaries. At 14 days, newly formed mineralized tissue was seen in the root canal that was adjacent to the existing wall at the apical portion (Figures 1I,J). The mineralized tissue contained cells in the matrix that resembled cementocytes or osteocytes. The newly formed mineralized tissue also appeared immediately beneath the MTA that had been placed at the top of the blood clot. The area of fibrous connective tissue became narrow and decreased in the root canal. On the 21st and 28th days, bone-like or cementum-like mineralized tissues were increased in the root canal and the cells were embedded into the matrix (Figures 1K–N). The root canal wall and the apical foramen became thicker over time. The mineralized tissue obliterated the root canal and apical closure was seen. We confirmed that the root canal wall and the mineralized tissue were continuous and filled with the mineralized tissue rather than the existing dentin wall. However, no pulp- or dentin-like structures characterized by the presence of polarized odontoblast-like cells adjacent to the dentin-like tissue that had dentinal tubes were seen.

### Immunohistochemical Observations

To analyze the healing process after regenerative endodontics, specimens at 3, 7, and 14 days were immunohistochemically investigated. Immunoreactivity for nestin, an odontoblast marker, was not observed (Figures 2A,B), whereas nestin-positive cells were clearly observed along with the odontoblast layer of the pulp tissue in the control group (Supplementary Figure 1). At 3 days, marked periostin-positive cells were observed in the PDL region around the M1 root canal (Figures 2C,D). The cells of the fibrous connective tissues at the apical portion of the root canal were immunoreactive for periostin. Some spindle-shaped cells were positive for CD31, a marker of endothelial cells, at the apical portion (Figures 2E,F). At 7 and 14 days, periostin expression was strongly observed in the narrow fibrous tissues in the root canal (Figures 3C,D, 4C,D), whereas nestin-immunoreaction was not seen in the root canal (Figures 3A,B, 4A,B). Periostin expression was also seen at the edge of the newly formed mineralized tissue, and cells embedded in the cementum-like mineralized tissue. CD31 expression was observed at the center area of the root canal (Figures 3E,F, 4E,F).



**FIGURE 1 |** Histological evaluation of the postoperative healing process. **(A)** One hour post the regenerative endodontics procedure. The pulp tissue of the mesial root was removed and no odontoblasts were found. The root canal was filled with blood clot-like structures containing erythrocyte components. Periodontal ligament tissue around the apex showed debris of torn tissue and partial hemorrhage. Dark brown granules are MTA cement (white asterisk). **(B)** Higher magnification of the black box area in **(A)**. **(C)** One day post the regenerative endodontics procedure. Compared with 1 h after the procedure, a decrease of blood clot-like structures was observed in the mesial root canal and around the apex. Although a decrease in erythrocytes was found around the apex, reticulated fibrin was partially observed. **(D)** Higher magnification of the black box area in **(C)**. Ingrowth of fibrous tissue from the apex is seen. **(E)** Three days after the regenerative endodontics procedure. Further reduction of blood clot-like structures was seen in the root canal and around the apex, and the root canal was filled with loose fibrous tissues. **(F)** Higher magnification of the black box area in **(E)**. Spindle-shaped fibroblast-like cells (black asterisk) were observed from the apex instead of blot clots. **(G)** Seven days post the regenerative endodontics procedure. No blood clot-like structures were observed in the root canal, and dense fibrous tissues with vessels were seen in the root canal. **(H)** Higher magnification of the black box area in **(G)**. Dilated vessels were observed in the root canal (arrowhead). **(I)** Fourteen days post the regenerative endodontics procedure. Newly formed mineralized tissue was observed in the root canal. Mineralized tissue was added adjacent to the apex and the root canal wall, and was also found directly beneath the MTA cement. The area of fibrous connective tissue in the root canal was narrowing. **(J)** Higher magnification of the black box area in **(I)**. The newly formed mineralized tissue in the root canal contained cellular components (black arrow). **(K)** Twenty-one days post the regenerative endodontics procedure. Compared with 14 days after the procedure, the mineralized tissue added to the root canal wall and the root width around the apex increased over time. **(L)** Higher magnification of the black box area in **(K)**. Extensive mineralized tissue was seen in the root canal and cellular components were present in the matrix. **(M)** Twenty-eight days post the regenerative endodontics procedure. Bone-like or cementum-like mineralized tissue was widespread in the root canal wall and apex over time. **(N)** Higher magnification of the black box area in **(M)**. The apical foramen was closed by the addition of mineralized tissue. Cells were embedded in the matrix of the mineralized tissue (white arrow) (Scale bars = 200  $\mu$ m in A, C, E, G, I, K, M; scale bars = 50  $\mu$ m in B, D, F, H, J, L, N).



