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題 名

Pulpal response to a triple antibiotic solution following intentionally delayed tooth replantation in mice

（マウス意図的歯の遅延再植後の三種抗菌性薬剤に対する歯髄反応）

第 1 報

Expression patterns of nestin and dentin sialoprotein during dentinogenesis in mice

(マウス象牙質形成におけるネスチンと象牙質シアロタンパク質の発現パターン)

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Use of a triple antibiotic solution affects the healing process of intentionally delayed replanted teeth in mice

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第 3 報

Effects of a triple antibiotic solution on pulpal dynamics following intentionally delayed tooth replantation in mice

（マウス意図的歯の遅延再植後の歯髄ダイナミクスへの三種抗菌性薬剤の効果）

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Expression patterns of nestin and dentin sialoprotein during dentinogenesis in mice

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Running headline: Nestin and Dsp in Dental Pulp

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ABSTRACT

Differentiated odontoblasts could not be identified by one unique phenotypic marker, but the combination of expression of dentin phosphoprotein (Dpp), dentin sialoprotein (Dsp), dentin matrix protein 1 (Dmp1), and nestin may be valuable for the assessment of these cells. However, the findings using these proteins remain controversial. This study aimed to compare two odontoblast differentiation markers: nestin and Dsp in the process of dentinogenesis in mice. We performed immunohistochemistry and/or *in situ* hybridization technique for nestin and Dsp using 3-week-old incisors as well as postnatal 1-day- to 8-week-old molars. Preodontoblasts began to express nestin and Dsp proteins and *Dsp* mRNA, which increased their intensity according to the progress of odontoblast differentiation in both incisors and developing molars. Nestin was consistently expressed in the differentiated odontoblasts even after the completion of dentin matrix deposition. The expression of *Dsp* mRNA coincided with the odontoblast secretory activity for dentin matrix deposition. In contrast, other pulpal cells, predentin matrix and dentinal tubules also showed a positive reaction for Dsp protein in addition to differentiated odontoblasts. In conclusion, nestin is valuable as a differentiation marker for odontoblasts, whereas *Dsp* mRNA is a functional marker for their secretory activity.

Dentin is a hard connective tissue that forms the bulk of the tooth, and it is a bone-like matrix characterized by multiple closely packed dentinal tubules that traverse its entire thickness and contain the cytoplasmic processes of odontoblasts, which are responsible for the formation and maintenance of the dentin. The cell bodies of the odontoblasts are aligned along the inner aspect of the dentin, beneath a layer of predentin, where they also form the peripheral boundary of the dental pulp (35). The odontoblasts are terminally differentiated ectomesenchymal cells that synthesize several collagenous and non-collagenous proteins (NCPs) (45). The morphologically discernible differentiation of the odontoblast begins with the dental papilla cells adjacent to the inner enamel epithelium (7). The dental papilla cells adjoining the acellular zone rapidly enlarge and elongate to become preodontoblasts first and then odontoblasts as their cytoplasm increases in volume to contain increasing amounts of protein-synthesizing organelles. The morphology of odontoblasts reflects their functional activity and ranges from an active synthetic phase to a quiescent phase (35). However, the preferred use of terminology regarding the classification of odontoblasts is controversial, because several terms for differentiated odontoblasts have been used by different researchers, *i.e.*, secretory, transitional, and aged odontoblasts (12), young and old odontoblasts (51), or immature and mature odontoblasts (39).

Rodent incisors are continuously growing teeth, for which all stages of odontogenesis – including amelogenesis and dentinogenesis – can be surveyed if the sections of the tooth are prepared from the apical end to the incisal edge (39, 48). Recent molecular biological studies have demonstrated the existence of a niche for self-renewing adult stem cells in these rodent incisors, which is referred to as an "apical bud" (21-23, 40, 56). Thus, continuously growing incisors are useful materials for analyzing the developmental process of dentinogenesis, although the incisal edge shows the unique feature that an inflammatory reaction is induced by the

attrition of the incisal tip (39). In contrast, rodent molars are teeth with limited growth similar to human teeth. The rate of dentin deposition increases in the early stage of coronal dentin formation and subsequently almost ceases, suggesting the transition from the active to quiescent stages of secretory odontoblasts (57). Hence, both rodent incisor and molar teeth should be compared for the exact understanding of dentinogenesis.

The selection of odontoblast differentiation markers is important for the proper assessment of the differentiation process of odontoblasts. Among these markers, NCPs have been widely studied, especially the small integrin binding ligand, N-linked glycoprotein (SIBLING) family, which is composed of dentin sialophosphoprotein (Dspp), dentin matrix protein 1 (Dmp1), osteopontin (Opn), bone sialoprotein (Bsp), and matrix extracellular phosphoglycoprotein (Mepe) (16). Dspp is proteolytically cleaved into dentin sialoprotein (Dsp) and dentin phosphoprotein (Dpp) (49, 55). However, differentiated odontoblasts could not be identified by a unique phenotypic marker; a combination of markers such as Dsp, Dpp, Dmp1, and nestin has been applied to identify these cells (18). Dspp and/or its cleaved products Dsp and Dpp are believed to be tooth-specific (11), although they are also expressed in preameloblasts (6, 8, 10, 15, 43, 44). Recent studies have shown the expression of Dspp in bone (41, 42), cementum (3), and some non-mineralized tissues (2, 37, 38), while its expression in non-dental tissues is lower than that in dentin. Dmp1 is an acidic phosphoprotein predominantly expressed in dentin, bone, and cementum (17, 30). A lower level of expression for these proteins has also been found in non-mineralized tissues such as the brain, kidney, pancreas, and salivary gland (53). Among them, the intermediate filament nestin is considered as a marker for differentiated odontoblasts in developing teeth and newly-differentiated odontoblast-like cells appearing following tooth injuries such as cavity preparation and tooth

replantation/transplantation (1, 24, 26, 28, 36, 46, 54). Regarding immunohistochemistry for Dmp1, we failed to obtain a specific immunoreaction in the differentiated odontoblasts in our preliminary experiments (data not shown). Thus, this study aimed to compare two odontoblast differentiation markers: nestin and Dsp in the process of dentinogenesis in mice, using immunohistochemistry and/or *in situ* hybridization technique for nestin and Dsp in 3-week-old incisors as well as postnatal 1-day- to 8-week-old molars.

MATERIALS AND METHODS

Tissue preparation. All experiments were reviewed by the Committee on the Guidelines for Animal Experimentation of Niigata University and performed according to the recommendations or under the conditions proposed by the committee. Materials were collected in groups of 2 to 5 ICR mice at intervals of 1 (n=3), 3 (n=2), 5 (n=2), 7 days (n=3) and 3 (n=5), 5 (n=3) and 8 weeks (n=2) after birth. At each stage, the animals were perfused with physiological saline transcardially followed by 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) under deep anesthesia by an intraperitoneal injection of chloral hydrate (the maximum dose of 350 mg/kg). The maxillae were removed *en bloc* and immersed in the same fixative for an additional 12 h at 4°C. Following decalcification in Morse's solution (10% sodium citrate and 22.5% formic acid) (47) for 1-4 days at 4°C, the specimens were dehydrated through ethanol series and embedded in paraffin, and sagittal sections of maxillae were cut at 4 μm. The paraffin sections were mounted on Matsunami adhesive silane (MAS)-coated glass (Matsunami Glass Ind., Osaka, Japan) slides and stained with H&E.

Immunohistochemical analysis. For the immuno-peroxidase procedure, the sections were processed for the EnVision method (DAKO Japan, Tokyo, Japan) using a mouse anti-nestin monoclonal antibody diluted 1:100 (Millipore, Temecula, CA, USA) and the avidin-biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories Inc., Burlingame, CA, USA) method using goat anti-Dsp polyclonal antibody diluted 1:50 or 1:500 (Santa Cruz Biotechnology, Inc., CA, USA), since the previous reports used the different dilutions of anti-Dsp antibody and we had to confirm the suitable concentration. The sections were counter-stained with hematoxylin. Immunohistochemical controls were performed by replacing the

primary antibodies with PBS. These immunostained sections contained no specific immunoreaction.

For double immunofluorescent staining for nestin and Dsp, frozen sections (40 μm in thickness) from 3-week-old incisors were treated by three consecutive incubations with a mouse anti-nestin monoclonal antibody diluted 1:50 (Millipore), biotinylated anti-mouse IgG diluted 1:100 (Vector), and FITC-conjugated streptavidin diluted 1:250 (Vector). After washing with PBS and blocking with Avidin/Biotin Blocking kit (Vector), they were then consecutively incubated with goat anti-Dsp polyclonal antibody diluted 1:25, biotinylated anti-goat IgG (Vector), and Texas red-conjugated streptavidin diluted 1:100 (Vector). The sections were examined with a confocal laser scanning microscope (FV300, Olympus, Tokyo, Japan).

In situ hybridization. Section *in situ* hybridization was conducted as previously described (34). A digoxigenin-labeled probe for *Dsp* mRNA (43) was prepared according to the manufacturer's protocol. Following the fixation, the specimens were decalcified with Morse's solution (10% sodium citrate and 22.5% formic acid) for 24 h (47), dehydrated through ethanol series and xylene, and embedded in paraffin. Five-μm-thick paraffin sections were mounted on MAS-coated glass slides, deparaffinized, dehydrated, and predigested with proteinase K. The sections were then acetylated with 0.25% acetic anhydride in triethanolamine for 10 min and incubated overnight at 70°C with hybridization buffer containing a digoxigenin-labeled probe for *Dsp* mRNA. After hybridization, the slides were washed in a series of sodium citrate–sodium chloride solution and treated by two consecutive incubations with blocking reagent (Roche Diagnostics Corp., Indianapolis, IN, USA) and anti-digoxigenin antibody (Roche). The sections were stained with 4-nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche).

RESULTS

Incisor at Week 3 (Table 1)

Nestin and Dsp were intensely expressed in odontoblasts, although other pulpal cells also showed weak positive reactions (Fig. 1a, b). The expression of *Dsp* mRNA coincided with the odontoblast secretory activity for dentin matrix deposition (Fig. 1c), judging from the previous data: the rate of dentin deposition in the medial coronal portion almost ceases after 60 days in rats (57) and the progress of dentinogenesis in mice is faster than that in rats (26, 27). Faint positive reactions of nestin and Dsp were observed in the dental papilla and follicle around an apical bud, respectively (Fig. 1d, e). Nestin was expressed in preodontoblasts and immature odontoblasts, and the apical end of their cytoplasm began to show intense immunoreactions according to the progress of odontoblast differentiation (Fig. 1j, m). Finally, mature odontoblasts contained intense immunoreaction in their entire cytoplasm including their cellular processes (Fig. 1p). Stellate reticulum in the enamel organ also showed faint immunoreaction for nestin (Fig. 1g). Intense Dsp immunoreactions were localized in the Golgi area of immature and mature odontoblasts as well as other pulpal cells (Fig. 1k, n, q). Dentinal tubules and predentin matrix also showed positive reactions for Dsp (Fig. 1q) in addition to stellate reticulum (Fig. 1h, k). Preodontoblasts began to express *Dsp* mRNA and increased their intensity according to the progress of odontoblast differentiation (Fig. 1l, o), and finally mature odontoblasts showed intense expression of *Dsp* mRNA in their cytoplasm (Fig. 1r).

Double immunofluorescent staining in incisor at Week 3

Nestin and Dsp were expressed in preodontoblasts and immature odontoblasts (Fig. 2a-f), and the apical end of their cytoplasm began to show intense immunoreactions for nestin according to

the progress of odontoblast differentiation (Fig. 2a, c, d, f). Finally, mature odontoblasts contained intense immunoreactions for nestin in their entire cytoplasm including their cellular processes (Fig. 2j, m, l, o). Stellate reticulum and stratum intermedium also showed faint immunoreactions for nestin and Dsp (Fig. 2a-f). Intense Dsp immunoreactions were localized in the Golgi area of immature and mature odontoblasts (Fig. 2d, e, j, k). Dentinal tubules and predentin matrix also showed positive reactions for Dsp (Fig. 2g, h, m, n) in addition to preameloblasts (Fig. 2d, e).

Molar at Day 1 (Table 2)

Odontoblasts consistently expressed nestin and Dsp proteins and *Dsp* mRNA (Fig. 3a-c). Other pulpal cells showed positive reaction for Dsp protein as well as bone cells (Fig. 3b, e, h, k, n). Intense expression of *Dsp* mRNA was observed in the part of the enamel organ (Fig. 3c). Preodontoblasts began to express nestin and Dsp proteins and *Dsp* mRNA (Fig. 3d-i), which increased their intensity according to the progress of odontoblast differentiation (Fig. 3j-o). Preameloblasts also showed immunoreaction for Dsp protein and expressed *Dsp* mRNA (Fig. 3k, l), and positive reaction for only Dsp protein was observed in the dentinal tubules (Fig. 3n).

Molar at Week 1 (Table 2)

Odontoblasts consistently expressed nestin and Dsp proteins and *Dsp* mRNA (Fig. 4a-c). Other pulpal cells showed a positive reaction for Dsp protein as well as bone cells (Fig. 4b, e, h, k). Dentinal tubules and predentin matrix in the coronal dentin showed intense expression of Dsp

protein (Fig. 4k). Immature and mature odontoblasts expressed intense nestin and Dsp proteins and *Dsp* mRNA in their cytoplasm (Fig. 4g-l).

Molar at Week 3 (Table 2)

Root formation progressed at this stage when odontoblasts continued to express nestin and Dsp proteins and *Dsp* mRNA (data not shown). Nestin immunoreactivity in the odontoblast cell processes in the predentin of coronal dentin was more intense than that in the root and pulp floor dentin, and this tendency continued until Week 5. Other pulpal cells and dentinal tubules showed intense positive reaction for Dsp protein (data not shown).

Molar at Weeks 5 and 8 (Table 2)

We adopted two different concentrations (1:50 and 1:500) for Dsp immunohistochemistry. The Dsp immunoreaction increased in intensity according to the progress of dentinogenesis and the use of different dilution of antibody was recommended to get the suitable stainability in dentin-pulp complex at each stage. Odontoblasts consistently expressed nestin and Dsp proteins during Weeks 5-8 (Fig. 5a-f), although the expression of *Dsp* mRNA in the odontoblasts became weak in intensity at Week 5 (data not shown) and almost disappeared except for some odontoblasts beneath the enamel-free area (9) and in the pulpal floor at Week 8 (Fig. 5g, h, i, k). Increased immunoreaction for Dsp protein was recognized in the coronal dentinal tubules and dental pulp according to aging (Fig. 5c, d), and intense expression of *Dsp* mRNA appeared in some cementoblasts at Week 8 (Figs. 5j, l).

DISCUSSION

Dsp

The present study clearly demonstrated that the expression of *Dsp* mRNA coincided with the odontoblast secretory activity for dentin matrix deposition in both 3-week-old incisors and developing molars. In contrast, other pulpal cells, predentin matrix and dentinal tubules showed positive reactions only for Dsp protein and increased in their intensity according to the progress of tooth development. Previous *in situ* hybridization experiments showed that *Dsp* and *Dspp* mRNA were expressed only by odontoblasts and not by other cell types except for preameloblasts in the mouse incisor and molar at Day 11 (43) or developing rat incisors and molars during embryonic day 20 to postnatal day 20 (4, 19) and developing mouse molars during Day 1 to Week 8 (5, 58). These observations are consistent with the results from immunohistochemical experiments demonstrating the highly specific manner of expression of Dsp protein in developing rat molars during Days 0-17 (4, 10, 13) and developing mouse incisors or molars during Day 1 to 13.5 months (5, 20, 58). However, our immunostaining for Dsp gradually increased in intensity in predentin matrix, dentinal tubules and odontoblasts, and appeared in many cells of the dental papilla or pulp. The discrepancy between the expressions of *Dsp* mRNA and Dsp protein could be explained from three possible perspectives. First, the antibodies may detect epitopes for other proteins similar to Dsp that are synthesized by pulpal cells (43). Second, pulpal cells may take up Dsp protein into their cytoplasm that has been secreted by differentiating and differentiated odontoblasts. Lastly, it is probable that *Dsp* mRNA is expressed at undetectable levels in the pulpal cells, and subsequently Dsp protein gradually accumulates in their cytoplasm according to the progress of dentinogenesis. The last idea is

supported by the findings of a recent study that demonstrated that DSPP is also expressed in lower levels in bone and non-mineralized tissue (58).

Regarding the regenerative process after tooth injuries such as cavity preparation and pulpal capping following pulp exposure, newly differentiated or surviving odontoblast-like cells express Dsp protein in addition to the immunoreaction in the tertiary dentin (14, 25), suggesting that this protein is a reliable marker for regenerated odontoblasts and tertiary dentin. However, immunohistochemistry for Dsp is unable to determine the up-regulation of Dsp production in the afflicted odontoblasts, because Dsp protein has already been accumulated in their cytoplasm. Analysis for *Dsp* mRNA is necessary for precise distinction between the accumulation of protein and up-regulation of its production. The present study demonstrated that some odontoblasts beneath the enamel-free area expressed intense *Dsp* mRNA at Week 8. Tertiary dentin is formed under this area according to aging (31), because this area lacking the enamel covering is directly exposed to the oral environment. Thus, the continuous irritation via the enamel-free area could induce the up-regulation of Dsp synthesis in the odontoblasts beneath this area. The intense expression of *Dsp* mRNA in the odontoblasts adjacent to the pulpal floor at Week 8 suggests that their secretory activity correlates with the continuous deposition of secondary dentin in this area.

Nestin

The present immunohistochemistry for nestin confirmed that nestin is valuable as a differentiation marker for odontoblasts (26). Furthermore, our double immunohistochemistry for nestin and Dsp proteins clearly demonstrated that the timing of their expressions in the odontoblast-lineage cells was synchronized: preodontoblasts began to express both proteins and increased their intensity according to the progress of odontoblast differentiation, and finally

mature odontoblasts showed intense expression of both proteins in their cytoplasm. However, the immunoreaction for Dsp was accumulated in the other pulpal cells, whereas nestin was exclusively expressed in the differentiated odontoblasts in the mature dental pulp. Thus, nestin is a more reliable marker for differentiated odontoblasts compared with Dsp protein. Although the expression patterns of *nestin* mRNA in the dental pulp of developing and aged teeth remain to be elucidated at the present, it is assumed that *nestin* mRNA is consistent with nestin protein judging from the cytoskeletal characteristics of intermediate filament nestin and the facts demonstrated by the previous study focusing on the pancreatic cancer (29). Furthermore, our preliminary study has confirmed the usefulness of nestin as a marker for differentiated odontoblast-like cells even in the environment of *in vitro* culture (author's unpublished data). Further study is needed to clarify the exact nature of nestin as a marker for differentiated odontoblasts.

Classification of odontoblast-lineage cells (Table 3)

Based on the present results, we reconsider the terminology for classification of odontoblast-lineage cells. Preodontoblasts began to express nestin and Dsp proteins and *Dsp* mRNA, which increased their expression intensities according to the progress of odontoblast differentiation. Since the matrix-producing cells lack their proliferative activity and acquire a differentiation marker such as heat-shock protein 25 during odontogenesis in rats (32, 33), proliferating cells are defined as "dental papilla cells" and the polarized cells beginning to acquire their differentiation markers such as nestin and Dsp are named "preodontoblasts". Matrix-producing cells increasing the expression of their nestin and Dsp are referred to as odontoblasts, and these cells are further divided into subgroups. In our previous study using continuously growing rat incisor teeth, we

have divided these cells into three types: immature, mature, and post-odontoblasts, according to their morphology, the rate of dentin deposition, and their nutritional supply (the location and ultrastructure of the odontoblastic capillaries) (39). The cells beginning to produce predentin are characterized by immature features including minute projections on their distal ends and are engaged in the production of mantle dentin where no capillaries are present in the odontoblast layer. These immature cells are referred to as "immature odontoblasts". Subsequently, they increase in height and develop a thick cellular process during active dentin formation resulting in the pseudostratified layer and fenestrated capillaries are always located close to the predentin. Based on their mature morphology and capacity for dentin deposition and sufficient nutritional supply, these odontoblasts can be described as "mature odontoblasts". The intense expression of *Dsp* mRNA in mature odontoblasts in this study was precisely correlated with their functional activity. In contrast, the term "post-odontoblasts" is not suitable for the general terminology used in the process of dentinogenesis, because their appearance is a peculiar status due to an inflammatory reaction caused by the attrition of the incisal tip (39, 50, 52). The expression of *Dsp* mRNA in the odontoblasts became weak in intensity at Week 5 and almost disappeared at Week 8 in this study, suggesting the existence of odontoblasts that have vacated an active synthetic phase and committed to a quiescent phase. The term "resting odontoblasts" seems suitable for the definition of these cells (13, 14).

In conclusion, nestin is valuable as a differentiation marker for odontoblasts, whereas *Dsp* mRNA is a functional marker for their secretory activity. Regarding the classification of odontoblasts, immature, mature, and resting odontoblasts are universal terminology in teeth with limited growth including human teeth from morphological and functional perspectives. Further study should continue to provide the information on the additional molecular markers that starts

to be expressed in mature odontoblasts (or ceases to be expressed in immature odontoblasts) to clarify the precise distinction among different types of odontoblasts.

