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ORIGINAL ARTICLE

Proliferation, migration and apoptosis of periodontal ligament cells after tooth replantation

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OBJECTIVE: The aim of this study was to investigate the proliferation, migration and death of periodontal ligament (PDL) cells after tooth replantation.

MATERIALS AND METHODS: Maxillary first molars were extracted from 4-week-old male (*n* = 28) Sprague–Dawley rats and immediately replanted, after which, proliferation, migration and death of PDL cells were investigated.

RESULTS: At 3 days after tooth replantation, many proliferative cell nuclear antigen (PCNA)-positive PDL cells were observed on the alveolar bone side, but fewer on the root side. However, while a gradual decrease was observed in number of PCNA-positive PDL cells on the alveolar bone side until 7 days, an increase was seen on the root side. At 3 weeks, cells labeled with PKH26 (fluorescent dye into plasma membrane) were located in the middle of the PDL space. However, these PKH26-labeled cells did not spread to the surface of the cementum or the alveolar bone. TUNEL-positive cells were observed on both the bone and root sides at 3 days. Number of apoptotic cells increased until 7 days on the bone sides, but decreased on root sides.

CONCLUSION: These results suggest that both cell proliferation and apoptosis occur in different patterns and at different times to maintain regular spacing of the PDL after tooth replantation.

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Keywords: periodontal ligament; tooth replantation; proliferative cell nuclear antigen; migration; apoptosis

Introduction

Tooth replantation is sometimes required in cases such as tooth dislocation caused by trauma, intractable periapical lesion, root fracture and where intentional tooth replantation is to be carried out. While numerous clinical studies have demonstrated synthesis of alveolar bone and the healing processes of the periodontal ligament (PDL) after tooth replantation(Andreasen *et al*, 1995; Krasner and Rankow, 1995), few studies have attempted to clarify the precise underlying mechanism of PDL regeneration following tooth replantation (Lin *et al*, 2000; Iqbal and Bamaas, 2001; Kawanami *et al*, 2001).

Success or failure of tooth replantation is associated with viability of PDL (Andreasen, 1981) and the presence of intact and viable PDL cells on the root surface is one of the most important factors in the success of tooth replantation. Therefore, various methods have been used to determine the viability of PDL cells after tooth replantation such as direct counting of cells identified by fluorescent diacetate (Patel *et al*, 1994), 5-bromo-2′-deoxyuridine (BrdU) (Mine *et al*, 2005), radioactive DNA precursors (Hupp *et al*, 1997), and tetrazolium-based colorimetric (MTT) assay (Kim *et al*, 2007). Mine *et al* (2005) investigated proliferative activity of PDL after tooth replantation immunohistochemically, and found a higher proliferation of PDL cells within 1 week after tooth replantation, which then decreased over time (Mine *et al*, 2005). However, change in number of viable PDL cells with time and at specific sites after tooth replantation remains to be fully characterized. Furthermore, to the authors' knowledge, no studies have reported change in distribution of superficial PDL cells attached to the root surface, and the migration of proliferating cells after tooth replantation remains to be elucidated.

Tooth extraction for replantation results in wounding of the PDL, and subsequent wound healing is believed to be directed by a homeostatic regulatory mechanism (Schroeder, 1986). After tooth replantation, regeneration of the PDL can regulate the cell population to maintain regular spacing, and it is hypothesized that both cell proliferation and apoptosis participate in maintaining the homeostasis of the PDL after tooth replantation. Cell proliferation and migration in the

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PDL have been observed under physiological (McCulloch and Melcher, 1983) and orthodontically-induced conditions (Roberts and Chase, 1981). Apoptosis and regulation of the cell population have also been investigated in normal PDL (McCulloch *et al*, 1989) and under orthodontically-induced conditions (Mabuchi *et al*, 2002). However, little is known about the mechanisms involved in regulating cell proliferation and apoptosis in the PDL after tooth replantation.

In this paper, we investigate the proliferation, migration and apoptosis of PDL cells after tooth replantation, and discuss homeostatic function in the PDL.

Materials and methods

We used young (4-week-old) Sprague-Dawley male rats ($n = 28$) in this study by reference to Mine *et al* (2005). Animal experiments were carried out in accordance with the Guidelines for the Treatment of Animals at Tokyo Dental College. All experiments were performed with the rats placed under anesthesia using intraperitoneal injection of sodium thiopental (Ravonal, Tanabe Pharma Co., Osaka, Japan).

Experimental model and tissue preparation

To investigate proliferation and death of PDL cells, the right maxillary first molars (M1) of 12 and 8 rats, respectively, were replanted according to the method described by Kvinnsland *et al* (1991). Teeth were extracted using a dental explorer rotated once anteriorly so that the roots came out of their sockets, after which, they were immediately repositioned. Adhesive resin (Super Bond C&B, Sun Medical, Moriyama, Japan) was applied for postoperative splinting.

To investigate migration of PDL cells attached to the root surface, the PKH26 red fluorescence kit (Sigma-Aldrich, St. Louis, MO, USA) was used according to the manufacturer's protocol. Teeth were extracted ($n = 8$) and stained with PKH26 red for 5 min at room temperature. The teeth were then washed three times with phosphate-buffered saline (PBS) by centrifugation at 400 ***g*** for 3 min and returned to the original sockets and splinted.

Rats were killed at 3, 5 or 7 days after replantation to investigate proliferation and to characterize cell death, and at 3 weeks to track migration. Perfusion-fixation with 10% neutral-buffered formalin solution was performed transcardially, and maxillary tissues were removed and fixed for 24 h in the same fixative. Samples were decalcified for 2 weeks in 10% ethylenediaminetetraacetic acid (EDTA) containing 7% sucrose. The contralateral first molar served as a control in each animal.