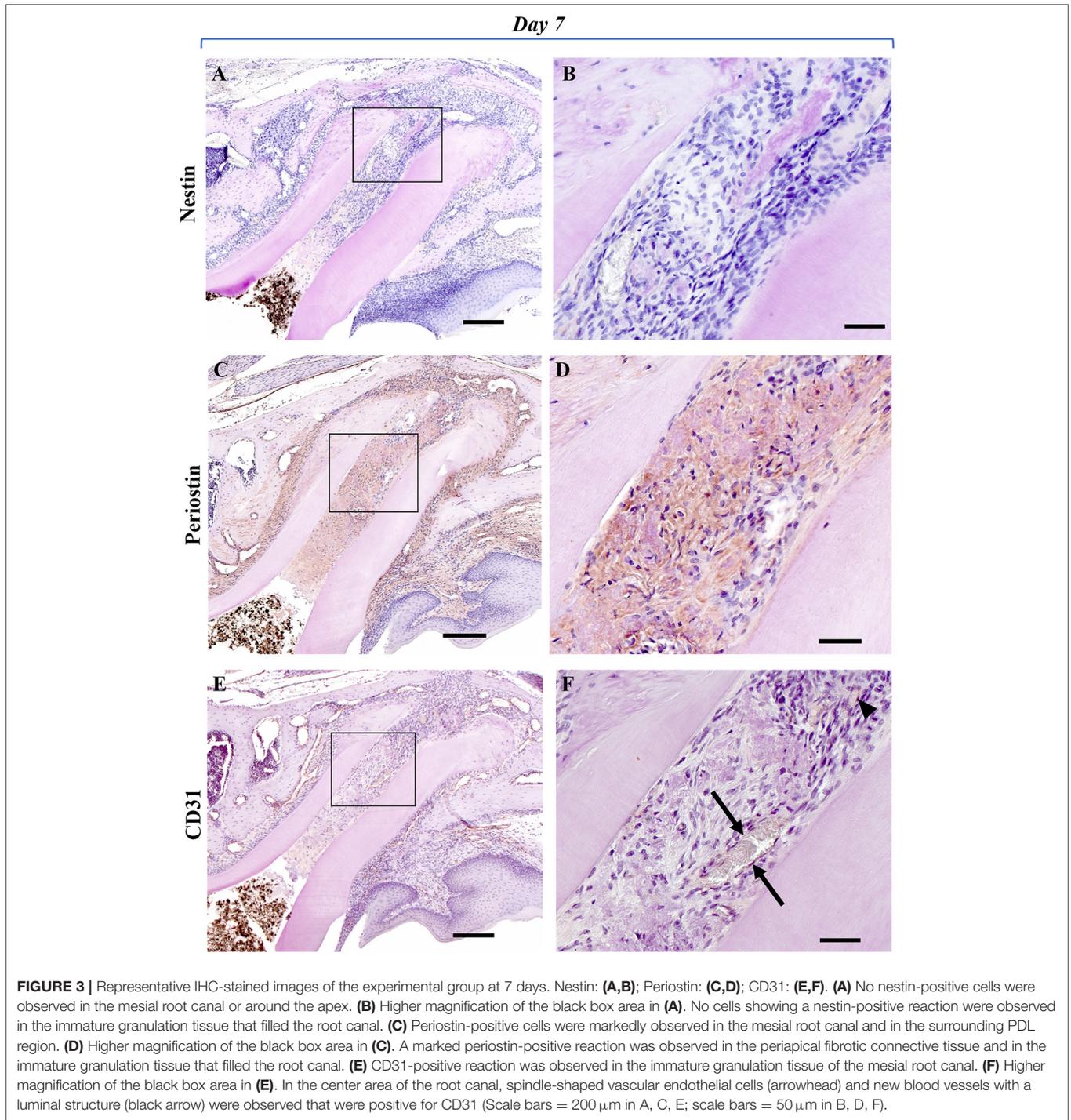
## Root Length

In both the control and the experimental groups, gradual root development was observed over time (Figure 5). The root length of the untreated control group became elongated from 890 to 950  $\mu\text{m}$  over time. On the other hand, the root length of the experimental group became elongated from 860 to 940  $\mu\text{m}$  over time. The root length in the control group was significantly

higher than in the experimental group at 7 and 14 days but there was no significant difference between the two groups at 21 and 28 days.

## Root Width

In both the control and the experimental groups, a gradual increase in root width was observed over time (Figure 6).