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Table 1 *Comparison of nestin and dentin sialoprotein (Dsp) immunoreactivity and Dsp mRNA expression during dentinogenesis in a 3-week-old incisor*

				odontoblasts	
	apical bud	dental papilla	preodontoblasts	immature	mature
nestin	+/-	-	+	++	+++
Dsp	+	-	+/-	++	+++
Dsp mRNA	-	-	+/-	++	+++

-: negative, +/-: very weak, +: weak positive, ++: medium positive, +++: intensely positive

Table 2 *Comparison of nestin and Dsp immunoreactivity and Dsp mRNA expression during dentinogenesis in developing molars*

	Day 1			Week 1			Week 3			Week 5			Week 8		
	DP	OB		DP	OB		DP	OB		DP	OB		DP	OB	
		C	R		C	R		C	R		C	R		C	R
nestin	-	+	N/A	-	++	+	+/-	+++	++	-	++	++	-	++	++
Dsp	+	+	N/A	+	++	+	++	+++	++	+++	+++	+++	+++	+++	+++
Dsp mRNA	+/-	++	N/A	+/-	+++	++	+/-	+++	++	+/-	+	+	-	+/-	-

-: negative, +/-: very weak, +: weak positive, ++: medium positive, +++: intensely positive, N/A: not available

C: coronal dentin, DP: dental pulp, OB: odontoblasts, R: root dentin

Table 3 *Summary of nestin and Dsp immunoreactivity and Dsp mRNA expression in each cell type during dentinogenesis in developing molars*

	dental papilla	preodontoblasts	immature odontoblasts	mature odontoblasts	resting odontoblasts
nestin	+/ -	+	++	++	++
Dsp	+/ -	+	++	+++	+++
Dsp mRNA	-	+	++	++	+/ -

-: negative, +/-: very weak, +: weak positive, ++: medium positive, +++: intensely positive

figure legends

Fig. 1 Expression of nestin (**a, d, g, j, m, p**) and Dsp proteins (**b, e, h, k, n, q**), and *Dsp* mRNA (**c, f, I, l, o, r**) in a 3 week-old incisor. Figures **d, g, j, m**, and **p** are higher magnified views of the boxed areas in **a**. Figures **e, h, k, n,** and **q** are higher magnified views of the boxed areas in **b**. Figures **f, i, l, o,** and **r** are higher magnified views of the boxed areas in **c**. Nestin and Dsp proteins are intensely expressed in odontoblasts, although other pulpal cells also show weak positive reactions (**a, b**). The expression of *Dsp* mRNA coincides with the odontoblast secretory activity for dentin matrix deposition (**c**). Faint positive reactions for nestin and Dsp are observed in the dental papilla and follicle around the apical bud, respectively (**d, e**), whereas there is no *Dsp* mRNA in this area (**f**). Nestin is expressed in preodontoblasts and immature odontoblasts, and the apical end of their cytoplasm begins to show intense immunoreactions according to the progress of odontoblast differentiation (**j, m**). Finally, mature odontoblasts show intense immunoreactions in their entire cytoplasm including their cellular processes (**p**). Stellate reticulum also shows faint immunoreactions for nestin (**g**). Intense Dsp immunoreactions are localized in the Golgi area of immature and mature odontoblasts as well as other pulpal cells (**k, n, q**). Dentinal tubules and predentin matrix also show positive reactions for Dsp (**q**) in addition to preameloblasts, ameloblasts, and stellate reticulum (**h, k, n**). Preodontoblasts begin to express *Dsp* mRNA and increase in their intensity according to the progress of odontoblast differentiation (**i, l, o**), and finally mature odontoblasts intensely express *Dsp* mRNA in their cytoplasm (**r**). Arrows: enamel matrix, arrowheads: odontoblastic capillaries, AB: ameloblasts, B: bone, D: dentin, DP: dental pulp, E: enamel, IEE: inner enamel epithelium, iOB: immature odontoblasts, mOB: mature

odontoblasts, pAB: preameloblasts, PD: predentin, pOB: preodontoblasts, SR: stellate reticulum *Bars* 500 μm (a-c), 50 μm (d-f), 25 μm (g-r)

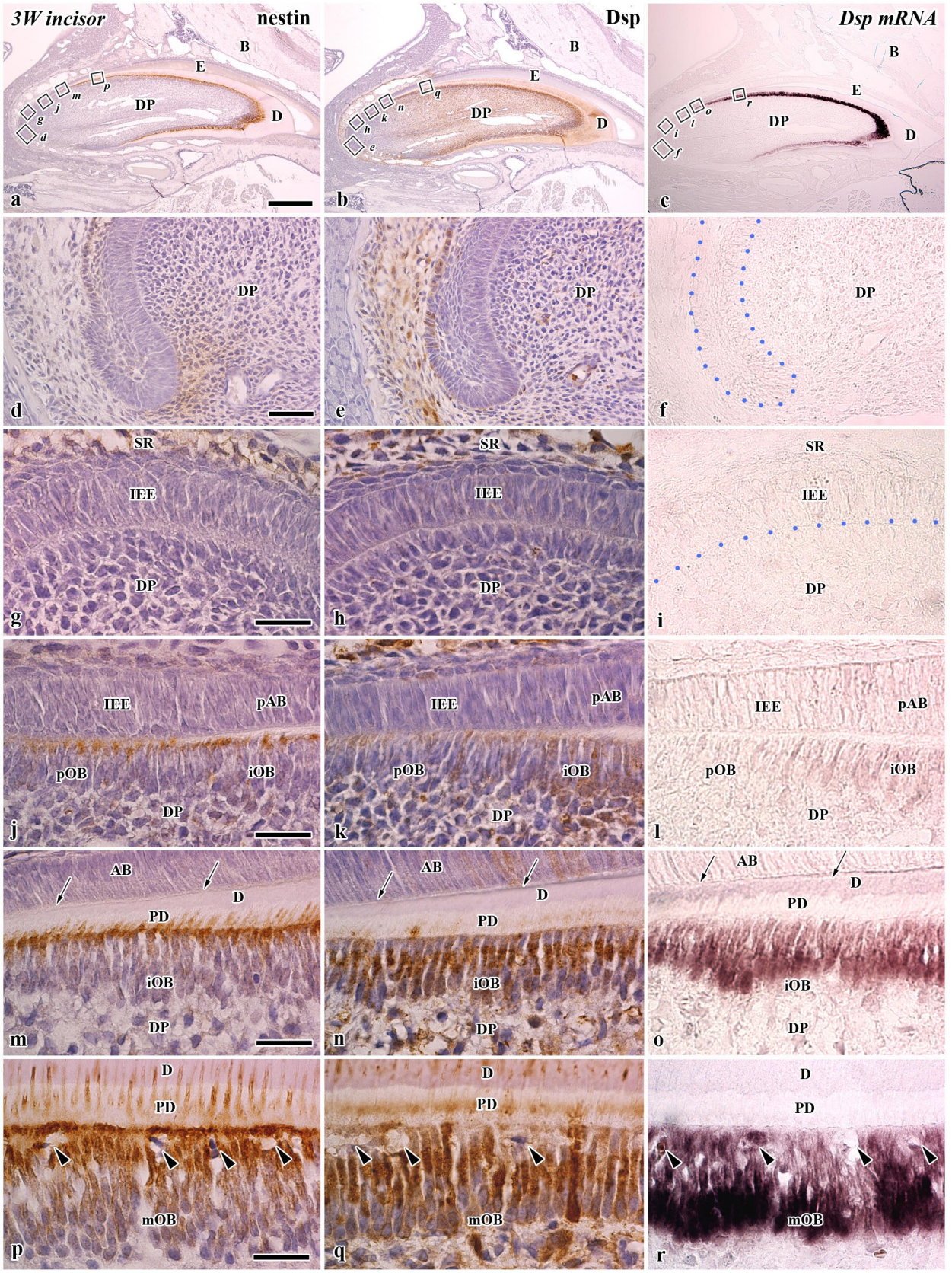
Fig. 2 Expression of both nestin and Dsp (**a, d, g, j, m**), Dsp (**b, e, h, k, n**), and nestin (**c, f, i, l, o**) in a 3 week-old incisor. Second and third rows are differently sliced views in the same section as well as fourth and fifth rows. The first row is the position including preodontoblasts, immature odontoblasts, and inner enamel epithelium. The second row is the position including immature odontoblasts and preameloblasts. The fourth row is the position including mature odontoblasts. The first row is near and the fourth and fifth rows are far from the apical bud, whereas the second and third rows are the positions between them. Nestin and Dsp are expressed in preodontoblasts and immature odontoblasts (**a-f**), and the apical end of their cytoplasm begins to show intense immunoreactions for nestin according to the progress of odontoblast differentiation (**g, i**). Finally, mature odontoblasts contain intense immunoreactions for nestin in their entire cytoplasm including their cellular processes (**j, m, l, o**). Stellate reticulum and stratum intermedium also show faint immunoreactions for nestin and Dsp (**a-f**). Intense Dsp immunoreactions are localized in the Golgi area of immature and mature odontoblasts (**d, e, j, k**). Predentin matrix and dentinal tubules also show positive reactions for Dsp (**g, h, m, n**) in addition to preameloblasts (**d, e**). D: dentin, DP: dental pulp, IEE: inner enamel epithelium, iOB: immature odontoblasts, mOB: mature odontoblasts, pAB: preameloblasts, PD: predentin, pOB: preodontoblasts, SI: stratum intermedium, SR: stellate reticulum *Bar* 100 μm.

Fig. 3 Expression of nestin (**a, d, g, j, m**) and Dsp proteins (**b, e, h, k, n**) and *Dsp* mRNA (**c, f, i, l, o**) in a 1 day-old molar. Figures **d, e** and **f** are higher magnified views of cervical areas of Figures **a, b,** and **c**, respectively. Figures **g, j,** and **m** are higher magnified views of the boxed areas in **a**. Figures **h, k,** and **n** are higher magnified views of the boxed areas in **b**. Figures **i, l,** and **o** are higher magnified views of the boxed areas in **c**. Odontoblasts consistently express nestin and Dsp proteins and *Dsp* mRNA (**a-c**). Other pulpal cells show positive reaction for Dsp protein as well as bone cells (**b, e, h, k, n**). Intense expression of *Dsp* mRNA is observed in the part of the enamel organ (**c**). Preodontoblasts begin to express nestin and Dsp proteins and *Dsp* mRNA (**d-i**), and increase their intensity according to the progress of odontoblasts differentiation (**j-o**). Preameloblasts also show immunoreactions for Dsp protein and *Dsp* mRNA (**k, l**), and positive reaction for only Dsp protein is observed in the dentinal tubules (**n**). Arrows: enamel matrix, AB: ameloblasts, B: bone, D: dentin, DP: dental pulp, EO: enamel organ, IEE: inner enamel epithelium, iOB: immature odontoblasts, pAB: preameloblasts, PD: predentin, pOB: preodontoblasts *Bars* 500 μm (a-c), 50 μm (d-f), 25 μm (g-o).

Fig. 4 Expression of nestin (**a, d, g, j**) and Dsp proteins (**b, e, h, k**) and *Dsp* mRNA (**c, f, i, l**) in a 1 week-old molar. Figures **d, e,** and **f** are higher magnified views of cervical areas of Figures **a, b,** and **c**, respectively. Figures **g** and **j** are higher magnified views of the boxed areas in **a**. Figures **h** and **k** are higher magnified views of the boxed areas in **b**. Figures **i** and **l** are higher magnified views of the boxed areas in **c**. Odontoblasts consistently express nestin and Dsp proteins, and *Dsp* mRNA (**a-c**). Other pulpal cells show positive reaction for Dsp protein as well as bone cells (**b, e, h, k**). Dentinal tubules in the coronal

dentin show intense expression of Dsp protein (**k**). Immature and mature odontoblasts express intense nestin and Dsp proteins and *Dsp* mRNA in their cytoplasm (**d-l**). Arrowheads: odontoblastic capillaries, AB: ameloblasts, B: bone, D: dentin, DP: dental pulp, E: enamel, EO: enamel organ, iOB: immature odontoblasts, mOB: mature odontoblasts, pAB: preameloblasts, PD: predentin *Bars* 250 μm (a-c), 50 μm (d-f), 25 μm (g-l)

Fig. 5 Expression of nestin (**a, b**) and Dsp proteins (**c, d, e, f**) and *Dsp* mRNA (**g-l**) in an 8 week-old molar. Figures **b, d, f,** and **h** are higher magnified views of the boxed areas in **a, c, e,** and **g**, respectively. Odontoblasts consistently express nestin protein (**a**) and Dsp protein which is demonstrated at two different antibody concentrations, 1:50 (**c**) and 1:500 (**e**). The Dsp immunoreaction increases in intensity according to the progress of dentinogenesis and the use of different dilution of antibody produces the suitable stainability in dentin-pulp complex at each stage. The expression of *Dsp* mRNA almost disappears (**g**) except for some odontoblasts beneath the enamel-free area (EFA) (**i**) and in the pulpal floor (**k**). Increased immunoreactions for Dsp protein are recognized in the coronal dentinal tubules and dental pulp (**c, e**), and intense expression of *Dsp* mRNA appears in some cementoblasts (**j, l**). B: bone, CB: cementoblasts, D: dentin, DP: dental pulp, JE: junction epithelium, OB: odontoblasts, PDL: periodontal ligament, rOB: resting odontoblasts *Bars* 250 μm (a, c, e, g), 50 μm (j), 25 μm (b, d, f, h, i, k, l)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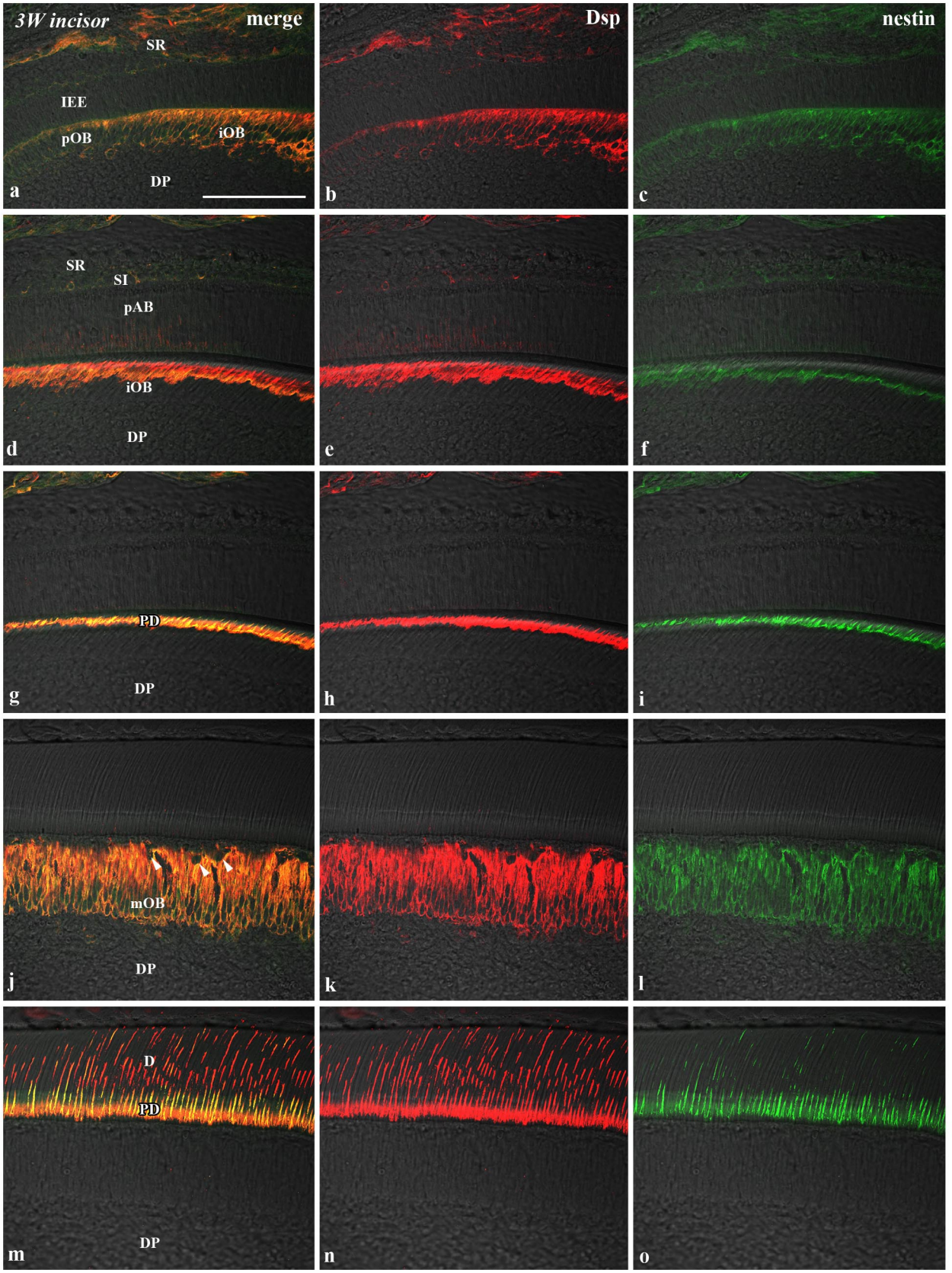
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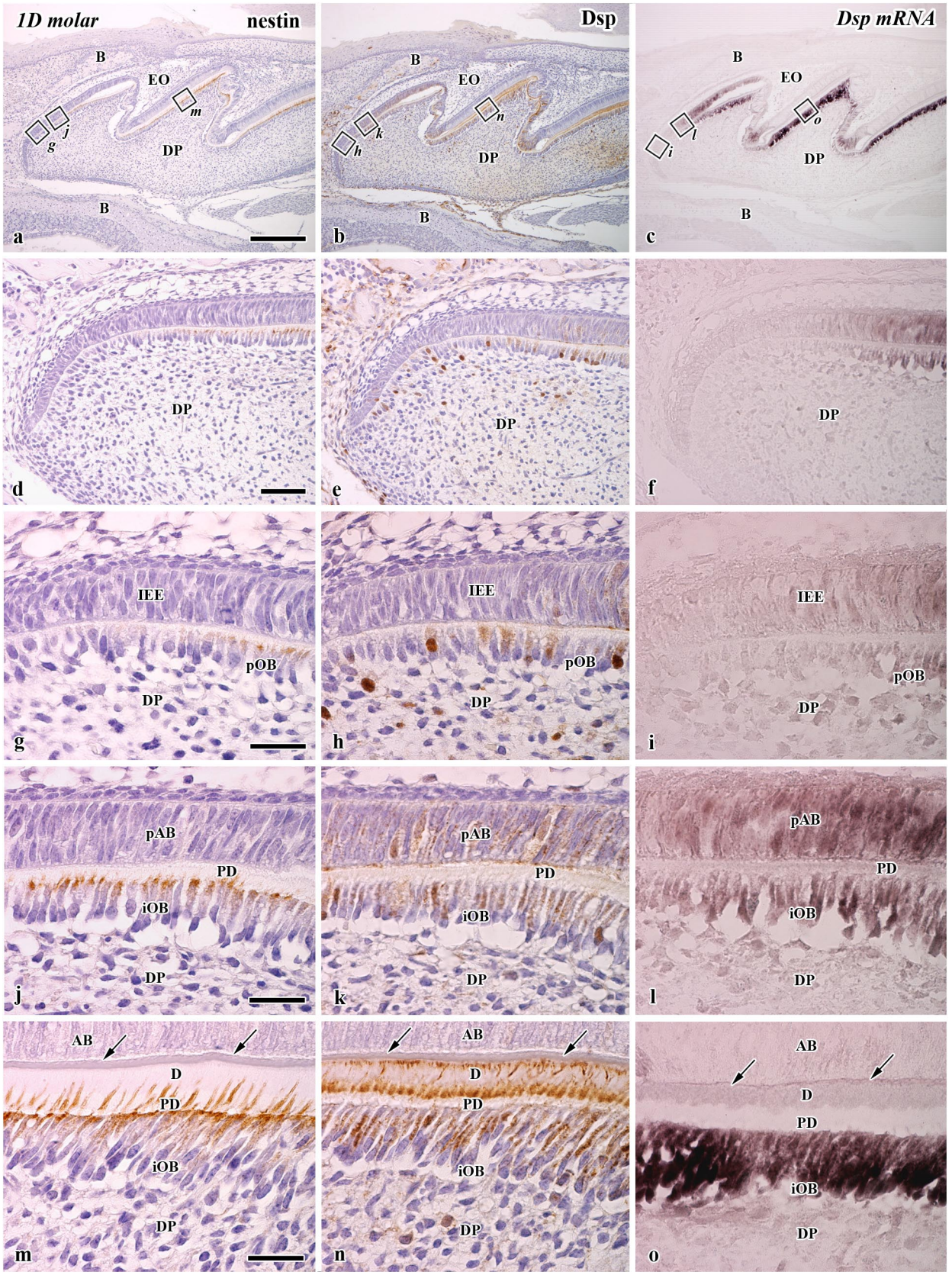
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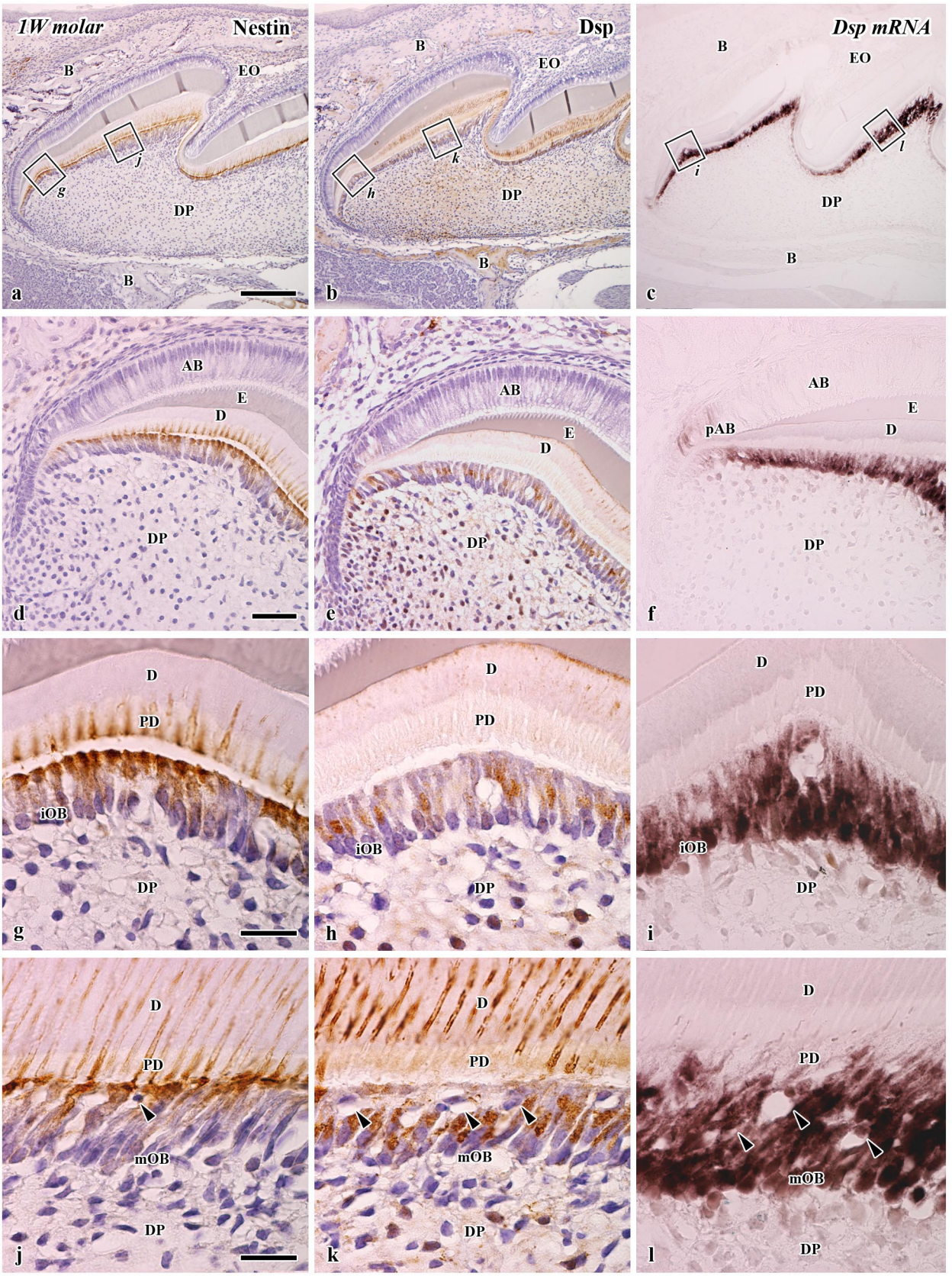
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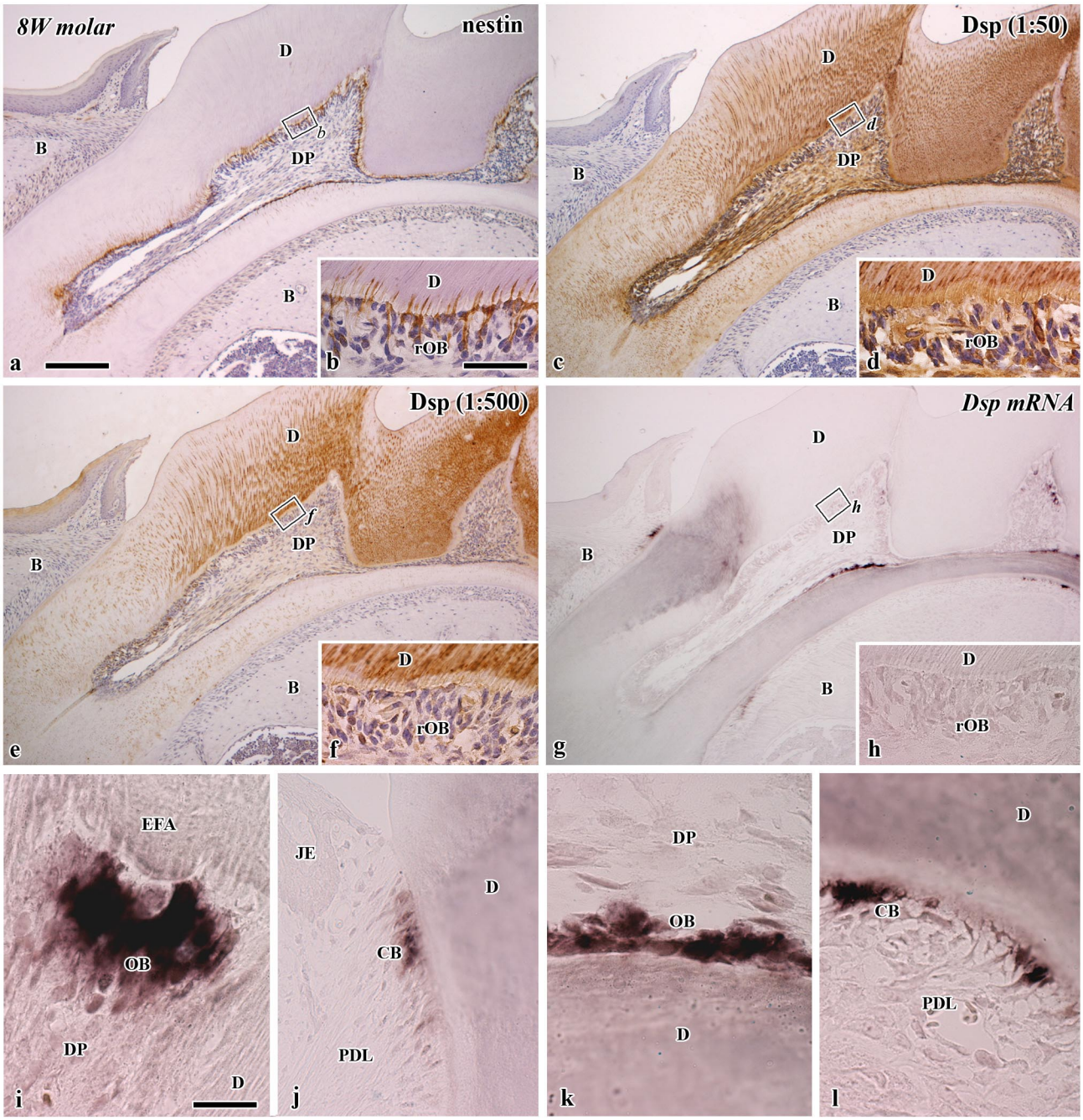
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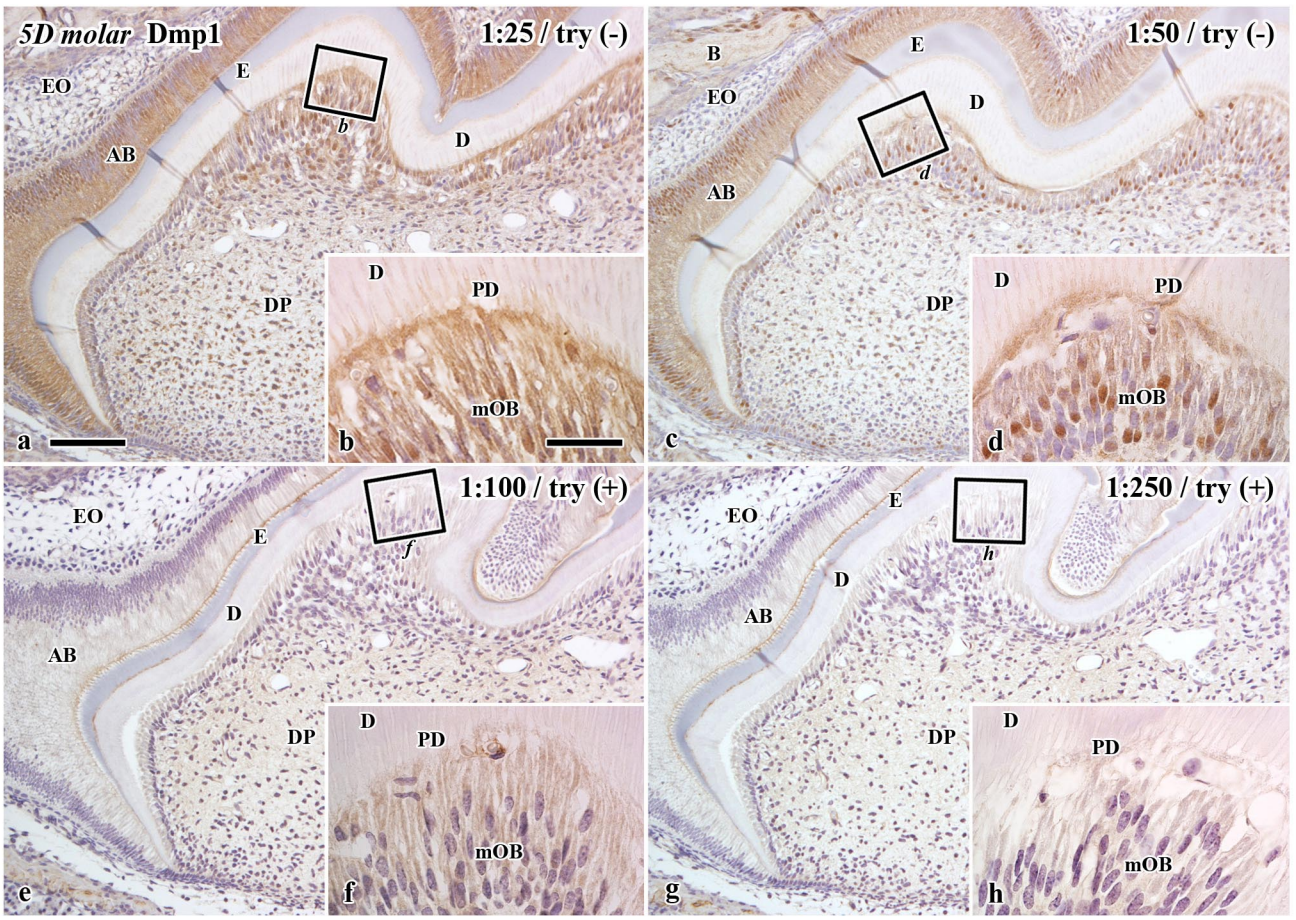
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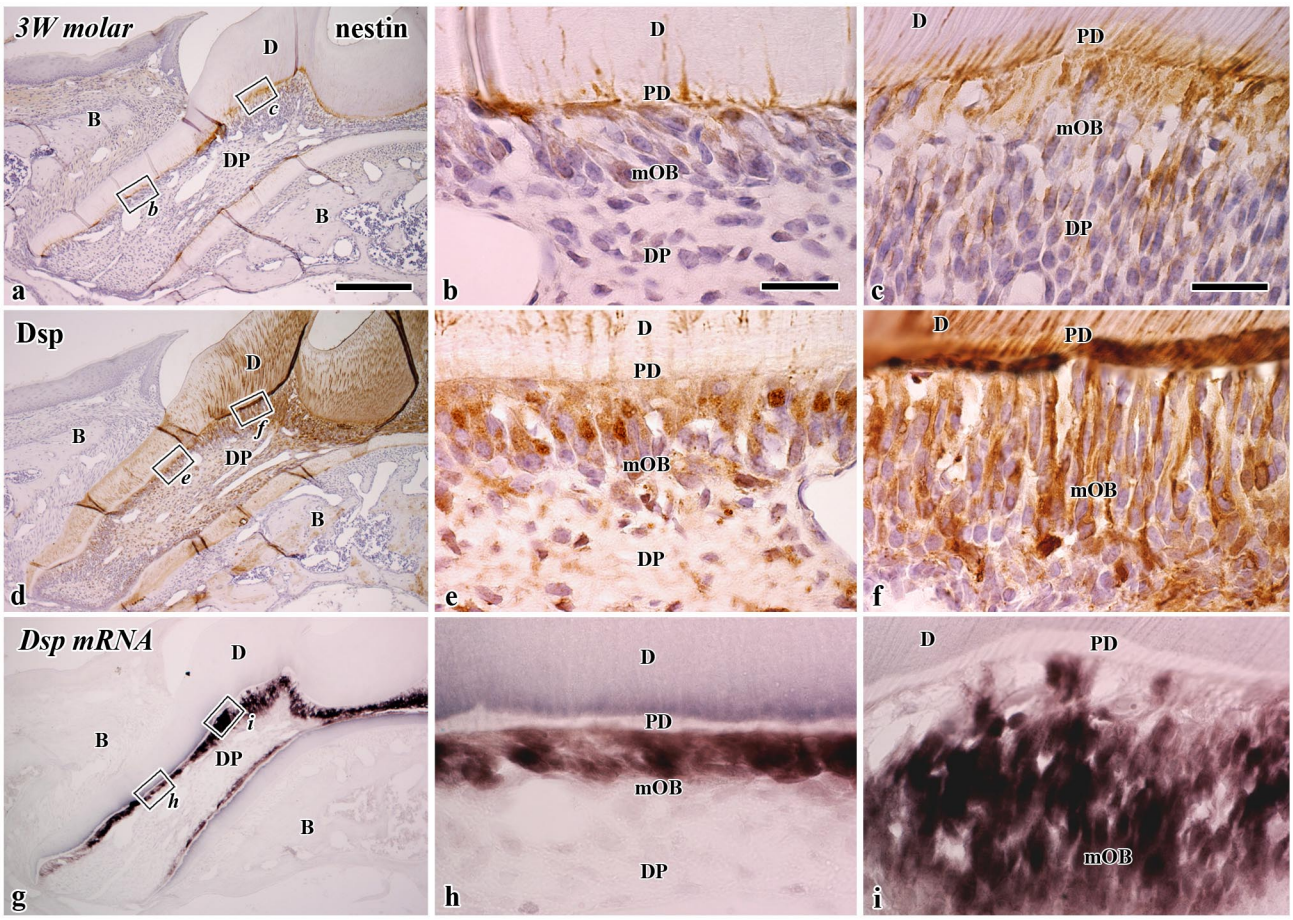
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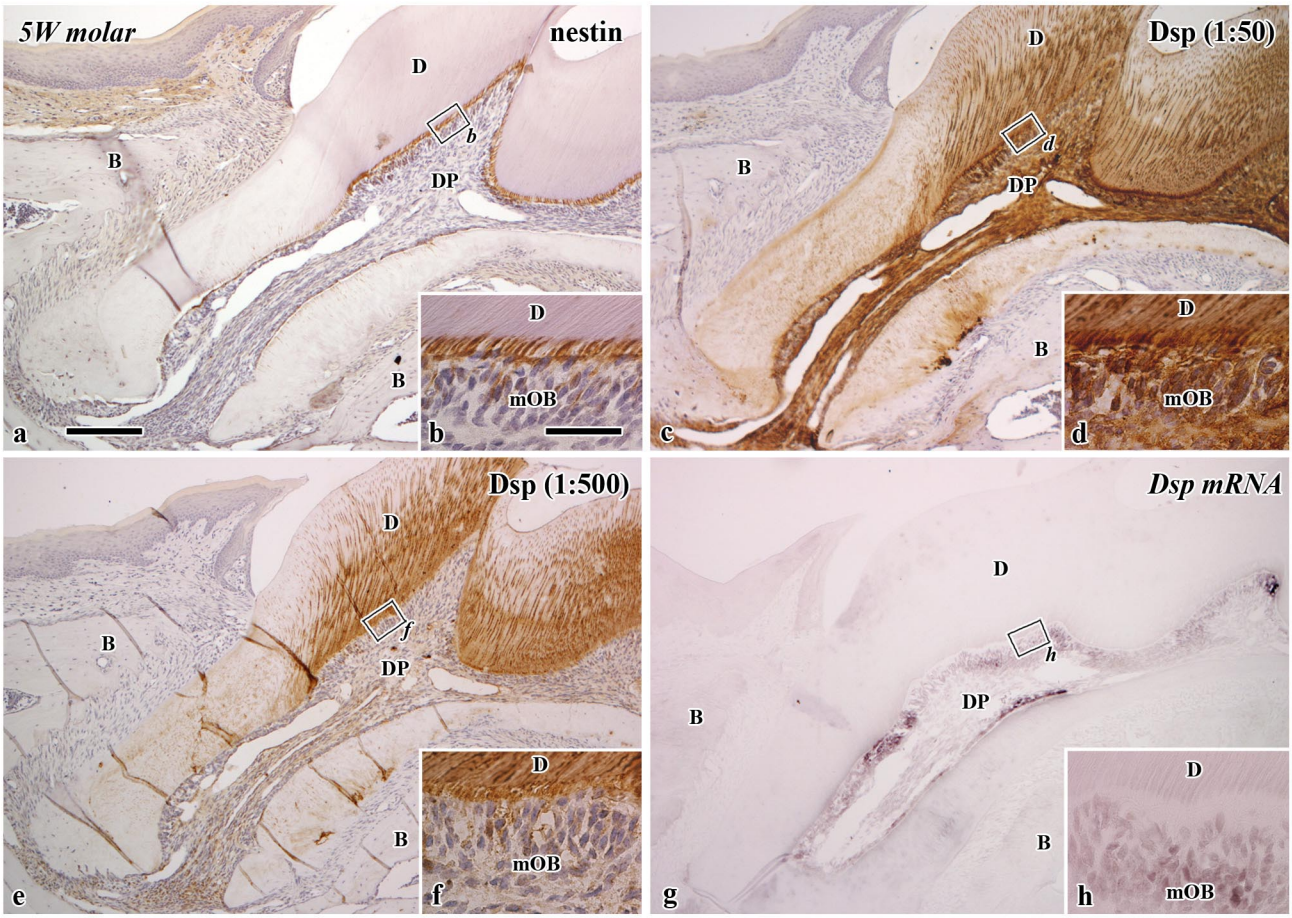
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Use of a triple antibiotic solution affects the healing process of intentionally delayed replanted teeth in mic

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Key words: Antimicrobials, Odontoblasts, Dental pulp, Tooth replantation, Immunohistochemistry, Mice (ICR).

Objective: A mixture of ciprofloxacin, metronidazole, and minocycline (3Mix) has been reported to be effective against oral bacteria from carious and endodontic lesions *in vitro* and *in vivo*. This study aimed to establish an animal model using mice for the application of 3Mix combined with the intentionally-delayed tooth replantation and investigate the effects of 3Mix on the healing process of dental pulp and periodontal tissues.

Methods: Upper first molars of ICR mice were extracted, immersed in 3Mix solution at different concentrations for 5 to 60 minutes with or without the use of a transfer solution (phosphate buffer solution: PBS) in addition to PBS alone, and subsequently repositioned in the sockets. Immunohistochemistry for nestin and Ki-67, histochemistry for TRAP, and TUNEL assay were performed to assess pulpal healing during Days 7-21.

Results: Increased apoptosis was observed in the PBS group at Week 1 followed by cell proliferation at Week 2 and tertiary dentin and/or bone-like tissue formation at Week 3. In contrast, nestin-positive newly-differentiated odontoblast-like cells began to align along the pulp-dentin border following the appearance of Ki-67- and TUNEL-positive cells during Weeks 1-2 in the 3Mix groups, suggesting the acceleration of pulpal healing. Severe root ankylosis was exclusively recognized in these groups. Rinsing with PBS before replantation partially rescued the viability of the periodontal ligament, although pulpal healing was delayed.

Conclusions: The application of 3Mix promotes pulpal regeneration of intentionally-delayed replanted teeth, although its use may induce severe damage to periodontal tissues.

1. Introduction

The combination of antibacterial drugs such as ciprofloxacin, metronidazole, and minocycline – referred to as 3Mix – has been reported to be effective against bacterial flora of humans in carious dentin, dental plaque and necrotic pulp [1-3]. Extensive evidence based on *in vivo* [4-8] and clinical studies [9-13] supports the use of 3Mix for carious dentinal lesions and for the regenerative endodontic treatments of primary and immature permanent teeth. However, the clinical use of 3Mix for the treatment of traumatized teeth remains limited.

Tooth avulsion implies the total displacement of the tooth out of its socket [14], resulting in the occurrence of attachment damage and pulp necrosis [15]. Tooth replantation is defined as a therapeutic method in which the avulsed tooth is replaced in its original socket. At least, two types of healing patterns are recognized in the replanted teeth: dentin and bone-like tissue formation [16-22]. In successful cases, pulpal regeneration has been shown to occur in experimental animal studies [16-22] and humans [23-25]. In contrast, when an inflammatory reaction remains for a long time, a bone-like tissue may occupy the pulp chamber in replanted teeth [16, 17, 20]. Thus, it is important to control the healing patterns of replanted teeth, since root ankylosis easily occurs when a bone-like tissue appears in the pulp chamber.

Determination of the healing pattern after tooth replantation may be directly linked to the death or survival of odontoblast-lineage cells [26]. A proper oxygenated medium is a decisive factor for the survival of odontoblast-lineage cells. Intentionally prolonged operating time for tooth replantation induces the total death of odontoblast-lineage cells. Furthermore, the occlusal forces significantly worsen the occurrence rate of dentin

formation [20]. In addition, the presence of bacteria on the root surface appears to affect the proper healing of the periodontal ligament. Thus, it is essential to limit these bacteria from the accident site and those tracking down the blood clot in the socket [14].

Positive effects of topical application of antibiotics, such as doxycycline and/or minocycline on replanted teeth have been reported in a series of experimental studies using dog, monkey, and rat models [27-31]. The use of 3Mix applied for the regenerative endodontic treatment of traumatized immature permanent teeth has been recently reported in a clinical case [25]. However, these studies lack analyses on the cellular responses such as cell proliferation, differentiation, and apoptosis to antibiotics following tooth replantation.

Several *in vivo* animal studies have been conducted to determine the optimal conditions for assuring the viability of the periodontal tissues in replanted teeth [32-36]. Nonetheless, there have been few investigations on the pulpal regeneration. This study aimed to establish an animal model using mice for the application of 3Mix combined with intentionally-delayed tooth replantation, and to investigate the effects of 3Mix on the survival of dental pulp and periodontal tissues using immunohistochemical and histochemical analyses and TUNEL assay.