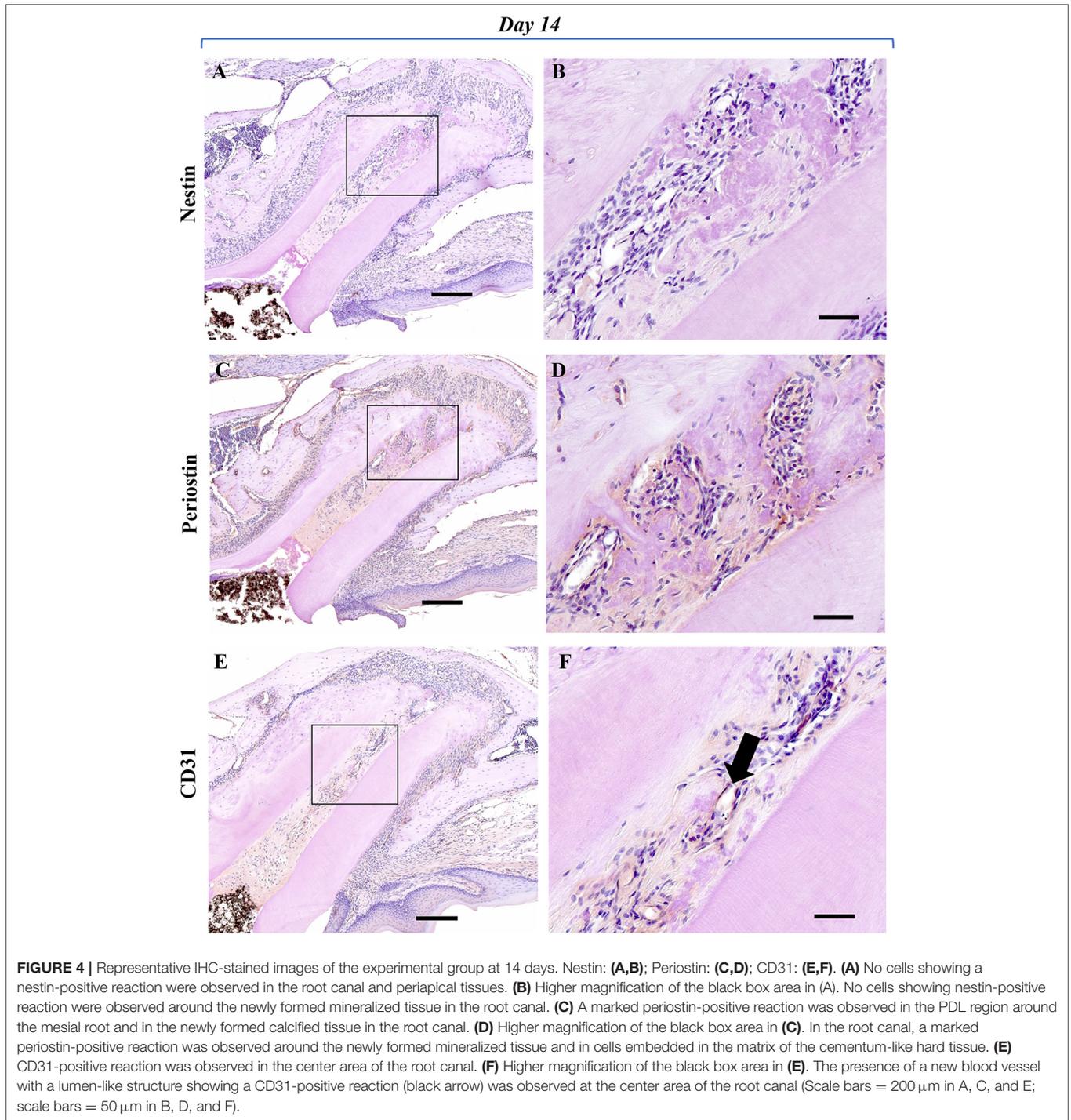


The root width in the untreated control group increased from 380 to 450  $\mu\text{m}$ , and the root width in the experimental group increased from 410 to 530  $\mu\text{m}$ . There was not a statistically significant difference in the root width between the control group and the experimental group at 7 days but the root width in the experimental group was significantly thicker than in the control group at 14, 21, and 28 days.