2. Materials and methods

2.1. Tooth replantation

All experiments were reviewed by the Committee on the Guidelines for Animal Experimentation of Niigata University and performed according to the recommendations or under the conditions proposed by the Committee. One hundred and thirteen Crlj:CD1 (ICR) mice (3 weeks old) were obtained for the completion of this study. The animals were divided into two major experimental groups: (1) phosphate buffer saline (PBS) group and (2) 3Mix group, using the combination of ciprofloxacin, metronidazole, and minocycline as a solution in the following concentrations.

a) Standard concentration: 0.1 mg ciprofloxacin, 0.2 mg metronidazole, and 0.1 mg minocycline diluted in 10 ml distilled water (DW).
b) High concentration: 25 mg ciprofloxacin, 25 mg metronidazole, and 25 mg minocycline diluted in 10 ml propylene glycol: the modified solvent of the clinical protocol for the use of 3Mix in the carious lesion (ciprofloxacin : metronidazole : minocycline = 1:1:1 in the mixture of one part propylene glycol and the same volume of macrogol).
c) Reduced concentration: three types (50%, 25%, and 10%) compared to the standard concentration were tested.

The upper right first molar of each animal was extracted under deep anesthesia by an intraperitoneal injection of chloral hydrate (the maximum dose of 350 mg/kg), with a pair of modified dental forceps and then repositioned in its original socket after immersion in PBS and/or the 3Mix solution. The non-treated left upper first molar of the same animal was used as a control in each group. The extracted teeth were immersed in different

solutions as shown in Table 1. The alveolar sockets were untreated during the immersion of teeth after stopping the bleeding, and the part of blood clot was removed before tooth replantation. No further treatments such as fixation of teeth and relief of occlusion were performed after tooth replantation.

2.2. Tissue preparation

Materials were collected from groups of 3-5 animals at intervals of 7, 14, and 21 days after tooth replantation. At each stage, the animals were transcardially perfused with physiological saline followed by 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) under deep anesthesia by an intraperitoneal injection of chloral hydrate. The maxillae were removed *en bloc* and immersed in the same fixative for an additional 12 hours at 4°C. Following decalcification in Morse's solution (10% sodium citrate and 22.5% formic acid) for 4-6 days at 4°C, the specimens were dehydrated through ethanol series and embedded in paraffin, and sagittal sections of maxillae were cut at 4 μm. The paraffin sections were mounted on Matsunami adhesive silane (MAS)-coated glass (Matsunami Glass Ind., Osaka, Japan) slides and processed for hematoxylin and eosin (H&E) staining and immunohistochemistry.

2.3. Immunohistochemical analysis and TUNEL assay

For the immuno-peroxidase procedure, sections were processed according to the EnVision method (DAKO Japan, Tokyo, Japan) using a mouse anti-nestin monoclonal antibody diluted to 1:100 (Millipore, Temecula, CA, USA). It has been clearly demonstrated that nestin is valuable as a differentiation marker for odontoblasts [37, 38].

Cell proliferation was quantified by Ki-67 immunohistochemical analysis. Apoptosis was quantified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Massachusetts, MA, USA). The procedures are described in detail in a previous study [39].

2.4 Histochemical analysis

For the histochemical demonstration of TRAP activity, the Azo-dye method was utilized with slight modification [20]. The paraffin sections were incubated for 60 min at 45 $^{\circ}$C in a medium comprising 0.01% naphtol AS-BI phosphatase (Na salt; Sigma Chemical, St. Louis, Mo., USA), 0.06% fast red violet LB salt (Sigma Chemical), and 50 mM L-(+)-tartaric acid in 0.2 M acetate buffer (pH 5.3). The sections were counter-stained with 0.5% methyl green.

2.5. Cell count and statistical analysis of Ki-67 and TUNEL positive cells

The numbers of Ki-67, and TUNEL-positive cells in the pulp horns and roots of each specimen (3.4 x 10^4 grid was selected) were calculated separately. All data were presented as the means and standard deviations (SD) of each group. Furthermore the number of cells in the pulpal horns and roots among different times after tooth replantation (7-21 days) and between experimental groups were compared by Bonferroni's test (one-way analysis of variance; ANOVA) using statistical software (SPSS 16.0J for Windows; SPSS Japan, Tokyo, Japan).

2.6. Statistical analysis of root ankylosis and root resorption

The percentage of the ankylosed or TRAP-positive areas was calculated on the base of the total perimeter of the mesial and distal roots and using image processing software (Image J 1.45s, NIH, USA). All data were presented as the means and SD of each group. The occurrence percentage of root ankylosis and root resorption at different times after tooth replantation (7-21 days) and between experimental groups were compared by Bonferroni's test using statistical software (SPSS 16.0J for Windows).

3. Results

3.1. Control and Group 1 (Figure 1)

In the untreated control teeth, nestin was exclusively expressed in the odontoblast and subodontoblastic layers (Fig. 1A). In Group 1, the dental pulp included abundant cell debris, scarce neutrophils, and/or hemorrhage at Week 1, when nestin-positive filamentous structures were observed in the apical side of the root pulp (Fig. 1B, C). Intense TRAP activity was frequently observed in the mesial side of the distal root (Fig. 1D) as well as around the apical areas in both mesial and distal roots (data not shown). Cell debris-derived matrices remained in the coronal pulp surrounded by nestin-positive filamentous structures and newly-differentiated odontoblast-like cells began to align along the pulp-dentin border at Week 2 (Fig. 2E), and subsequently tertiary dentin and/or bone-like tissue were observed at Week 3 (Fig. 2F-H).

3.2. Groups 2 and 3 (Figure 2)

The use of 3Mix solution at standard concentration combined with prolonged immersion times in PBS after extraction improved the healing process by accelerating the odontoblasts differentiation compared with Group 1 (Fig. 2 A-F). Blood vessels were clearly observed in the root pulp of Groups 2 and 3 compared with those in the coronal portion, where cell debris, scarce blood cells, and neutrophils remained in the pulpal horns at Week 1. Nestin immunoreaction was recognized in the newly-differentiated odontoblasts and subodontoblastic layer (Fig. 2A, D), while TRAP-positive reactions were observed in the cervical areas in both groups in addition to intense TRAP activity in the mesial side of the distal root and around the root apexes (Fig. 2B). At Week 2, immunohistochemistry for

nestin provided the evidence of tertiary dentin formation in the dental pulp of both groups (Fig. 2C, E), although the mixed healing pattern consisting of tertiary dentin and bone-like tissue was observed in 50% of samples from Group 3 (Table 2). In addition, total root ankylosis occurred in 100% of samples of both groups (Fig. 2F). Statistical analyses are presented in Fig. 2G, H.

3.3. Groups 4 to 7 (Figure 3)

The prolonged immersion times in 3Mix solution at different concentrations induced the diversity of healing patterns. 3Mix solution using equal amounts of each antibiotic (Group 4) led to the total degeneration of the dental pulp (Fig. 3A) and the intense TRAP activity in the apical areas of the mesial and distal roots at Week 1 (Fig. 3B), and further bone-like tissue formation at Week 2 in all samples (Fig. 3C). The use of 3Mix solution at reduced concentrations did not improve pulpal healing. At Week 1, the dental pulp of Groups 5 and 6 included the presence of abundant cell debris and scarce neutrophils, except for the apical pulp where nestin-positive filamentous structures were detected (Fig. 3D, J). At Week 2, a mixed form of tertiary dentin and bone-like tissue was recognized in almost all samples from Groups 5-7. However, fibrous and/or bone-like tissues were also recognized in the dental pulp regardless of the 3Mix concentrations (Fig. E-I, K, L). Severe root ankylosis was also observed in Groups 4-7 at Week 2.

3.4. Groups 8 to 12 (Figure 4)

The PBS rinse for 1 min following immersion in 3Mix solution and before tooth replantation was tested. At Week 1, nestin-positive immunoreactions were recognized in

the newly-differentiated odontoblast-like cells and subodontoblastic layer in Groups 8, 9 (data not shown), and 10 (Fig. 4A, B). In contrast, Groups 11 and 12 showed delayed pulpal healing with degenerated areas in the coronal pulp (Fig. 4F). At Week 2, the occurrence of the mixed form of tertiary dentin and bone-like tissue increased in number in all groups (Table 2). The occurrence rate of tertiary dentin was higher in Groups 8, 10, and 12 (Fig. 4E, H), although the healing of the whole dental pulp was also delayed (see Table 2). TRAP activity was decreased in intensity at Week 2 for all the groups, especially in Groups 10 and 12 (Fig. 4C, I), and the occurrence of root ankylosis was improved (Fig. 4D, G). Statistical analyses are presented in Fig. 2J-L.

3.5. Cell proliferation in the dental pulp of Groups 1-12 using Ki-67 immunoreaction (Figure 5)

Analysis of the Ki-67 positive cells showed that active cell proliferation took place at Week 2 and subsequently proliferative cells were decreased in number at Week 3 in Group 1 (Fig. 5 A, E, F, J). In the groups using 3Mix standard concentration, in contrast, the cell proliferation was significantly accelerated at Week 1 compared with 3Mix at reduced or high concentration (Fig. 5B, C, D, K). At Week 2, the statistical analysis demonstrated a significant reduction in the number of proliferative cells for all the 3Mix groups (Fig. 5L).

3.6. Apoptosis in the dental pulp of Groups 1-12 using TUNEL assay (Figure 6)

Analysis of the TUNEL-positive cells showed that the apoptotic activity was decreased at Week 2 and 3 in both coronal (Fig. 6A, E, F) and root pulp (data not shown) in

Group 1. At Week 1, an increase in apoptotic activity was observed in the coronal and root pulp of 3Mix groups (Fig. 6B-D, J, K). Group 3 showed the lowest average of TUNEL-positive cells, and significant differences were found between this group and Groups 6-12 as well as between Group 1 and Groups 2 and 3 in the coronal (Fig. 6J) and/or root dental pulp (Fig. 6K). The average of TUNEL-positive cells sharply decreased at Week 2 for all 3Mix groups in the coronal and root pulp (Fig. 6G-I, L, M). However, significant differences were observed between Group 12 and Groups 5, 8, and 11 in the coronal pulp (Fig. 6L) and between Groups 4 and 12 and the rest of the 3Mix groups in the root pulp (Fig. 6M).

4. Discussion

This study is the first report of an animal model using mice for the evaluation of therapeutic reagents on replanted teeth, since previous studies on tooth replantation testing antibiotics or bisphosphonates have been conducted using dogs, monkeys or rats [27-31]. The current results clearly demonstrate the positive effect of 3Mix solution to improve the final outcome of replanted teeth. Previous studies have shown that the contamination of dental pulp affects the healing pattern due to the movement of bacteria along the blood clot that develops between the roots of replanted teeth and their sockets [27, 28, 30]. Minocycline increases the rate of pulp revascularization in replanted teeth compared with doxycycline or saline solution [30]. Metronidazole is highly effective against the anaerobes prevalent in the necrotic dental pulp [40], and ciprofloxacin significantly reduces the inflammatory reactions in the pulp tissue of monkeys mechanically exposed and contaminated [41]. Thus, the mixture of ciprofloxacin, metronidazole, and minocycline seems to be sufficiently effective to avoid the penetration of bacteria in the root canal, causing an aseptic environment that favors pulpal regeneration.

Different healing patterns were observed in the intentionally-delayed replanted teeth [20]. The current results are similar to those in the previous studies using minocycline and doxycycline, where reactionary dentin, osteodentin, and dense fibrous connective tissue were recognized after Weeks 2 and 3 [27-30]. The healing patterns seem to depend on the variations in the concentrations of the antibiotics. Our recent studies demonstrated the importance of maintaining putative dental pulp stem/progenitor cells for the regeneration of odontoblast-like cells in the healing process following tooth replantation and transplantation [42, 43]. In addition, a recent study showed that high concentrations of

antibiotics have a detrimental effect on the survival of stem cells of the apical papilla [44]. Hence, higher amounts of antibiotics seem to be detrimental to the survival of odontoblast-like cells of replanted teeth. In contrast, reduced concentrations of 3Mix solution caused an increase in the mixed form of tertiary dentin and bone-like tissue in the replanted teeth. The phenomenon may be related with a decrease in the antibacterial effectiveness of 3Mix solution.

Despite improvement of the pulp healing process, 3Mix induced severe root ankylosis. Previous studies showed the effectiveness of antibacterial agents such as tetracycline to stimulate fibroblast attachment to the root surface, favoring periodontal ligament regeneration [33, 45]. Interestingly, we noticed that a rinse with PBS decreased the rate of root ankylosis. Nevertheless, the pulpal healing process was delayed in most of the samples. Thus, direct exposure to the mixture of antibiotics may be highly cytotoxic for the survival of the periodontal ligament cells, and/or 3Mix can efficiently stimulate the osteoblast differentiation in the supporting bone. The use of DW as a solvent also may seem unsuitable for cell viability compared with PBS from the view point of osmotic pressure.

The immersion of the avulsed tooth in a transfer solution followed by a short immersion time in 3Mix solution at standard concentration with a PBS rinse seems to be the optimal condition for improvement of both pulpal and periodontal tissue healing. Since the incidence of root ankylosis might be an important drawback for its application, further experimental animal studies should be conducted to improve the viability of the periodontal tissues following the topical application of 3Mix through *in vivo* and *in vitro* studies. Regarding the clinical use of 3Mix in the replanted teeth, we should consider that the

cellular events in the human dental pulp could not be readily extrapolated from the mouse experiments due to the big gap in the size of teeth between mice and humans.

Conflict of interest

No potential conflicts of interest are disclosed.

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Figure Legends

Fig. 1 Nestin- (A, C, E, G, H), H&E- (B, F), and TRAP-stained sections (D) in the control (A) and replanted teeth at 1 (A-D), 2 (E), and 3 weeks (F-H) after the operations in Group 1 (B-H). (A) Odontoblasts show pseudostratified features in both the coronal and root pulp. (Inset) Higher magnification of the boxed area in (A). Nestin is exclusively expressed in the odontoblast and subodontoblastic layers, and blood capillaries (arrowheads) are located in the odontoblast layer. (B, C) The dental pulp includes abundant cell debris, scarce neutrophils, and hemorrhage, and nestin-positive filamentous structures are observed in the apical side of the root pulp. (D) Intense TRAP activity is observed in the mesial side of the distal root. (E) Cell debris-derived matrices remain in the coronal pulp surrounded by nestin-positive filamentous structures. (Inset) Higher magnification of the boxed area in (E). Newly-differentiated odontoblast-like cells align along the pulp-dentin border. (F-H) Nestin-positive odontoblast-like cells are arranged beneath the tertiary dentin, and bone-like tissue is observed in the pulp floor. (G, H) Higher magnification of the consecutive section comparable to the boxed areas in (F). AB, alveolar bone; B, bone-like tissue; D, dentin; DP, dental pulp; OB, odontoblasts or odontoblast-like cells; PD, predentin; TD, tertiary dentin. Scale bars, 250 μm (A-C, E, F); 100 μm (D); 50 μm (H); 25 μm (Insets, G).

Fig. 2 H&E- (A, D, F), and nestin- (A, C, E), and TRAP-stained sections (B) in the replanted teeth at 1 (A, B, D) and 2 weeks (C, E, F) after the operations in Groups 2 (A-C) and 3 (D-F) and quantitative analysis of the percentage occurrence of root ankylosis at 1 (G) and 2 weeks (H). (A, D) Blood vessels are clearly observed in the

root pulp, and cell debris and scarce neutrophils remain in the pulpal horns. (Inset) Higher magnification of the consecutive section comparable to the boxed area in (A). Nestin immunoreaction is observed in the newly-differentiated odontoblast-like cells located along the pulp-dentin border and subodontoblastic layer. (B) TRAP-positive reactions are observed in the mesial side of the distal root. (C, E) Nestin immunoreaction is recognizable throughout the dental pulp. (Inset) Higher magnification of the boxed area in (E). Nestin-positive odontoblast-like cells are arranged beneath the tertiary dentin. (F) Ankylosis occurs over the whole root surface. (G, H) At postoperative Week 1, a significant difference is noticed between Group 1 and Group 2 as well as between Group 2 and Group 4 (G). At Week 2, the occurrence of root ankylosis in the 3Mix groups excluding Groups 2, 8, 10, and 12 is significantly higher than that in Group 1 (H). AB, alveolar bone; D, dentin; DP, dental pulp; OB, odontoblast-like cells; TD, tertiary dentin. Scale bars, 250 μm (A, C, D, E); 100 μm (B, F); 25 μm (Insets).

Fig. 3 H&E- (A, D-F, H-L), nestin- (C, D, G, K), and TRAP-stained sections (B) in the replanted teeth at 1 (A, B, D, J) and 2 weeks (C, E-I, K, L) after the operations in Groups 4 (A-C), 5 (D-I), 6 (J, K), and 7 (L). (A) 3Mix solution in Group 4 leads to the total degeneration of the dental pulp. (B) Intense TRAP activity is observed in the apical area of the mesial root. (C) Bone-like tissue is recognizable (arrows). (D, J) The dental pulp of Groups 5 and 6 includes abundant cell debris and scarce neutrophils, except for the apical pulp. (Inset) Higher magnification of the root pulp. Nestin-positive filamentous structures are detected. (E-I, K, L) A mixed form of tertiary dentin and bone-like tissue is recognized in Groups 5-7. Fibrous and/or bone-like tissues

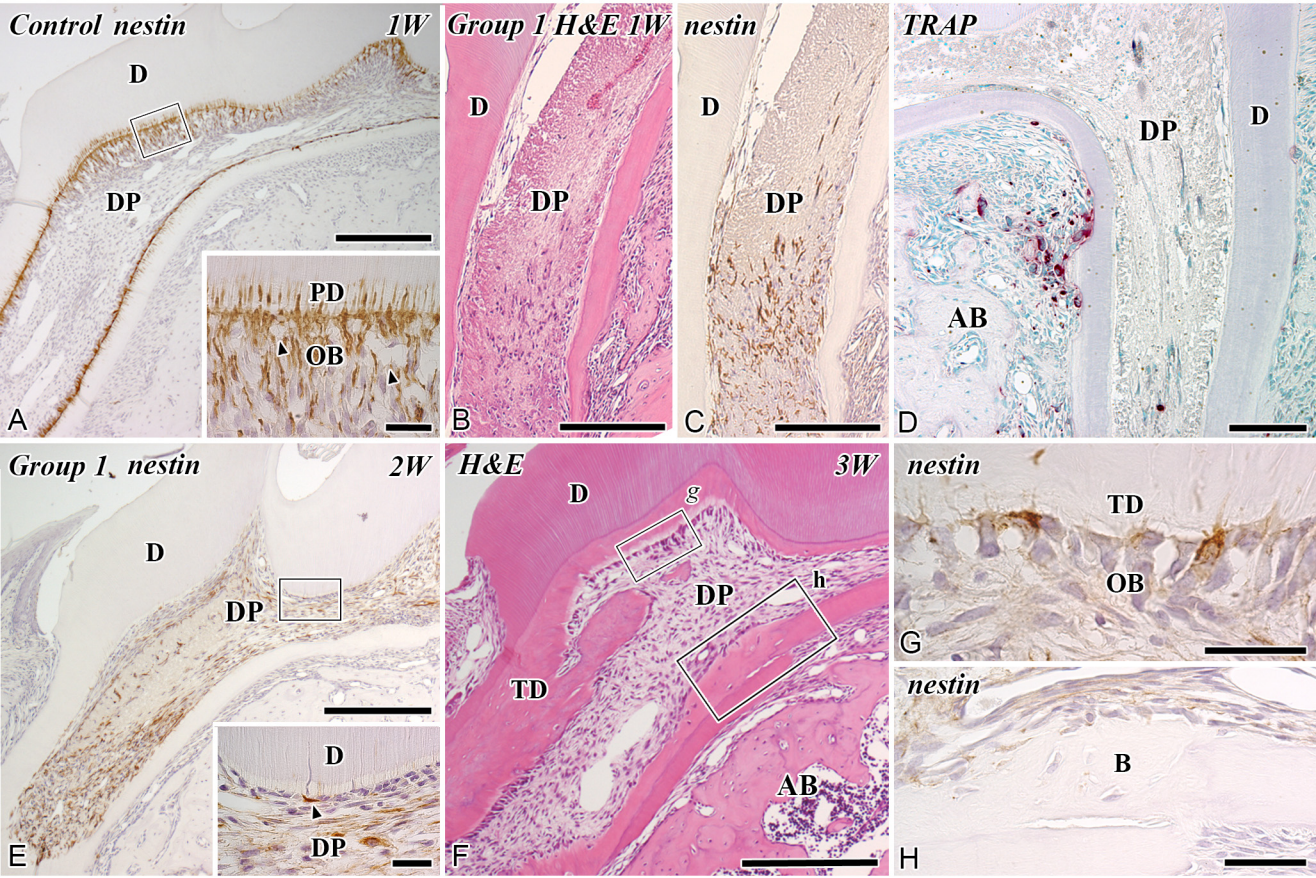
including bone marrow and expanded inflammatory reactions are also recognized in the dental pulp regardless of the 3Mix concentration. (F, H, I) Higher magnification of the boxed areas in (E). (G) Higher magnification of the pulp horn. AB, alveolar bone; B, bone-like tissue; D, dentin; DP, dental pulp; FH, fibrous tissue; OB, odontoblast-like cells; TD, tertiary dentin. Scale bars, 500 μm (E); 250 μm (A-D, J-L, Insets); 50 μm (F-I).

Fig. 4 Nestin- (A, B, E, F, H), H&E- (D, G), and TRAP-stained sections (C, I) in the replanted teeth at 1 (A, B, F) and 2 weeks (C-E, G-I) after the operations in Groups 10 (A-E) and 12 (F-I) and quantitative analysis of the percentage of TRAP-positive areas in Group 1 (J) and at 1 (K) and 2 weeks (L) in all Groups. (A, B) Nestin-positive immunoreactions were observed in the newly-differentiated odontoblast-like cells along the pulp-dentin border and subodontoblastic layer. (B) Higher magnification of the boxed area in (A). (C, J) TRAP activity is decreased in intensity at Week 2. (D, G) The occurrence of root ankylosis becomes improved. (E, H) The tertiary dentin is observed in the pulp tissue. (J) The statistical analysis of the percentage of TRAP-positive areas following tooth replantation in Group 1 shows a significant difference between Weeks 1 and 2. (K) At postoperative Week 1, a significant difference is noticed between Groups 3 and 4 as well as between Groups 4 and 5. (L) At Week 2, the occurrence of root resorption is consistently decreased in all groups. AB, alveolar bone; D, dentin; DP, dental pulp; OB, odontoblast-like cells; TD, tertiary dentin. Scale bars, 250 μm (A, C-I); 25 μm (B).

Fig. 5 Ki-67-stained sections (A-I) in the replanted teeth at 1 (A-D), 2 (E-I), and 3 weeks (F) after the operations in Groups 1 (A, E, F), 2 (B, G), 5 (C, H), and 10 (D, I) and

quantitative analysis of the number of Ki-67-positive cells/3.4 x 10^4 μm^2 in Group 1 (J) and at 1 (K) and 2 weeks (L) in all Groups. (A, E, F, J) Active cell proliferation takes place at Week 2 after operation and subsequently the proliferative cells are decreased in number at Week 3. (B-D, G-I, K, L) In the groups using 3Mix standard concentration, the cell proliferation is significantly accelerated at Week 1 compared with that in the control or the 3Mix groups at reduced or higher concentration. At Week 2, there is a significant reduction in the number of proliferative cells between the control and the 3Mix groups. DP, dental pulp. Scale bars, 50 μm.

Fig. 6 TUNEL-stained sections (A-I) in the replanted teeth at 1 (A-D), 2 (E-I), and 3 weeks (F) after the operations in Groups 1 (A, E, F), 2 (B, G), 5 (C, H), and 10 (D, I) and quantitative analysis of the number of TUNEL-positive cells/3.4 x 10^4 μm^2 in the coronal (J, L) and root pulp (K, M) at 1 (J, K) and 2 weeks (L, M) in all Groups. (A-C) Apoptotic activity is decreased in the coronal pulp at Weeks 2 and 3 in Group 1. (B-D, J, K) At Week 1, an increase in the apoptotic activity is observed in the coronal and root pulp of 3Mix groups. Group 3 shows the lowest average of TUNEL-positive cells, and significant differences are found between this group and Groups 6-12 as well as between Group 1 and Groups 2 and 3 in the coronal and/or root pulp. (G-I, L, M) The average of TUNEL-positive cells sharply decreased at Week 2 in the coronal and/or root pulp in all groups except for Groups 4 and 12. Significant differences are observed between Group 12 and Groups 5, 8, 10, and 11 in the coronal pulp and between Groups 4 and 12 and the other groups in the root pulp. DP, dental pulp. Scale bars, 50 μm.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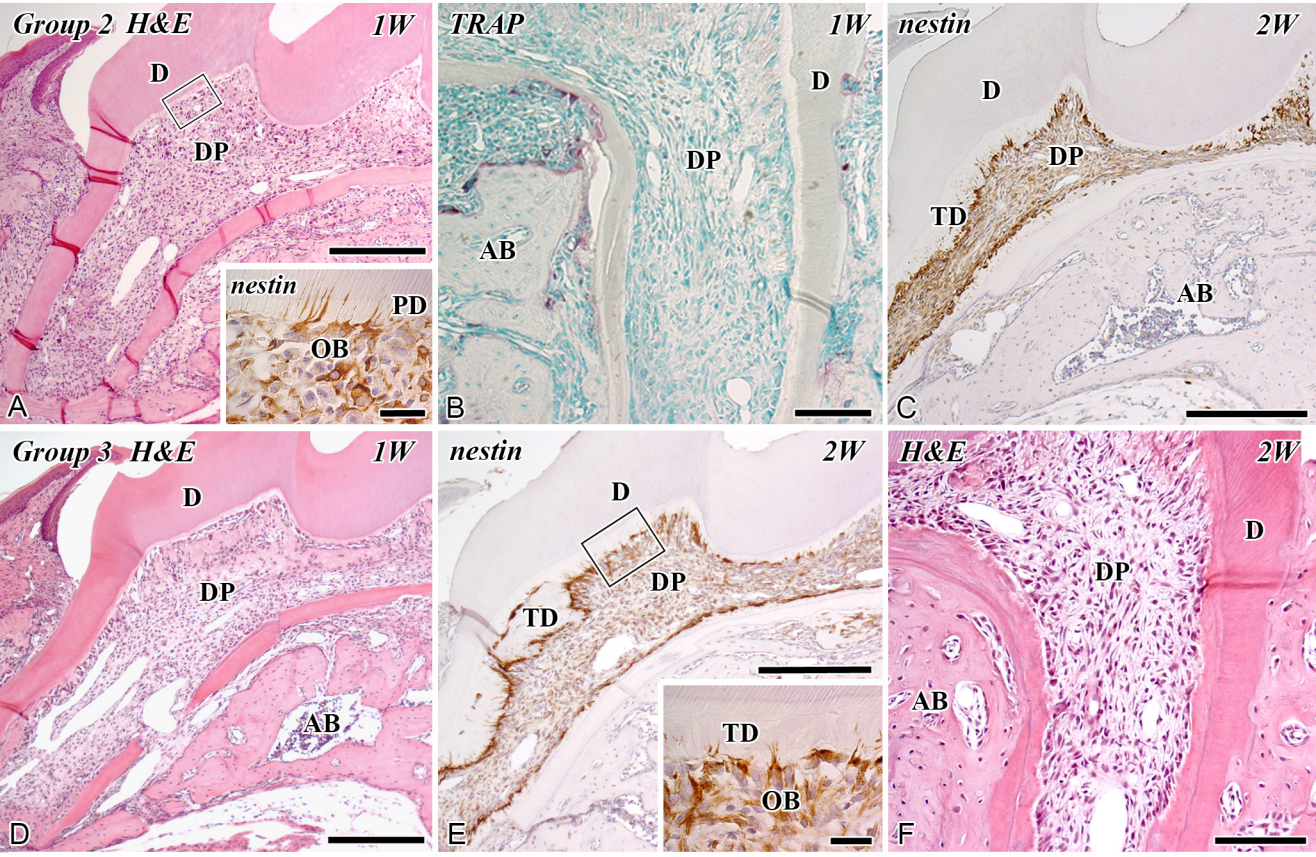
2W
D
DP
D
DP
E
H&E
3W
D
g
DP
h
TD
AB
F
nestin
TD
OB
G
nestin
B
H

Figure 1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D
DP
nestin
PD
OB
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TRAP
1W
D
DP
AB
B
nestin
2W
D
DP
TD
AB
C
Group 3 H&E
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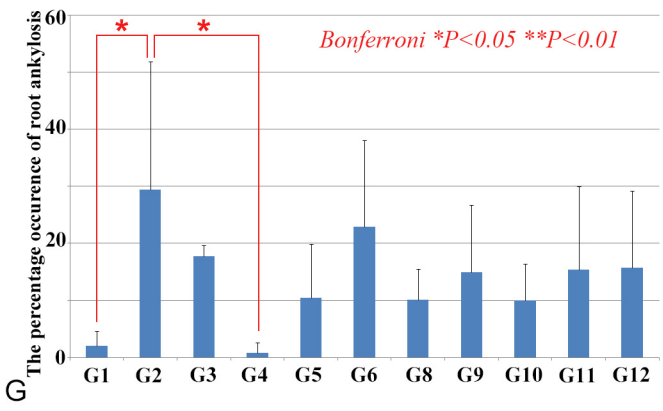
2W
D
DP
AB
F


Bonferroni *P<0.05 **P<0.01
The percentage occurence of root ankylosis
60
40
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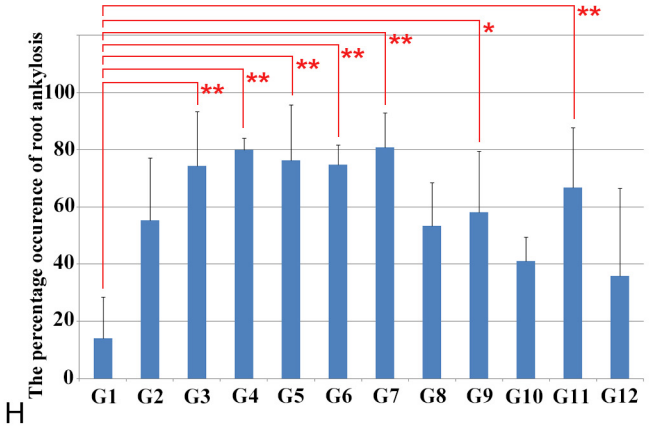
G1
G2
G3
G4
G5
G6
G8
G9
G10
G11
G12
G


The percentage occurence of root ankylosis
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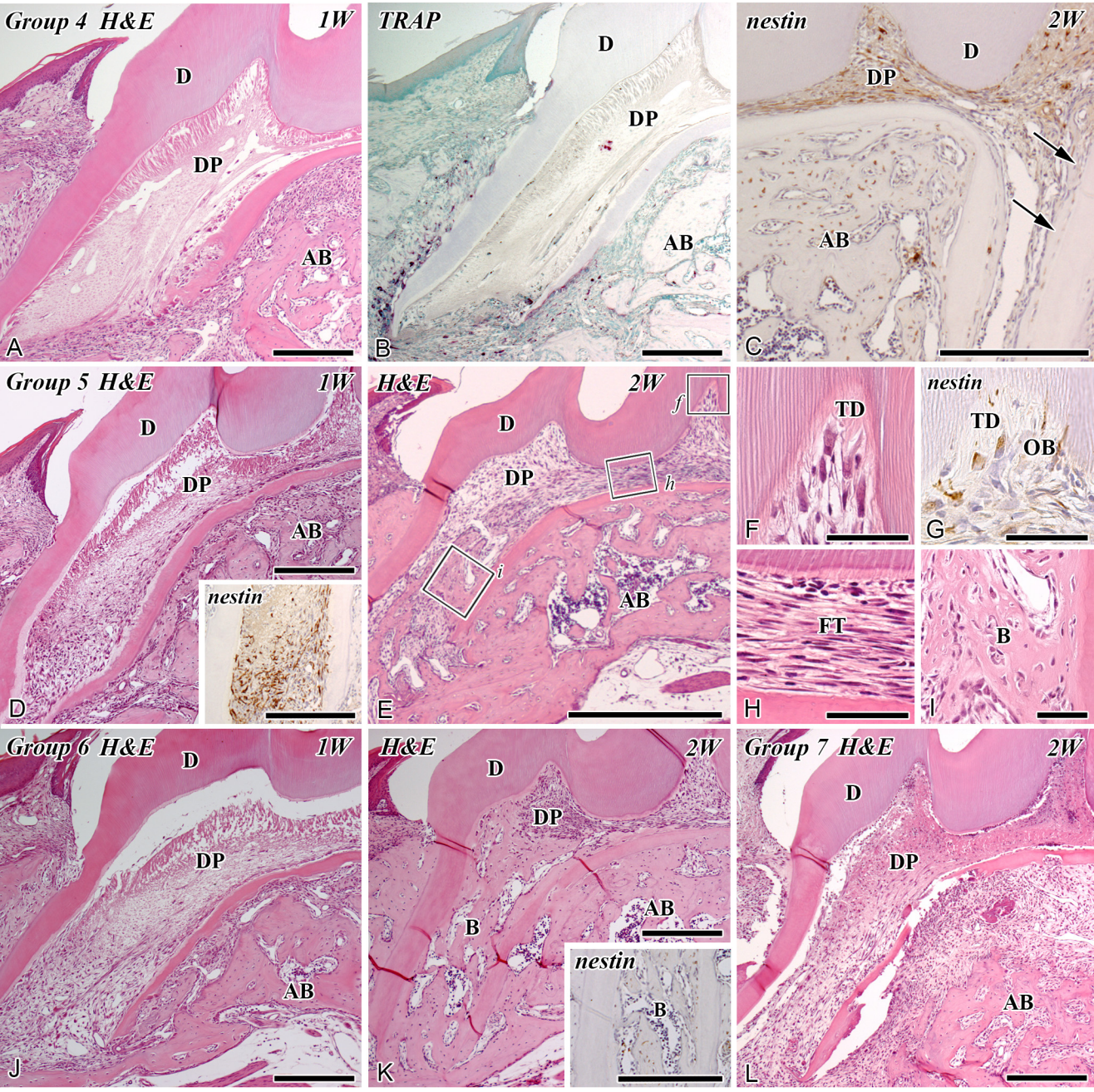
80
60
40
20
0
G1
G2
G3
G4
G5
G6
G7
G8
G9
G10
G11
G12
H

Figure 2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AB
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TRAP
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DP
AB
B
nestin
2W
D
DP
AB
C
Group 5 H&E
1W
D
DP
AB
nestin
D
H&E
2W
f
D
DP
h
i
AB
E
TD
F
nestin
TD
OB
G
FT
H
B
I
Group 6 H&E
1W
D
DP
AB
J
H&E
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D
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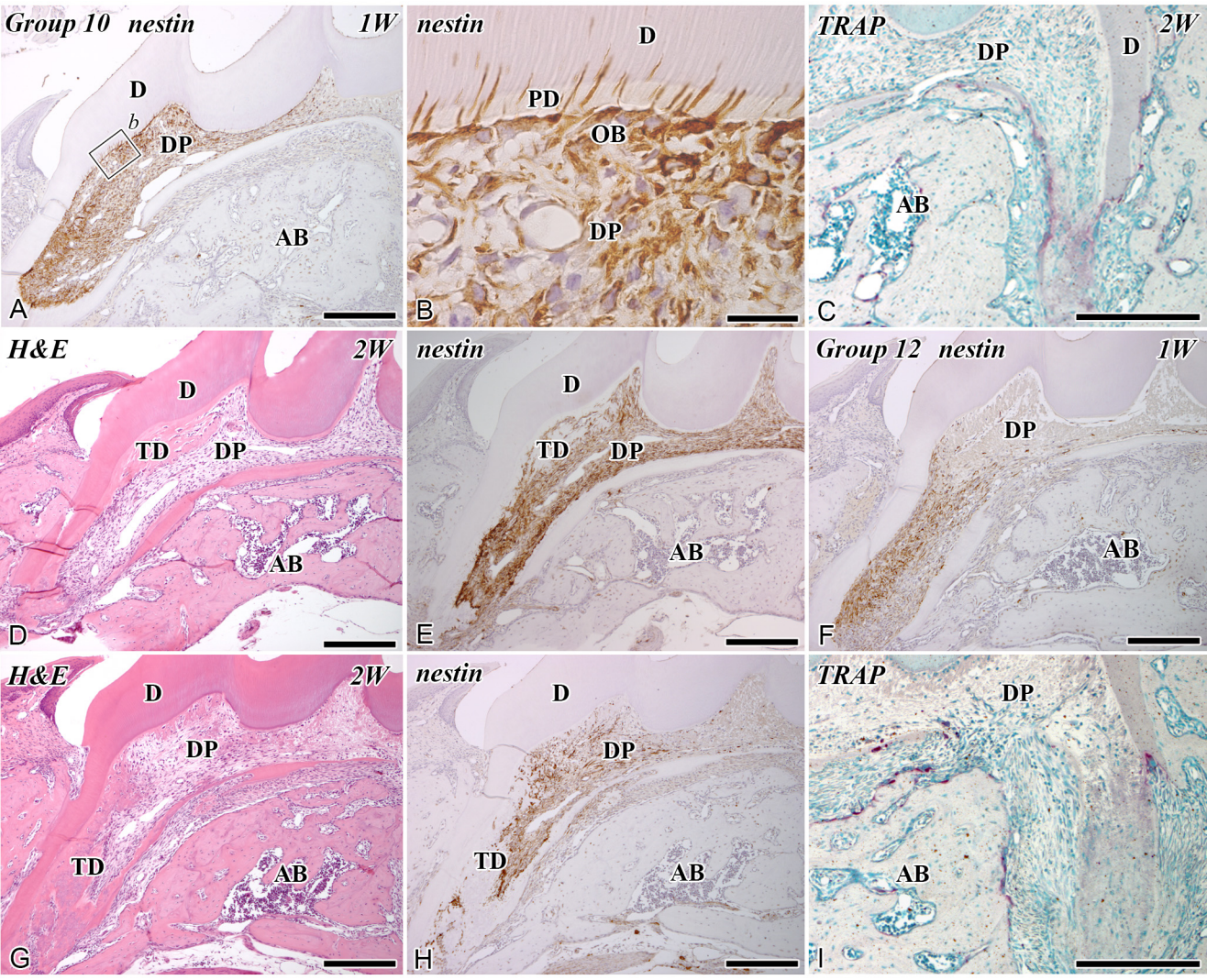
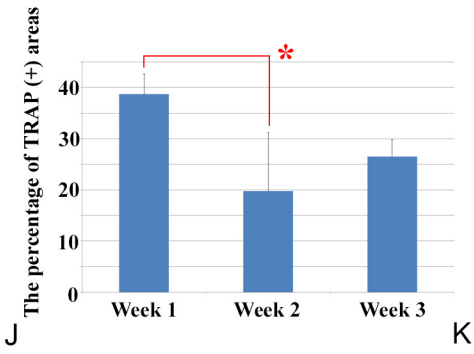
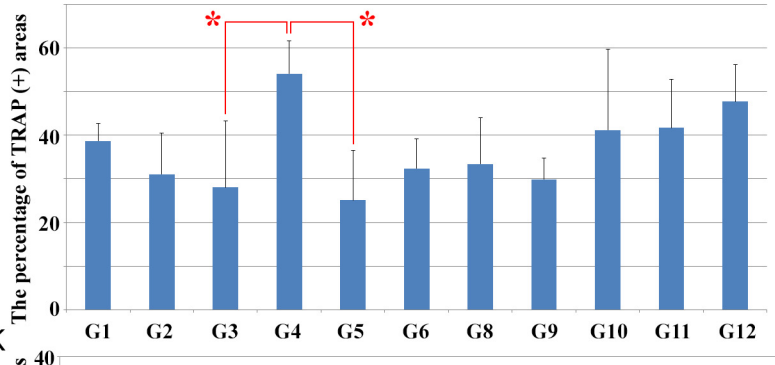
B
AB
nestin
B
K
Group 7 H&E
2W
D
DP
AB
L

Figure 3







* *Bonferroni P*<0.05

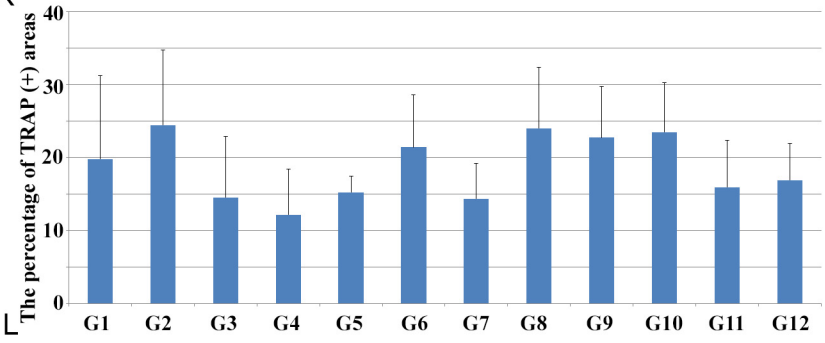
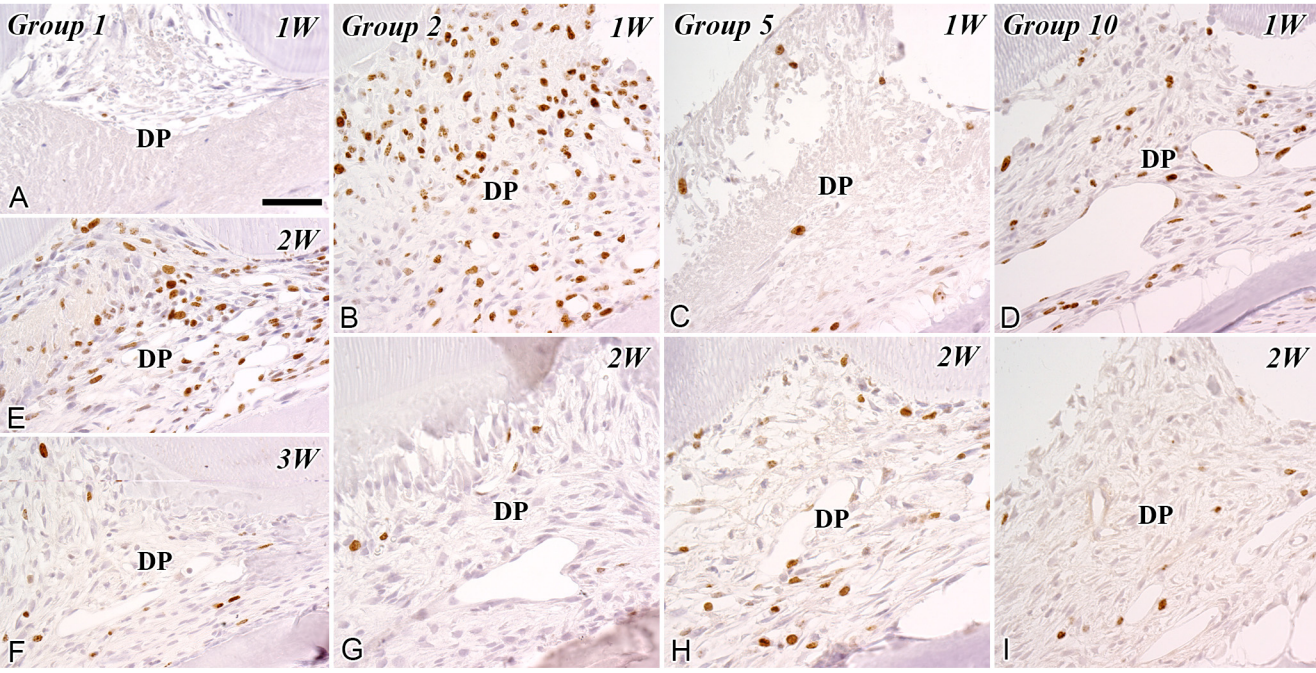