## DISCUSSION

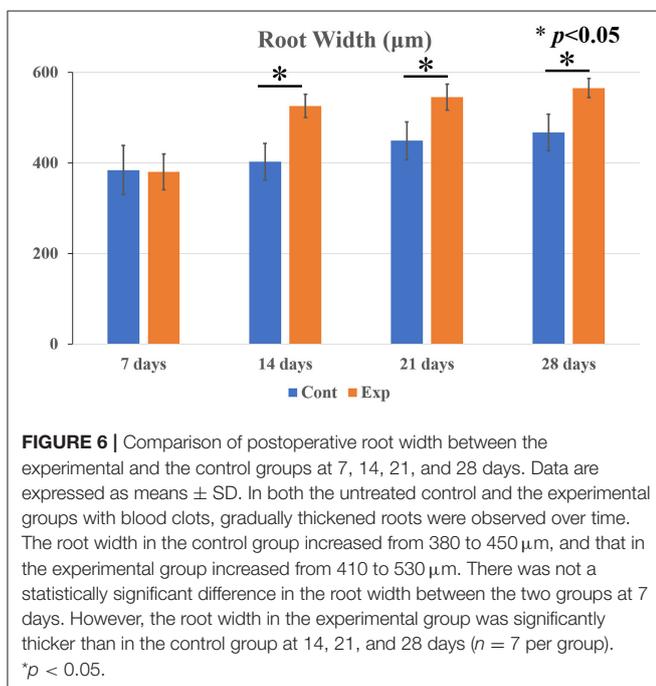
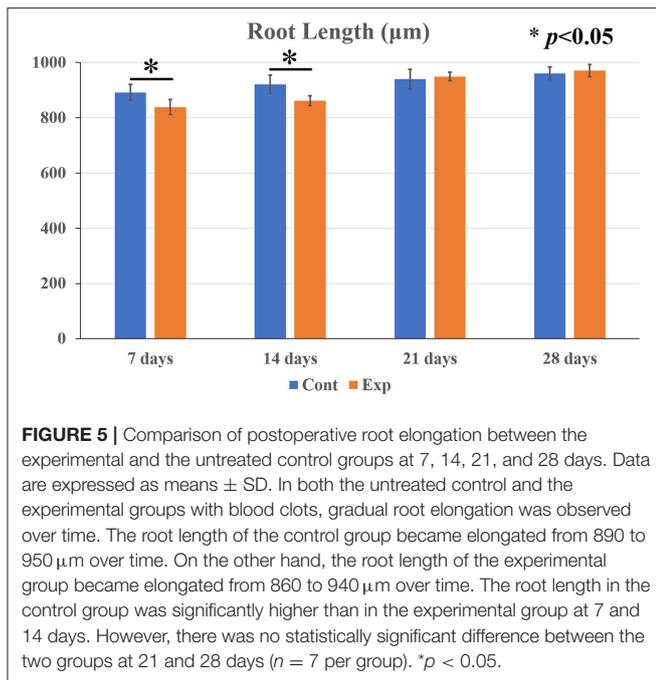
### Experimental Model of Regenerative Endodontics Using Mice

In this study, we established a new experimental animal model of regenerative endodontics using mice. Earlier reports described experimental models using larger animals such as dogs (22–26) and ferrets (27, 28) because there are technical difficulties



involved in performing regenerative endodontics with smaller animals. However, it has been difficult to investigate the histologically detailed analysis of the healing process over time using large animal models. Furthermore, immunohistochemical analysis has been challenging, because antibodies specific to targets in larger animals are limited. Recently, a rat experimental model was reported, and histological analysis was performed,

but only one time point was shown (31). Experimental models using mice have advantages to overcome the difficulties described aforementioned, and therefore, we decided to employ mice in this study. Our results showed detailed histological findings such as blood clot formation, fibrous tissue, and bone-like or cementum-like mineralized tissues over time. These histological findings are similar to those of earlier studies using larger animals such as



dog models (22–26) and in human cases (18, 20, 32). This is the first report detailing histological findings during the healing process of regenerative endodontics over time as far as we know. These results should allow the identification of the origin of cells associated with the healing process using gene-altered mice in the future.

## Origin of the Newly Mineralized Tissue

In this study, we observed newly mineralized tissue formation in the root canal. Earlier studies demonstrated various types of newly mineralized tissues such as bone-like or cementum-like mineralized tissue with various combinations (18, 20, 32). On the contrary, one case showed dentin-like tissue formation after regenerative endodontics (33, 34). One recent report demonstrated that the type of hard tissue formation depends on the remnant pulp (31). Our results suggest that we definitely removed the dental pulp tissue in the root canal during the procedure. Our immunohistochemical analysis also showed that no nestin-positive cells were detected in the root canal and around the mineralized tissue but the localization of periostin-positive cells was observed. These findings suggest that the newly formed mineralized tissue is not derived from odontoblasts but rather from PDLs. However, it remains unclear whether the newly formed mineralized tissue is bone-like or cementum-like hard tissue because the periostin is thought to be a marker of PDLs that can differentiate into both cementoblasts and osteoblasts (35). In our histological findings, the mineralized tissue contains cells in the matrix and would be osteoid but the tissue is continuous with the cellular cementum at the apex along the root canal wall. Furthermore, cementum does not contain vessels in the matrix, and our immunohistochemical finding also shows the newly formed mineralized tissue does not contain CD31 positive blood vessels in the matrix. Therefore, our results suggest that the mineralized tissue is cementum-like hard tissue.