Figure 4


Group 1
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DP
A
Group 2
1W
DP
B
Group 5
1W
DP
C
Group 10
1W
DP
D
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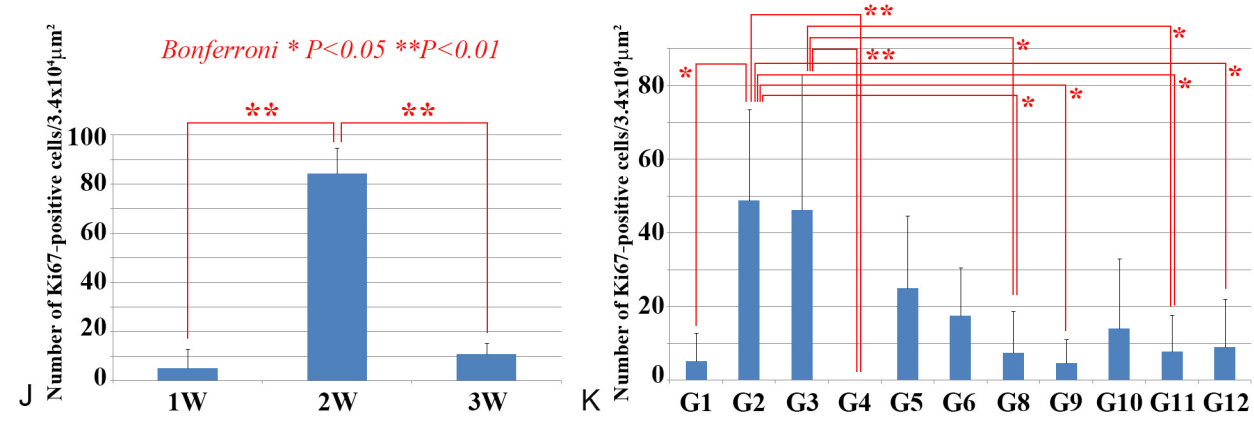
2W
DP
H
2W
DP
I
3W
DP
F


Bonferroni * P<0.05 **P<0.01
Number of Ki67-positive cells/3.4x10⁴μm²
J
1W
2W
3W
K
G1
G2
G3
G4
G5
G6
G8
G9
G10
G11
G12

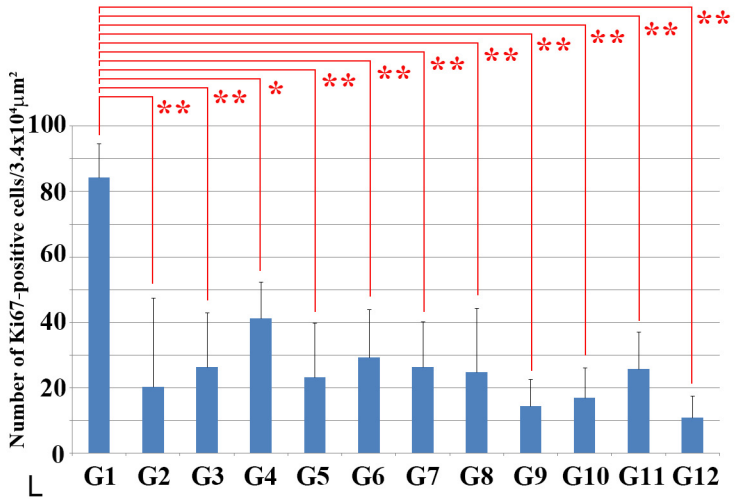
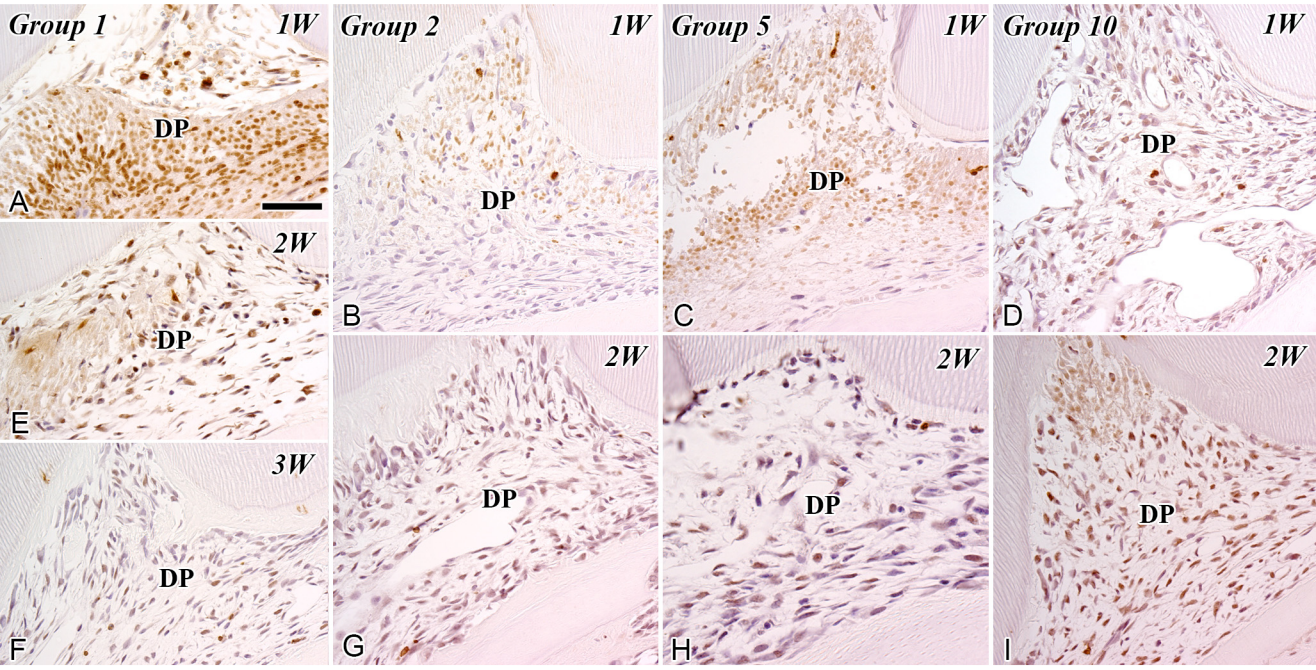

Number of Ki67-positive cells/3.4x10⁴μm²
L
G1
G2
G3
G4
G5
G6
G7
G8
G9
G10
G11
G12

Figure 5


Group 1
1W
DP
A
Group 2
1W
DP
B
Group 5
1W
DP
C
Group 10
1W
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D
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2W
DP
G
2W
DP
H
2W
DP
I
3W
DP
F

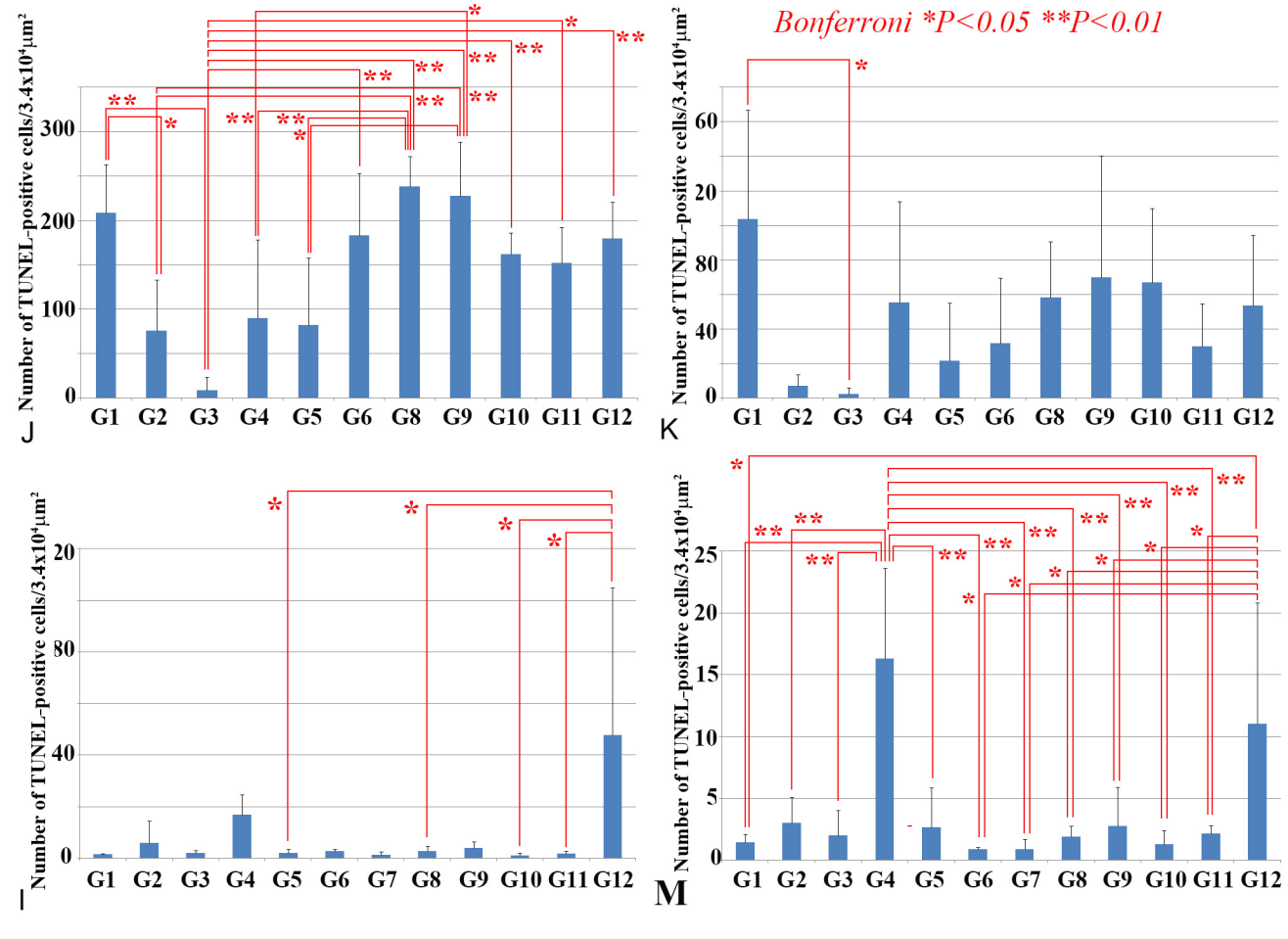
Bonferroni *P<0.05 **P<0.01
Number of TUNEL-positive cells/3.4x10⁴μm²
G1 G2 G3 G4 G5 G6 G8 G9 G10 G11 G12
J
K
G1 G2 G3 G4 G5 G6 G7 G8 G9 G10 G11 G12
I
M

Figure 6

Effects of a Triple Antibiotic Solution on Pulpal Dynamics Following Intentionally Delayed Tooth Replantation in Mice

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TEL +81-25-227-2812
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Abstract

Introduction: This study analyzed the detailed biological events underlying pulpal dynamics evoked by 3Mix (the mixture of ciprofloxacin, metronidazole, and minocycline) solution following intentionally delayed tooth replantation, since 3Mix improves pulpal healing after tooth injuries. **Methods:** The maxillary first molars of 3-week-old mice were extracted and immersed in 3Mix solution for 30 minutes, in comparison to phosphate buffer saline (PBS) alone. Cell proliferation, apoptosis, and differentiation were assessed in extracted/replanted teeth during Days 0-14 using immunohistochemistry, apoptosis assay, and reverse transcriptase-polymerase chain reaction. **Results:** 3Mix solution accelerated odontoblast differentiation in the coronal pulp at Day 7 and tertiary dentin formation at Day 14, whereas the regenerative process was delayed in the PBS group. Cell proliferation and apoptosis occurred in the pulp of the 3Mix group during Days 5-7 and subsequently decreased from Days 7-14. At Day 5, *dentin sialophosphoprotein* and *nestin* were first recovered in the 3Mix group, while expression levels for *alkaline phosphatase*, *osteopontin*, and *osteocalcin* increased in the PBS group. The expression levels for *octamer-binding factor 3/4A* and *3/4B* reached maximum at Day 1 and were sharply decreased at Day 3 in both groups. High expression levels of *Cd11c* were first observed in the 3Mix group at Day 1, and later at Days 5 and 7. **Conclusions:** The results suggest that the application of 3Mix may suppress osteoblast differentiation by migration of dendritic cells to the injury site and via the activation of stem/progenitor cells, resulting in the acceleration of odontoblast-like cell differentiation.

Key Words

Antimicrobial, apoptosis, cell differentiation, cell proliferation, dental pulp, odontoblasts, regeneration, tooth replantation, ICR mice

Dental pulp is a connective tissue uniquely situated within the rigid encasement of mineralized dentin (1). Dental pulp not only provides nutritional and sensory properties to dentin, but also has its own reparative capacity. Dental caries, attrition, abrasion, or restorative treatments, such as cavity preparation, lead to local dentin formation in the pulp chamber (2). Tooth injuries such as cavity preparation and tooth replantation induce destructive changes in the odontoblasts at the affected site and an acute inflammatory reaction (3-5). Following odontoblast cell death, stem or progenitor cells residing in the adult dental pulp replace the degenerated cells to differentiate into odontoblast-like cells. Numerous clinical studies have shown that pulp may heal after tooth injuries even if a complete severance of the neurovascular supply takes place. Hence, the mode of pulpal responses may vary according to the origin of the progenitor cells involved and also by the extent of tissue injury (6).

Tooth replantation, defined as a therapeutic method in which the avulsed or extracted tooth is replaced in its original socket, has become widely utilized in clinical dentistry. However, this procedure causes interruption of the nerve and vascular supply to the dental pulp. Pulpal responses to tooth replantation can be divided into at least two types of healing patterns: dentin and/or bone-like tissue formation in the pulp tissue (7-12). Although the mechanisms for determining the divergent healing processes after tooth replantation remain to be fully clarified, they may be directly linked to the death or survival of odontoblast-lineage cells. Intentionally prolonged operating time for tooth replantation induces total death of odontoblast-lineage cells due to the lack of a properly oxygenated medium (12). The presence of bacteria in the root surface as well as bacteria associated with the blood clot in the socket appears to worsen the chances of a successful outcome (6). Therefore, the establishment of an adequate environment that regulates these external factors seems to be critical for the regeneration of the afflicted dental pulp following tooth replantation.

The combination of antibacterial drugs such as ciprofloxacin, metronidazole, and minocycline–referred to as 3Mix–is currently widely used as an intracanal medicament in the regenerative endodontic/revascularization procedures for the treatment of immature teeth with pulpal necrosis (13). The high antibacterial effect and biological compatibility of 3Mix as well as the clinical outcomes have been reported elsewhere (14-31). However,

there are some drawbacks related to the clinical application of 3Mix (32). These include the risk of developing antibiotic resistance in certain strains of root canal bacteria (33, 34), the fear of triggering an allergic reaction in sensitive patients (35-37), and the discoloration of the tooth crown caused by tetracycline (38-41). Recently, the use of calcium hydroxide has been reported in regenerative endodontic procedures with successful outcomes (42, 43). Despite of the several concerns raised about the use of calcium hydroxide in revascularization (13, 44, 45), this could become a safe alternative in patients with sensitivity to one of the 3Mix components. Our recent paper demonstrated the usefulness of 3Mix antibiotic solution in improving the healing process of intentionally delayed replanted teeth in mice. 3Mix solution effectively accelerated the pulpal regeneration process of replanted teeth in concentration- and immersion-time-dependent manners (46). Although the pulpal healing patterns have already been investigated in terms of cell proliferation and apoptosis in different types of 3Mix solutions, more detailed analysis of the biological events underlying the pulpal dynamics evoked by 3Mix solution is necessary for the proper understanding of its implications for dental therapy at the molecular level. This study analyzed the detailed biological events underlying the pulpal dynamics evoked by 3Mix solution following intentionally delayed tooth replantation. The progression of the pulpal healing was assessed by immunohistochemistry for nestin and Ki-67, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. Furthermore, we analyzed the gene expression profile by reverse transcription-polymerase chain reaction (RT-PCR) using *dentin sialophosphoprotein* (*Dspp*), *nestin*, *alkaline phosphatase* (*Alp*), *cyclinD1*, *caspase3*, *osteopontin* (*Opn*), *osteocalcin* (*Ocn*), *octamer-binding factor (Oct) 3/4A, Oct3/4B*, and *Cd11c* primers on each observation point.

Materials and Methods

Tooth replantation

All experiments were reviewed by the Committee on the Guidelines for Animal Experimentation of Niigata University and performed according to the recommendations or under the conditions proposed by the Committee. One hundred and five Crlj:CD1 (ICR) mice (3 weeks old) were divided into two groups: (1) using phosphate buffer saline (PBS) solution and (2) using 3Mix solution (ciprofloxacin 0.01 mg/mL, metronidazole 0.02 mg/mL, and minocycline 0.01 mg/mL diluted in distilled water). Under anesthesia with an intraperitoneal injection (IP) of chloral hydrate (maximum dose of 350 mg/kg), the maxillary right first molars of each animal were extracted and then repositioned in their original socket after immersion for 30 minutes in PBS or 3Mix solution. The current concentration of 3Mix solution as well as the immersion time was established based on the results provided in our previous publication (46).

Tissue preparation

Materials were collected from groups of 8-9 animals immediately after immersion (n = PBS: 8, 3Mix: 8) or at 1 (n = PBS: 9, 3Mix: 9), 3 (n = PBS: 8, 3Mix: 9), 5 (n = PBS: 9, 3Mix: 8), 7 (n = PBS: 9, 3Mix: 10) and 14 (n = PBS: 9, 3Mix: 9) days after tooth replantation. At each stage, the animals were transcardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia by IP injection of chloral hydrate. The maxillae were removed *en bloc* and immersed in the same fixative for an additional 12 hours at 4°C. Following decalcification in Morse's solution (10% sodium citrate and 22.5% formic acid) for 4-6 days at 4°C, the specimens were processed for embedding in paraffin, and cut sagittally at a thickness of 4 μm. Sections were processed for hematoxylin & eosin (H&E) staining and immunohistochemistry.

Immunohistochemistry and TUNEL assay

Immunohistochemistry was conducted using a mouse anti-rat anti-nestin monoclonal antibody diluted 1:100 (Millipore, Temecula, CA; catalog number: MAB353), and a rat anti-mouse Ki-67 monoclonal antibody diluted 1:100 (Dako Japan, Tokyo, Japan; catalog number: M7249). The Envision + Horseradish Peroxidase System (Dako Japan, catalog number: K5027) and the avidin-biotin peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) method using biotinylated anti-rat immunoglobulin G (Vector Laboratories; catalog number: BA-4000) were used for nestin and Ki-67 immunohistochemistry, respectively. For the final visualization of the sections, 0.05 mol/L Tris-HCl buffer (pH=7.6) containing 0.04% 3-3'-diaminobenzidine tetrahydrochloride and 0.0002% H_2O_2 was used. The immunostained sections were counterstained with hematoxylin. Apoptosis was quantified by TUNEL with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore; catalog number: S7100). Negative controls were performed by replacing the primary antibodies or terminal deoxynucleotidyl transferase (TdT) enzyme with PBS.

RT-PCR

Total RNA was isolated from the dental pulp tissue of replanted teeth at each observation stage (Days 0-14) using the Trisol system (Invitrogen; Life Technologies, Carlsbad, CA). cDNA was synthesized from the RNA with the SuperScript First-Strand Synthesis System (Invitrogen). The sequences of the PCR primer pairs for *β-actin*, *Dspp*, *nestin, cyclinD1*, *caspase3*, *Alp*, *Opn, Ocn, Oct3/4A, Oct3/4B* and *Cd11c* are listed in Supplemental Table 1. The thermocycling protocol during 30 or 34 amplification cycles was as follows: denaturation 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The amplified DNA fragments were separated by electrophoresis on 2% agarose gels. The relative densities of each band against those of *β-actin* on monochrome photographs were determined with Image J software (Image J 1.45, NIH, USA).

Cell counting

The numbers of Ki-67- and TUNEL-positive cells in the coronal and root pulp of each specimen (3.4 x 10^4 grid was selected) were calculated separately. All data were presented as the mean and standard deviation (SD) of each group. Furthermore, the number of cells in the coronal and root pulp at different times after tooth extraction or replantation (Days 0-14) and between groups were compared by Bonferroni's test (one-way analysis of variance; ANOVA) using statistical software (SPSS 16.0J for Windows; SPSS Japan, Tokyo, Japan).

Statistical analysis of nestin-positive perimeters and newly-formed hard tissue areas in the dental pulp following tooth replantation

The percentage of nestin-positive perimeters in the total perimeter of the pulp-dentin border was calculated at Days 7 and 14 using Image J software (Image J 1.45s, NIH, USA). Similarly, the percentage of newly-formed hard tissue areas in the total area of the pulp chamber was quantified at Day 14 using WinRoof image processing software (WinRoof Version 7.4, Mitani Corporation, Japan). All data were presented as the mean and SD of each group and analyzed by Student's *t*-test using statistical software (SPSS 16.0J for Windows).

Results

Nestin Immunoreaction in the Dental Pulp of Controls

Coronal odontoblasts showed pseudostratified features and blood capillaries were located in the odontoblast layer (Fig. 1*A*). Intense immunoreactivity for nestin was recognized in coronal (Supplementary Fig. 1*A*) and root odontoblasts (data not shown) within their cell bodies and processes.