## Vascular Growth and Angiogenesis

Angiogenesis is necessary for tissue regeneration or repair to supply oxygen and eliminate waste products. Although regenerative endodontics has been called pulp revascularization, angiogenesis during the procedure has not well been investigated histologically. We assessed the localization of CD31-positive cells to detect endothelial cells and confirmed that CD31-positive vascular cells had grown in from the apex. Furthermore, CD31-positive cells with a vascular luminal structure were observed in the root canal and around the mineralized tissue over time. The histological replacement of blot clots with fibrous tissues with vessels is well described in the earlier reports but was not characterized. The results of this study show histological evidence of that replacement process during the wound healing after the procedure.

Blood clot formation in the root canal plays an important role because blood clots serve as a scaffold and contain various growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), which signal endothelial precursor cells and perivascular progenitor cells (36) and promote angiogenesis. Furthermore, mesenchymal stem cells reside in the microenvironment (niche) that is located at perivascular sites, and the maintenance and regulation of stem cell populations are controlled at the sites of regeneration or repair of injured tissues (37–39). This study identified new vessels that had grown in from the apical tissue and were observed adjacent to newly mineralized tissue. These findings imply that the ingrowth of vessels contributes not only to angiogenesis but also to

the formation of newly mineralized tissue during the wound healing process. Further study is needed to further clarify the mechanism involved.

## Root Development

Apexification, which has conventionally been performed on immature teeth diagnosed with pulp necrosis and/or apical periodontitis, has various disadvantages such as a short root length and a thin root canal wall. A short root length makes a higher crown root ratio, and a thin root canal wall becomes fragile and prone to root fracture (4, 40). Regenerative endodontic procedures (REPs) are performed to overcome those two problems. An earlier *in vivo* study that used beagle dog showed that fracture resistance was significantly more than two times as high in the REP group compared to the control group (41). However, few studies have been reported that investigated root length and root width after the procedures (41, 42). Our results show that the root length in the control group was significantly higher than in the experimental group at 7 and 14 days after the procedure, but there was not a significant difference between those two groups at 21 or 28 days. Cementum-like mineralized tissue formation around the apex was histologically seen at the same time points. Regarding the root width, there was no significant difference between the experimental group and the control group 7 days after the procedure, whereas the root width of the experimental group was significantly higher than that of the control group since the 14th day. Narrowing or obliteration due to cementum-like mineralized tissue formation in the root canal was histologically observed at the same time points. These results suggest that the root length is complemented by the addition of newly mineralized tissue around the root apex and that the increase in root width is attributed to mineralized tissue formation in the root canal, resembling similar findings in human cases (18, 20, 32) and larger animal experiments (22–26). Increased root width ensures that the root is held firmly by the PDL and alveolar fossa. Therefore, we think that the new structure with an increase in root width results in resistance to external stress, which leads to the prevention of root fracture. However, we employed mice in this study, and the tooth size, shape, and occlusal force of the mice were different from those of humans and dogs. The point is a limitation of the fracture resistance.

## CONCLUSION

In conclusion, we have established a new animal model of regenerative endodontics using mice. We demonstrated histological and immunohistochemical findings after the procedure in detail. The results of this study suggest that blood

clots are replaced by PDL cells (periostin-positive cells) and that vessels around the apex have grown into the root canal. Furthermore, the fibrous tissue derived from PDL cells formed newly cementum-like mineralized tissue in the root canal and the apex over time. These results serve as a basis for understanding the healing process after the regenerative endodontics procedure in detail and should contribute to further investigations of the procedure in the future.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experiment Committee at Tokyo Dental College (approval number: 302101).

## AUTHOR CONTRIBUTIONS

TK, KM, MS, and TM: conceptualization. TK, TI, KM, H-SJ, MS, and TM: methodology. TK, TI, KM, and TM: investigation. TK, KM, H-SJ, and TM: data analysis. TK and KM: drafting original manuscript. TM: editing manuscript. All authors have read and agreed with submitting the article.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fdmed.2022.864406/full#supplementary-material>

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