Histological and Nestin Immunohistochemical Changes in the Dental Pulp of the PBS Group

In the PBS group, the odontoblasts maintained their pseudostratified features after 30-minute-immersion. Intense nestin immunoreactivity was observed in the odontoblast layer (Supplementary Fig. 1*B*). At Day 1, the dental pulp was occupied by numerous inflammatory cells such as neutrophils. The odontoblasts showed degenerative features and blood capillaries disappeared from the odontoblast layer (Supplementary Fig. 1*D*). At Day 3, the pulpal tissue contained eosinophilic amorphous matrices including many erythrocytes with a decreased number of inflammatory cells (Supplementary Fig. 1*E*). At Day 5, nestin-positive filamentous structures were first recognized in the apical third of the root pulp (Fig. 1*E* and 1*F*). At Day 7, nestin-positive filamentous structures were still observed in the root pulp (Supplementary Fig. 1*H* and 1*J*). The coronal pulp began to show some scattered nestin-positive cells (Fig. 1*I*). At Day 14, newly differentiated odontoblast-like cells were clearly aligned along the pulp-dentin border of the coronal pulp (Fig. 1*K*). Other pulpal cells also showed a positive reaction for nestin, showing the ongoing healing process. Tertiary dentin was observed in the root pulp of some samples, as revealed by nestin immunostaining (data not shown). The percentages of nestin-positive perimeters in the total perimeter of the pulp-dentin border and newly-formed hard tissue areas in the total area of the pulp chamber are shown in Fig. 1*M* and 1*N*, respectively.

Histological and Nestin Immunohistochemical Changes in the Dental Pulp of 3Mix Group

3Mix solution accelerated odontoblast differentiation and tertiary dentin deposition following intentionally delayed tooth replantation. Thirty minutes after immersion in the experimental solution, odontoblasts maintained their pseudostratified features (Fig. 1*B*), and nestin immunoreaction remained in the odontoblast layer (Supplementary Fig. 1*C*). During Days 1-3, the histological changes in the 3Mix group were the same as those in the PBS group (Fig. 1*C* and 1*D*). In some samples, ascending nestin-positive filamentous structures were observed in the apical third of the root dental pulp at Day 3 (data not shown). At Day 5, the dental pulp in the 3Mix group began its regenerative process from the apical area onwards. Although the coronal pulp still showed degenerative features, the root pulp began to exhibit normal characteristics and the revascularization occurred in this area. In addition, nestin immunoreactivity revealed the presence of newly differentiated odontoblast-like cells aligning on the pulp-dentin border in the apical third of the root pulp (Fig. 1*G* and 1*H*) and some nestin-positive filamentous structures were observed in the coronal pulp (Supplementary Fig. 1*F* and 1*G*). Seven days after replantation, 3Mix solution accelerated the odontoblast differentiation in the coronal pulp, and newly differentiated odontoblast-like cells with their odontoblast processes beneath the predentin showed a positive reaction for nestin. Other pulpal cells undergoing the regenerative processes also showed a positive reaction for nestin (Fig. 1*J*). In the root pulp, an intense nestin-immunoreaction was observed in the differentiated odontoblast-like and pulpal cells (Supplementary Fig. 1*I* and 1*K*). Consequently, a considerable amount of tertiary dentin was observed in the coronal and root pulp at Day 14. Some cellular inclusions were occasionally observed in the newly-secreted matrices, particularly at the coronal portion. The percentages of nestin-positive perimeters in the total perimeter of the pulp-dentin border and newly-formed hard tissue areas in the total area of the pulp chamber are shown in Fig. 1*M* and 1*N*, respectively. There were significant differences in nestin-positive perimeters between both groups at Day 7, in addition to different time points in both groups (Fig. 1*M*). There was a significant difference in newly-formed hard tissue areas between both groups at Day 14 (Fig. 1*N*).

Cell Proliferation in the Dental Pulp of 3Mix and PBS Groups Using Ki-67 Immunohistochemistry

The analysis of cell proliferation by Ki-67 immunostaining showed that in the PBS group the most active cell proliferation took place at Day 14 in the coronal and root dental pulp. In contrast, in the 3Mix group, the number of proliferative cells began to increase from Days 3 and 5 in the root and coronal dental pulp, respectively, reaching its peak around Day 7. A significant difference was found in the root dental pulp between 3Mix and PBS groups at Day 7. Proliferative cells decreased in number in the 3Mix group at Day 14 (Fig. 2*A*, 2*B*, 2*J*, 2*K*, 2*L*, and 2*M*).

Apoptosis in the Dental Pulp of 3Mix and PBS Groups Using TUNEL Assay

The apoptotic activity was analyzed by TUNEL assay. In both groups, TUNEL-positive cells were progressively decreased in number during Days 5-14 in the coronal pulp. An increasing number of apoptotic cells were observed during Days 5-7. Significant differences between 3Mix and PBS groups were found in the root pulp at Days 5 and 7. The lowest number of TUNEL-positive cells was observed in the dental pulp of the 3Mix group at Day 14 (Fig. 2*C*, 2*D*, 2*E*, 2*F*, 2*N*, 2*O*, 2*P*, and 2*Q*).

Related Gene Expression Levels in the PBS and 3Mix Groups during the Pulpal Healing Process

The gene expression was analyzed by RT-PCR using *Dspp*, *nestin*, *cyclinD1*, *caspase3*, *Alp*, *Opn*, *Ocn, Oct3/4A, Oct3/4B,* and *Cd11c* primers during the pulpal healing process in the PBS and 3Mix groups. Although there were no significant differences ($P > .05$) in both groups for the selected primers, a favorable tendency for the 3Mix group was noted along the chronological changes during the pulpal healing process. During Days 0-3, the mRNA levels of the odontoblast differentiation markers *Dspp* and *nestin* were

dramatically decreased in both groups. Their expressions first recovered at Day 5 in the 3Mix group, while they were found to increase from Day 7 onwards in the PBS group (Fig. 3*A*, 3*B*, and 3*C*). In addition, the expression levels of osteoblast differentiation markers *Alp*, *Opn* and *Ocn* increased in the PBS group at Day 5 (Fig. 3*A*, 3*D*, and 3*E*). The gene expression of *cyclinD1* in the 3Mix and PBS groups was considerably increased in intensity during Days 1-7, being sharply decreased by Day 14 only in the 3Mix group, whereas the PBS group maintained the high expression level (Fig. 2*G* and 2*H*). The *caspase3* mRNA levels were markedly high in the PBS group at Day 1, and subsequently these levels decreased in both groups (Fig. 2*G* and 2*I*). The expression levels for *Oct3/4A* and *Oct3/4B* reached maximum at Day 1 and were sharply decreased at Day 3 in the PBS and 3Mix groups (Fig. 3*A* and 3*F*). The expression of *Cd11c* was observed to reach its highest level at Days 1, 5, and 7 in the 3Mix group. In contrast, the expression levels in the PBS group were consistently increased in intensity during Days 1-7 (Fig. 3*A* and 3*G*).

Discussion

The present study clearly demonstrated that 3Mix antibiotic solution accelerates odontoblast-like cell differentiation and tertiary dentin formation in the dental pulp of intentionally delayed replanted teeth of mice. This study also provided new findings regarding the chronological changes in the gene expression during the pulpal healing process, although no significant difference was found between 3Mix and PBS groups. The healing process following luxation injuries begins apically and moves coronally as stated below in detail. For the analysis of the gene expression, the coronal and root pulp failed to be separated. Thus, the remnant necrotic coronal pulp could mask the original results during pulp healing, resulting in the lack of significant differences in the current work.

In a previous study, we successfully established an animal model using mice for the evaluation of therapeutic reagents on replanted teeth, to clarify the optimal conditions such as the immersion-time and the concentration of 3Mix antibiotic solution (46). Higher amounts of antibiotics seem to be detrimental to the survival of odontoblast-like cells of replanted teeth, whereas reduced concentrations of the 3Mix solution caused an increase in the mixed form of tertiary dentin and bone-like tissue in addition to the fibrous tissue or expanded inflammatory reactions in replanted teeth. Ruparel et al. (2012) has shown that high concentrations of ciprofloxacin, metronidazole, and minocycline in the triple antibiotic paste (TAP) have a detrimental effect on the survival of stem cells from the apical papilla (SCAP) (47-49), whereas low concentration such as 0.01 or 0.1 mg/mL have no direct effect on their survival and proliferation (24). A recent publication showed that the cytotoxicity of 3Mix and each antibiotic (except metronidazole) on human dental pulp cells and SCAP increased in concentration- and time-dependent manners (28). Thus, the healing patterns of the replanted teeth are affected by variations in the concentration of the antibiotic solution. Tooth immersion in 3Mix solution for 60 minutes, even at a low dose, modifies the dental pulp homeostasis, inducing the appearance of pulp stones (data not shown). Taking these findings together, the current concentration of 3Mix solution and the immersion time of 30 minutes used for the completion of this study are appropriate to comparatively analyze the responses of the dental pulp in both groups. 3Mix solution did

not show any deleterious effect on the pulpal cells, probably favoring the viability of dental pulp stem/progenitors cells including SCAP following tooth replantation.

Active cell proliferation was observed from Day 3 in the root dental pulp in the 3Mix group in the present study. The healing process following luxation injuries begins apically and moves coronally, and the phenomenon is highly dependent on the stage of root development of replanted teeth (50, 51). In contrast, in the PBS group active cell proliferation took place at Day 14 after operation. Since extensive cell proliferation in the dental pulp of injured teeth is expected to follow the cell division of adult stem cells (52, 53), the findings suggest that there could be a synergistic relationship between the effect of 3Mix solution and the dental pulp stem/progenitor cells including SCAP surviving in the root pulp after tooth replantation. Interestingly, at Day 14 the proliferative activity was sharply decreased in the 3Mix group, presumably due to the establishment of reparative dentin formation. Regarding the apoptotic activity in the replanted teeth, high levels of *caspase3* transcripts were observed at Days 0 and 1. The histological data of the TUNEL-positive cells did not precisely correlate with the RT-PCR data during Days 0-3. This could be explained by the evidence that the TUNEL assay recognizes the DNA fragments of cells undergoing the apoptotic process. In the current results, the number of TUNEL-positive cells progressively increased during Days 0-5, particularly in the coronal pulp. Cell apoptosis precedes the regeneration process including cell renewal and differentiation (54, 55). The current results clearly demonstrate that the timing between apoptosis and cell proliferation is well correlated especially in the 3Mix group. Thus, following the decrease of extensive apoptosis, dental pulp cells begin to actively proliferate, leading to pulpal regeneration.

The expression of *nestin* and *Dspp* mRNA levels correlated with the histological data during the chronological changes of the pulpal healing process. This evidence supports the use of nestin as a valuable odontoblast differentiation marker (56). Nestin-positive newly differentiated odontoblast-like cells were aligned in the root pulp in the 3Mix group at Day 5. Interestingly, at this stage, the mRNA levels of the bone-lineage related markers such as *Alp, Opn*, and *Ocn* showed an increased tendency in the PBS group. As previously reported, in the degenerated dental pulp tissue, numerous osteoclast-lineage cells may

appear in the pulp chamber and make contact with mesenchymal cells, which may differentiate into osteoblasts and deposit the bone-like matrix (11). Therefore, these results suggest that the treatment with 3Mix solution may suppress the osteoblast differentiation pathway in the dental pulp of severely injured teeth. In addition, a recent study has shown that minocycline helps to convert the osteoclastic-differentiation pathway of progenitor cells to produce dendritic cell-like cells (57, 58). Dendritic cells initiate and regulate the highly pathogen-specific adaptive immune responses (59, 60). Moreover, it has been extensively reported that the recruitment of dendritic cells along the pulp-dentin border precedes the appearance of newly differentiated odontoblast-like cells (8, 31, 61, 62). In the current study, high expression levels of *Cd11c* mRNA, a marker for dendritic cells, were first observed in the 3Mix group at Day 1, and later at Days 5 and 7; whereas in the PBS group, these levels were progressively increased during Days 1-7. Hence, 3Mix solution may promote the migration of dendritic cells in the dental pulp shortly after repositioning the tooth into the alveolar socket.

Oct3/4 is a key regulator in maintaining the pluripotency and self-renewal properties of embryonic stem cells (63, 64). *Oct3/4A* is related to the maintenance of stemness, while *Oct3/4B* is related to cell differentiation (65, 66). Lovelace et al (2011) demonstrated that the evoked-bleeding step after the use of TAP in regenerative procedures for the treatment of immature teeth with pulpal necrosis triggered the accumulation of undifferentiated stem cells into the root canal (22). In this study, the levels of *Oct3/4A* and *Oct3/4B* transcripts were greatly enhanced at Day 1 in both groups. We hypothesize that 3Mix solution activates dental pulp stem/progenitor cells including SCAP cells of the root pulp, which might trigger stem/progenitor cells-mediated cell differentiation into odontoblast-like cells to begin the reparative dentin matrix deposition. However, more detailed and extensive research should be conducted to clarify the effect of 3Mix drugs combination on the activation of stem/progenitor cells following tooth injuries.

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Figure Legends

Figure 1. (*A, B, C, D, E,* and *G*) Hematoxylin & eosin (H&E) staining and (*F, H, I, J, K,* and *L*) nestin immunohistochemistry in the (*A*) control, (*B*) extracted teeth immediately after immersion, and replanted teeth (*C*) 1, (*D*) 3, (*E-H*) 5, (*I* and *J*) 7, and (*K* and *L*) 14 days after operation in the (*E, F, I*, and *K*) PBS and (*B-D, G, H, J*, and *L*) 3Mix groups, and (*M* and *N*) quantitative analyses of the percentage of nestin-positive perimeters in the total perimeter of the pulp-dentin border and newly-formed hard tissue areas in the total area of the pulp chamber, respectively. (*A*) Odontoblasts show pseudostratified features in the coronal pulp and blood capillaries are located in the odontoblast layer. (*B*) Odontoblasts maintain their pseudostratified features. (*C*) The dental pulp is occupied by numerous inflammatory cells. The odontoblasts show degenerative features and blood capillaries disappear from the odontoblast layer. (*Inset*) Higher magnification of inflammatory cells including neutrophils located in the center of the pulp. (*D*) Eosinophilic amorphous matrices including many erythrocytes and a decreased number of inflammatory cells are observed in the pulp tissue. (*Inset*) Higher magnification of the pulpal cells in the center of the pulp tissue. (*E* and *F*) Ascending nestin-positive filamentous structures are recognized in the apical third of the distal root pulp. (*G* and *H*) Blood capillaries reappear in the pulp tissue and newly differentiated odontoblast-like cells align on the pulp-dentin border of the apical third of the root pulp. (*I*) The coronal pulp shows scattered nestin-positive cells. (*J*) Newly differentiated odontoblast-like cells with their odontoblast processes beneath the predentin show a positive reaction for nestin. (*K*) Newly differentiated odontoblast-like cells align along the pulp-dentin border of the coronal pulp. (*L*) Extensive areas of tertiary dentin are observed in the coronal pulp. (*M*) There are significant differences in perimeters between both groups at Day 7 in addition to different time points in both groups. (*N*) There is a significant difference in areas between both groups at Day 14. DP, dental pulp; OB, odontoblasts or odontoblast-like cells; TD, tertiary dentin. Scale bars = (*E-H*) 100 μm, (*A-D*, *K*, and *L*) 50 μm, (*I, J*, and *Insets*) 25 μm.

Figure 2. Cell proliferation and apoptosis in replanted teeth in PBS and 3Mix groups evaluated by (*A, B,* and *J-M*) immunohistochemistry, (*C-F* and *N-Q*) TUNEL assay, and (*G-I*) RT-PCR. (*A* and *B*) Quantitative analysis of the number of Ki-67-positive cells in the (*A*) coronal and (*B*) root dental pulp during Days 0-14 and (*J-M*) immunohistochemistry for Ki-67 at (*J* and *K*) Day 7 and (*L* and *M*) Day 14 in the (*J* and *L*) PBS and (*K* and *M*) 3Mix groups. (*C-F*) Quantitative analysis of the number of TUNEL-positive cells in the (*C* and *D*) coronal and (*E* and *F*) root dental pulp during Days 0-14 in the (*C* and *E*) PBS and (*D* and *F*) 3Mix groups. (*G*) Comparative mRNA expression levels for *cyclinD1* and *caspase3* molecules at different time points (Days 5, 7, and 14 after operation). (*H*) Chronological changes in relative amounts of *cyclinD1* to *β-actin*. (*I*) Chronological changes in relative amounts of *caspase3* to *β-actin*. Scale bars = (*J-Q*) 50 μm.

Figure 3. (*A*) Comparative mRNA expression levels for *Dspp*, *nestin*, *Alp*, *Opn*, *Ocn*, *Oct3/4A*, *Oct3/4B* and *Cd11c* between PBS and 3Mix groups at different time points (Day 0, 1, 3, 5, 7, and 14 after operation). (*B-G*) Relative amounts of each RT-PCR product to *β-actin*.

Supplementary Figure 1. (*A, B, C, G, H, I, J,* and *K*) nestin immunohistochemistry and (*D, E,* and *F*) H&E staining in the (*A*) control, (*B* and *C*) extracted teeth immediately after immersion, and replanted teeth (*D*) 1, (*E*) 3, (*F* and *G*) 5, (*H, I, J,* and *K*) 7 days after operation in the (*B, D, E, H,* and *J*) PBS and (*C, F, G, I,* and *K*) 3Mix groups. (*A*) Intense immunoreactivity for nestin is recognized in coronal odontoblasts. (*B* and *C*) Nestin immunoreaction remains invariable in the odontoblast layer. (*D*) The dental pulp is occupied by numerous inflammatory cells including neutrophils. The odontoblasts show degenerative features and blood capillaries disappear from the odontoblast layer. (*E*) Eosinophilic amorphous matrices including many erythrocytes and a decreased number of inflammatory cells are observed in the pulp tissue. (*F*) The coronal pulp still shows degenerative features. (*G*) Some nestin-positive filamentous

structures are observed in the coronal pulp. (*H* and *J*) Ascending nestin-positive filamentous structures are still observed in the root pulp. (*I* and *K*) In the root pulp, intense nestin-immunoreaction is observed in the differentiated odontoblast-like and pulpal cells. AB, alveolar bone; DP, dental pulp; OB, odontoblasts or odontoblast-like cells. Scale bars = (*H* and *I*) 250 μm; (*J* and *K*) 100 μm; (*A*-*G*) 50 μm.

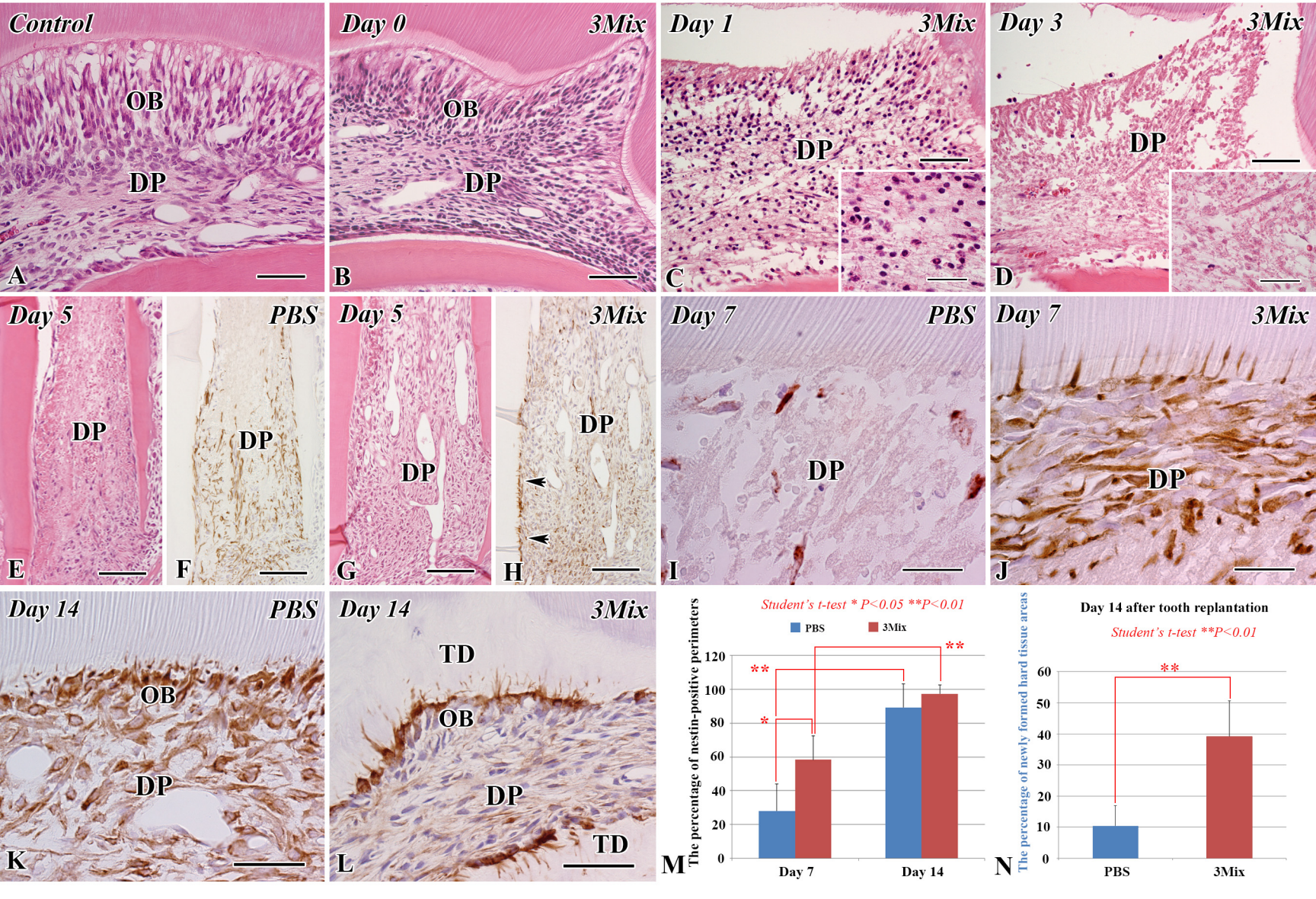

Control
OB
DP
A
Day 0
3Mix
OB
DP
B
Day 1
3Mix
DP
C
Day 3
3Mix
DP
D
Day 5
DP
E
PBS
DP
F
Day 5
3Mix
DP
G
DP
H
Day 7
PBS
DP
I
Day 7
3Mix
DP
J
Day 14
PBS
OB
DP
K
Day 14
3Mix
TD
OB
DP
TD
L
Student's t-test * P<0.05 **P<0.01
PBS
3Mix
The percentage of nestin-positive perimeters
120
100
80
60
40
20
0
**
**
*
Day 7
Day 14
M
Day 14 after tooth replantation
Student's t-test **P<0.01
The percentage of newly formed hard tissue areas
60
50
40
30
20
10
0
**
PBS
3Mix
N

Figure 1

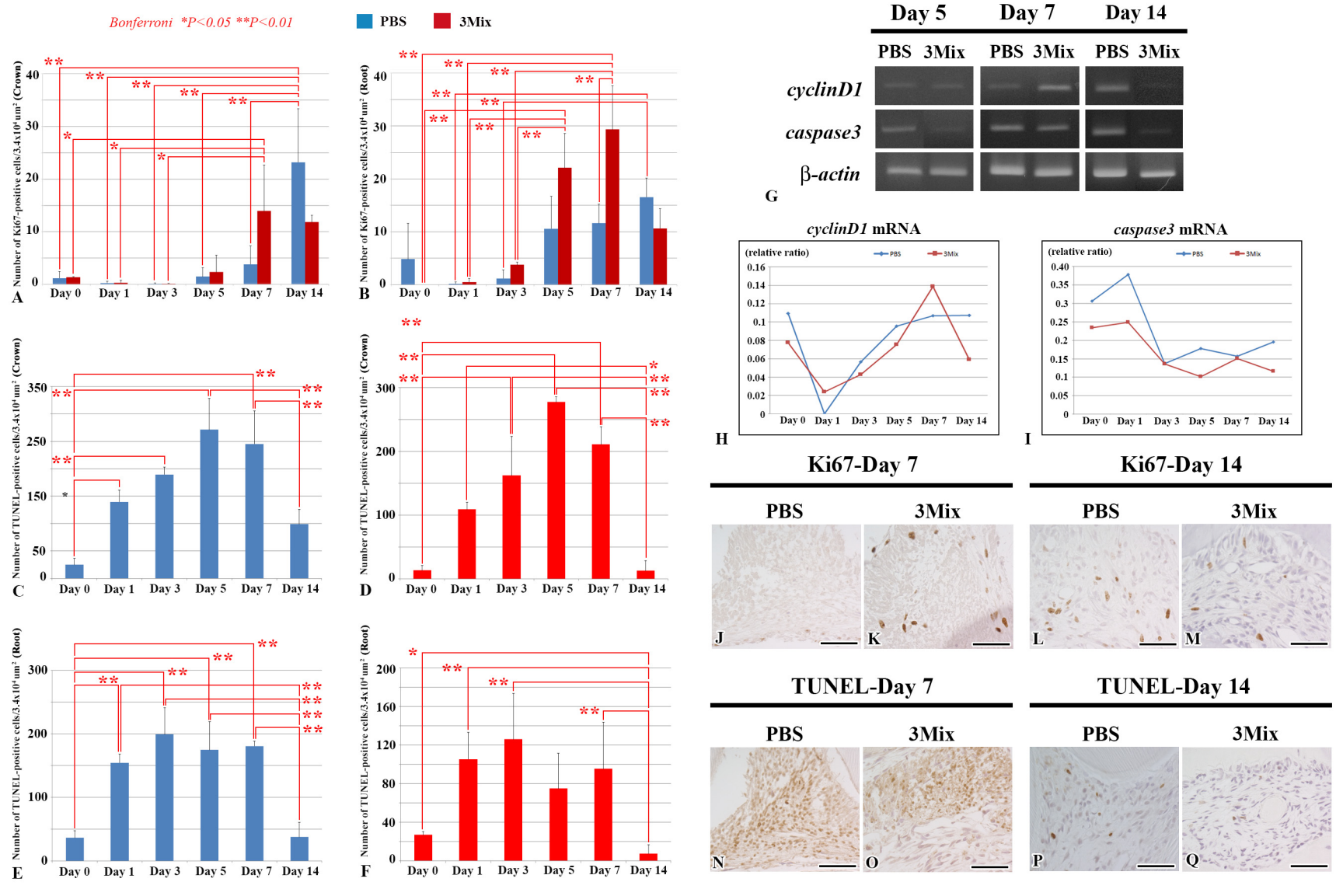
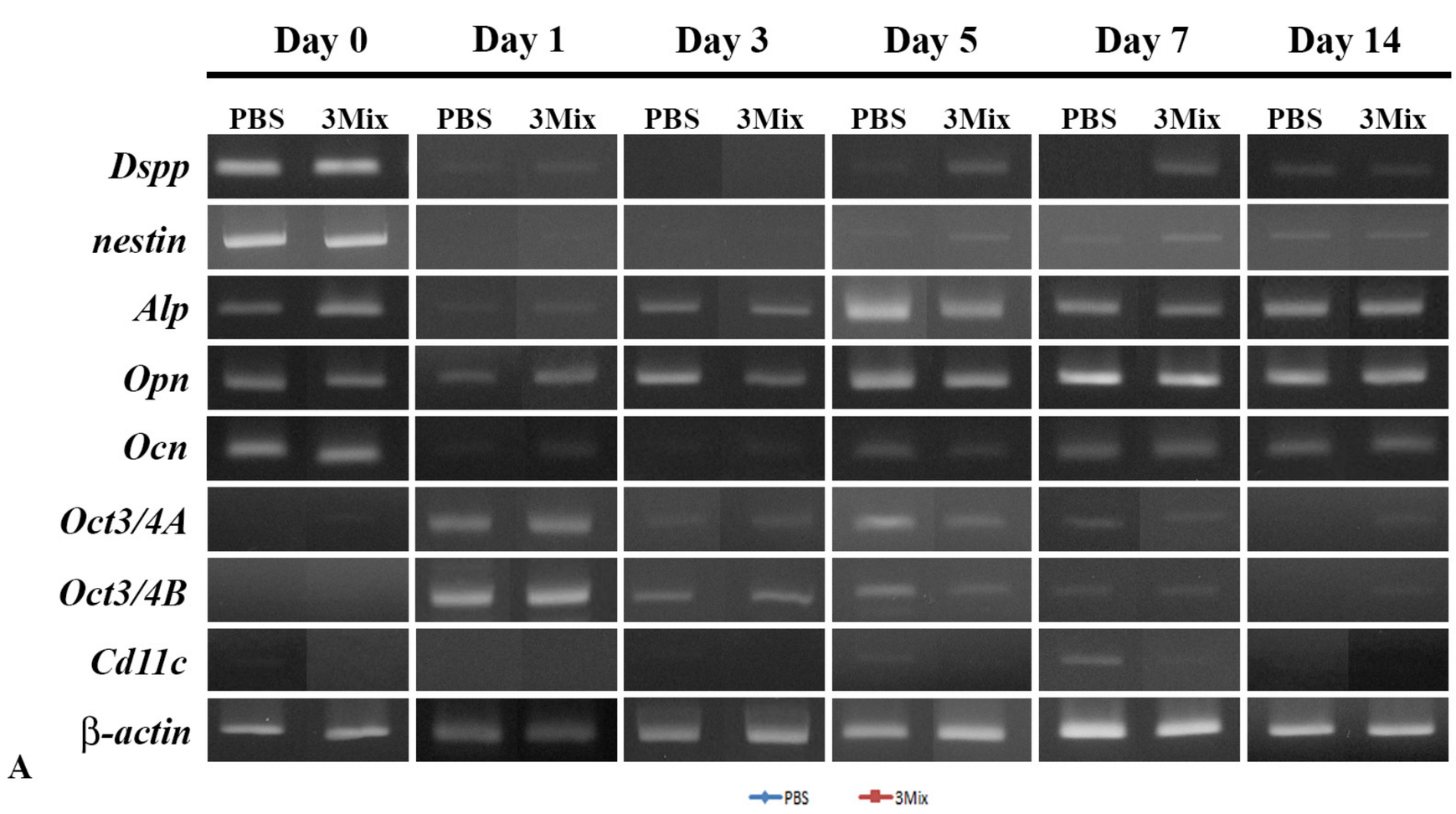
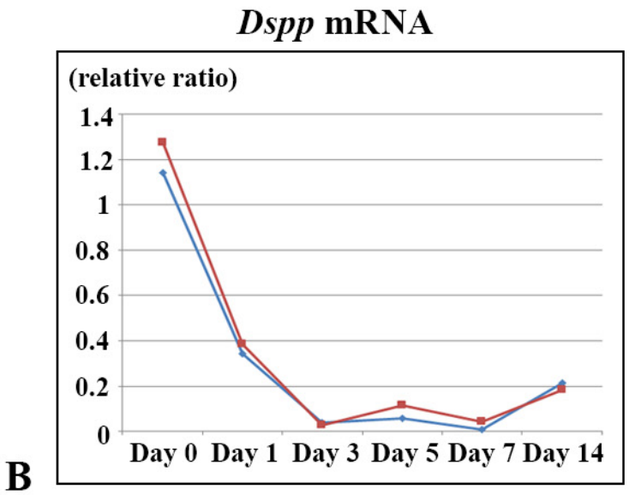

Bonferroni *P<0.05 **P<0.01
PBS
3Mix
Number of Ki67-positive cells/3.4x10⁴um² (Crown)
Number of Ki67-positive cells/3.4x10⁴um² (Root)
Number of TUNEL-positive cells/3.4x10⁴um² (Crown)
Number of TUNEL-positive cells/3.4x10⁴um² (Root)
Day 0
Day 1
Day 3
Day 5
Day 7
Day 14
A
B
C
D
E
F
Day 5
Day 7
Day 14
PBS 3Mix PBS 3Mix PBS 3Mix
cyclinD1
caspase3
β-actin
G
cyclinD1 mRNA
caspase3 mRNA
(relative ratio)
H
I
Ki67-Day 7
Ki67-Day 14
TUNEL-Day 7
TUNEL-Day 14
PBS
3Mix
J
K
L
M
N
O
P
Q

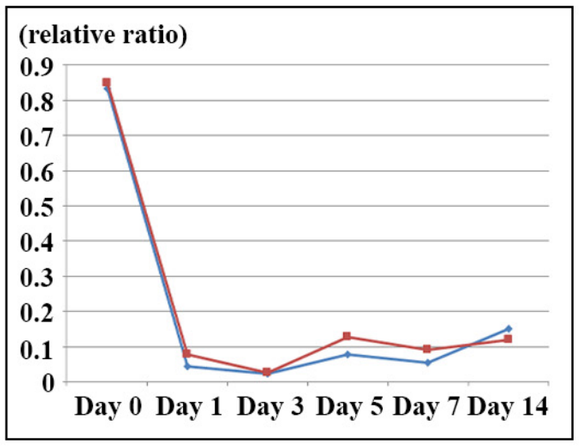
Figure 2



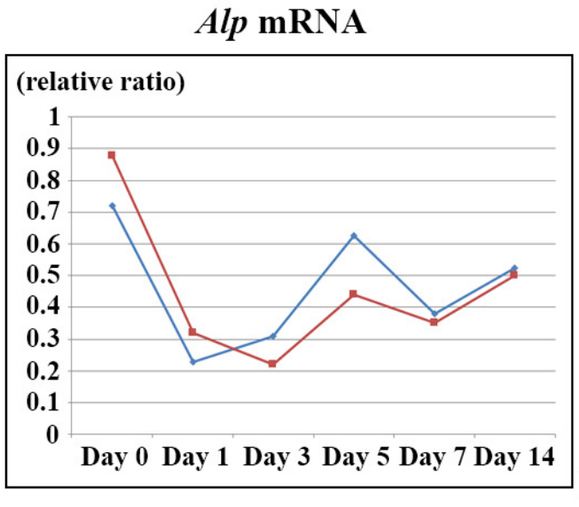
A



B



C



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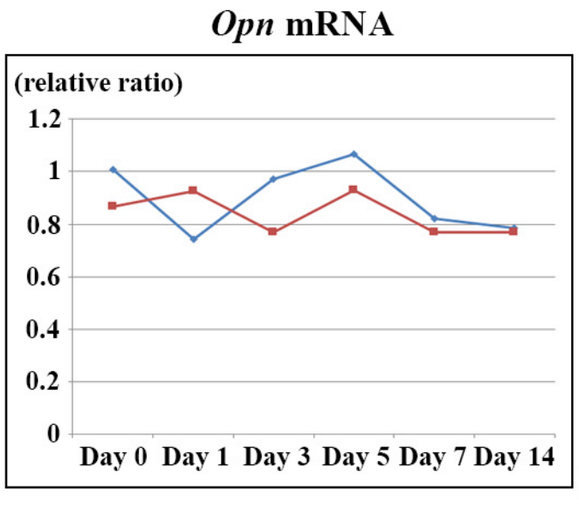

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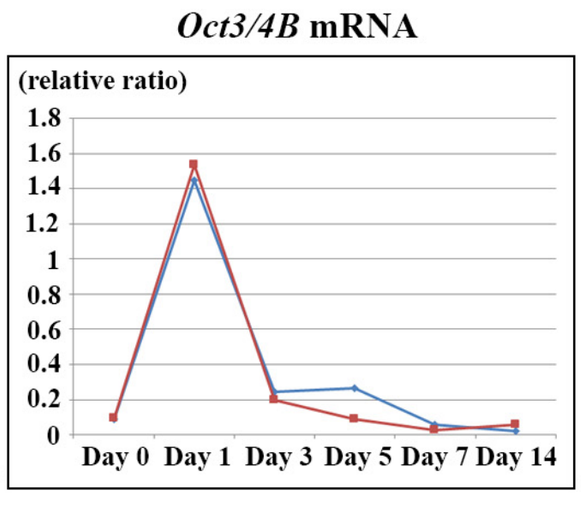

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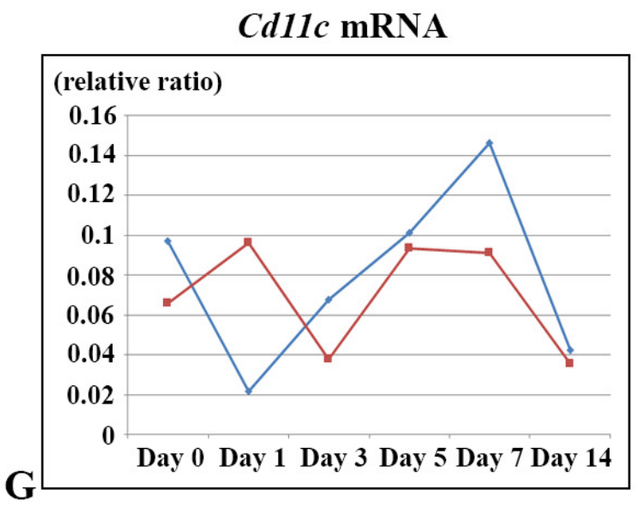
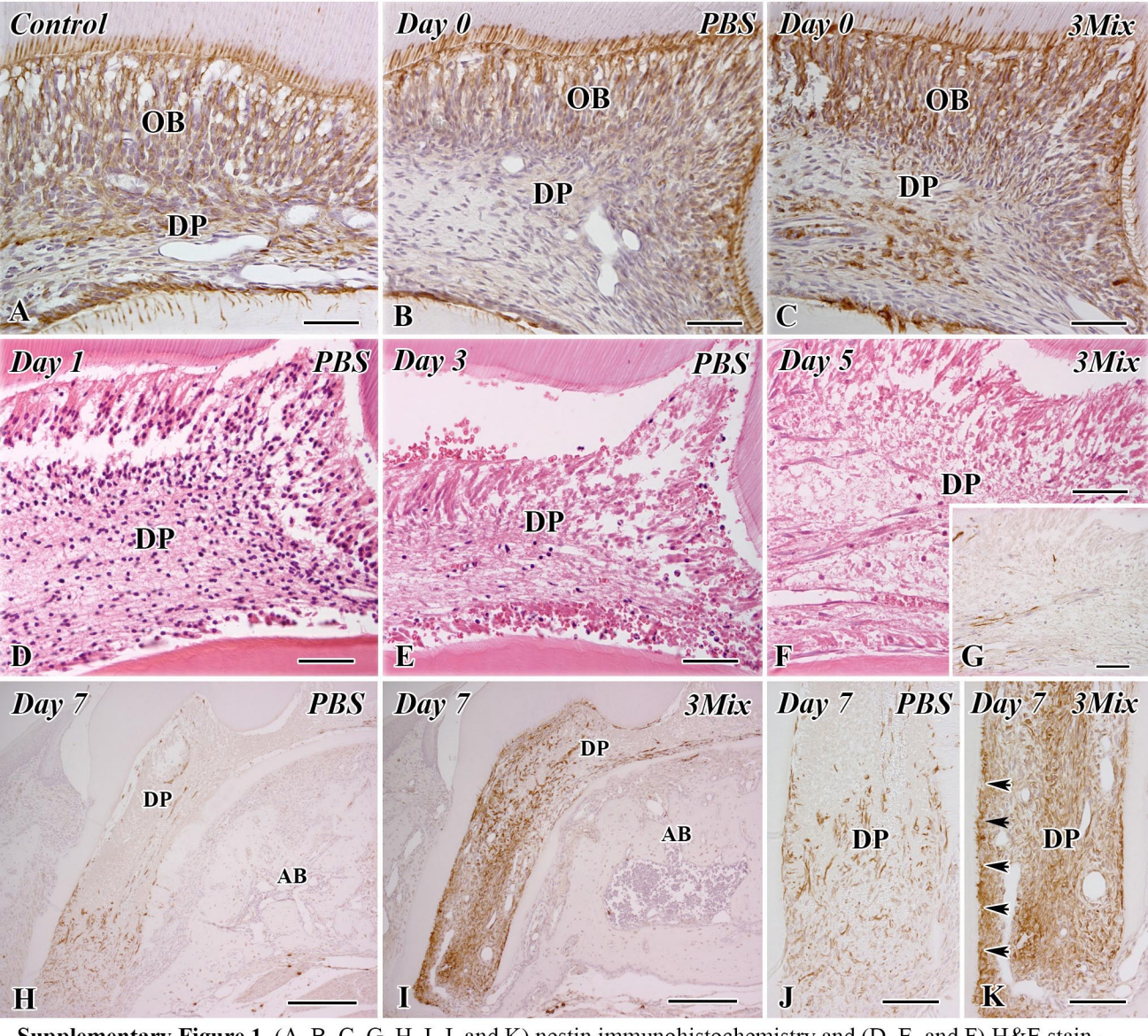


G

Figure 3

Supplementary Table 1. The oligonucleotide primers used for RT-PCR

molecule	primer sequence	amplicon size	genebank accession number
β-actin			
Forward	5'-TGGAATCCTGTGGCATCCATGAAAC-3'	348 bp	NM007393
Reverse	5'-TAAAACGCAGCTCAGTAACAGTCCG-3'		
Alp			
Forward	5'-CCAGCAGGTTTCTCTCTTGG-3'	239 bp	X13409
Reverse	5'-CTGGGAGTCTCATCCTGAGC-3'		
Dspp			
Forward	5'-TGAAAACTCTGTGGCTGTGC-3'	236 bp	NM010080.2
Reverse	5'-CAGTGTTCCCCTGTTCGTTT-3'		
cyclinD1			
Forward	5'-GCGTACCCTGACACCAATCT-3'	329 bp	NM007631
Reverse	5'-CACAACTTCTCGGCAGTCAA-3'		
caspase3			
Forward	5'-CCACAGTGCAGCTACCTCAA-3'	313 bp	NM009810
Reverse	5'-GTTCCAGCCTTTGACTCTGC-3'		
Oct3/4A			
Forward	5'- AAGTTGGCGTGGAGACTTTG-3'	204 bp	AB375278.1
Reverse	5'- TCTTCTGCTTCAGCAGCTTG-3'		
Oct3/4B			
Forward	5'-GCGTTCTCTTTTGGAAAGGTG -3'	211 bp	AB375271.1
Reverse	5'-CTCACACGGTTCTCAATGCT -3'		
Osteopontin			
Forward	5'-ATTTGCTTTTGCCTGTTTGG-3'	271 bp	AF515708
Reverse	5'-CTCCATCGTCATCATCATCG-3'		
Osteocalcin			
Forward	5'-CTTGGTGCACACCTAGCAGA-3'	152 bp	NM031368.4
Reverse	5'-ACCTTATTGCCCTCCTGCTT-3'		
Cd11c			
Forward	5'-GGAGGAGAACAGAGGTGCTG-3'	292 bp	NM021334
Reverse	5'-CACCTGCTCCTGACACTCAA-3'		
nestin			
Forward	5'-AGCAGGTGAACAAGACTCCG-3'	531 bp	NM016701
Reverse	5'-AGTGCTTCAGTCCCAGCTTC-3'		



Supplementary Figure 1. (A, B, C, G, H, I, J, and K) nestin immunohistochemistry and (D, E, and F) H&E staining in the (A) control, (B and C) extracted teeth immediately after immersion, and replanted teeth (D) 1, (E) 3, (F and G) 5, (H, I, J, and K) 7 days after operation in the (B, D, E, H, and J) PBS and (C, F, G, I, and K) 3Mix groups. (A) Intense immunoreactivity for nestin is recognized in coronal odontoblasts. (B and C) Nestin immunoreaction remains invariable in the odontoblast layer. (D) The dental pulp is occupied by numerous inflammatory cells including neutrophils. The odontoblasts show degenerative features and blood capillaries disappear from the odontoblast layer. (E) Eosinophilic amorphous matrices including many erythrocytes and a decreased number of inflammatory cells are observed in the pulp tissue. (F) The coronal pulp still shows degenerative features. (G) Some nestin-positive filamentous structures are observed in the coronal pulp. (H and J) Ascending nestin-positive filamentous structures are still observed in the root pulp. (I and K) In the root pulp, intense nestin-immunoreaction is observed in the differentiated odontoblast-like and pulpal cells. AB, alveolar bone; DP, dental pulp; OB, odontoblasts or odontoblast-like cells. Scale bars = (H and I) 250 μm; (J and K) 100 μm; (A-G) 50 μm.