Immunohistochemistry

Tissues were embedded in paraffin and 4-μm sections cut. The streptavidin–biotin immuno-peroxidase method was employed using the Histofine MAX-PO (MULTI) kit (Nichirei Co., Ltd, Tokyo, Japan). Sections were deparaffinized with xylene, washed with 100% alcohol and then washed again with distilled water. Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 in methanol for 30 min. Sections were then microwaved for 20 min at 65°C in 0.01 M citrate buffer (pH 6.0), cooled to room temperature and washed in PBS three times for 5 min each. To prevent non-specific reactions, the sections were incubated with 10% goat serum for 10 min. Proliferative cell nuclear antigen (PCNA) antibody (PC10; Dako, Glostrup, Denmark) was incubated at a dilution of 1:50 at room temperature for 1 h. As a negative control, PBS was used instead of the primary antibody. After the primary antibody reaction, the sections were rinsed in PBS three times for 5 min each. The secondary antibody (horse radish peroxidase conjugated anti-mouse IgG or anti-rabbit IgG) was then incubated at room temperature for 30 min. After washing in PBS three times for 5 min each, 3,3′diaminobenzidine-tetrahydrochloride Tris–HCl buffer (pH 7.6) was used to visualize reactivity. Finally, sections were counterstained with Mayer's hematoxylin, examined using a light microscope (Axiophot 2; Carl Zeiss, Oberkochen, Germany) and photographed.

PKH26 labeling

The decalcified tissues were embedded in O.C.T. compound and 6 μm cryosections were prepared. After washing three times each in PBS for 5 min, the sections were incubated with Alexa fluor488-conjugated phalloidin (dilution 1:20; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After washing in PBS, the sections were incubated in 4′,6-diamino-2-phenylindole (DAPI, 10 ng ml^{-1}; Molecular Probes, Eugene, OR, USA) for 5 min and observed using a fluorescent microscope (Axiophot 2).

TUNEL staining

TUNEL staining was carried out using the ApopTag Plus peroxidase *in situ* apoptosis detection kit (Chemicon International, Temecula, CA, USA) according to the method of an earlier study (Kichi *et al*, 2005). Sections were deparaffinized, washed for 5 min in PBS, incubated in 20 μg ml^{-1} of proteinase K (Wako Pure Chemical Industries Ltd, Osaka, Japan) in PBS for 15 min, and then washed in PBS. Endogenous peroxidase was inactivated with 3% H_2O_2 in PBS for 5 min. Sections were rinsed twice with PBS for 5 min, then immersed in equilibration buffer and incubated with TdT in a humidified chamber at 37°C for 1 h. Reactions were terminated by incubation in PBS. The sections were then incubated with anti-digoxigenin conjugate for 30 min, and finally incubated with 3,3′-diaminobenzidine for 3–6 min and counterstained with Mayer's hematoxylin. For the positive control, specimens were treated in DN buffer (30 mM Tris–HCl buffer, pH 7.2, 4 mM $MgCl_2$ and 0.1 mM dithiothreitol) and DNase (bovine pancreas, 0.1 g ml^{-1}; Amresco Inc, Solon, OH, USA) for 10 min.

Observation areas

Observation areas are shown in Figure 1. Briefly, the width of the PDL space around the mesial root was

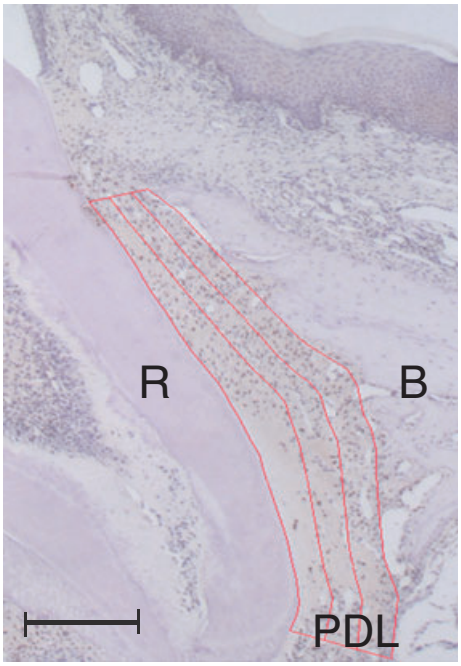

Figure 1 Observation areas and calculation of proliferative cell nuclear antigen-positive ratios. PDL space around mesial root is divided into 3 areas vertically, termed root side, center and bone side. B, alveolar bone; PDL, periodontal ligament; R, root; bar = 300 μm

divided into three areas, termed the root side, the center and the bone side. The positive ratio was calculated for each area about PCNA and TUNEL investigation as follows;

$$\text{Positive ratio} = \frac{\text{Positive cells}}{\text{Total cells}} \times 100(\%)$$

Statistical analysis
Statistical significance of multiple comparisons was evaluated using a repeated measures using ANOVA. Statistical significance was set at $P < 0.01$.

Results

Cell proliferation (PCNA-positive cells)
Nuclei of proliferating PDL cells were stained immunohistochemically with anti-PCNA antibody. At 3 and 5 days after tooth replantation, 15–25% of PDL cells on the root side were PCNA-positive. This ratio was lower than that on the bone side, but increased gradually at 7 days after tooth replantation (Figure 2a). The ratio on the root side was higher than that on the bone side at 7 days (Figure 2b). Statistically significant differences were observed between 3 and 5 days and between 3 and 7 days after root replantation on the root side ($P < 0.01$; Figure 2c). The PCNA-positive ratio on the bone side was higher than that on the root side at 3 days after tooth replantation. The ratio on the bone side decreased gradually until 7 days after replantation. A statistically significant difference was observed between 3 and 7 days after tooth replantation on the bone side ($P < 0.01$; Figure 2c).

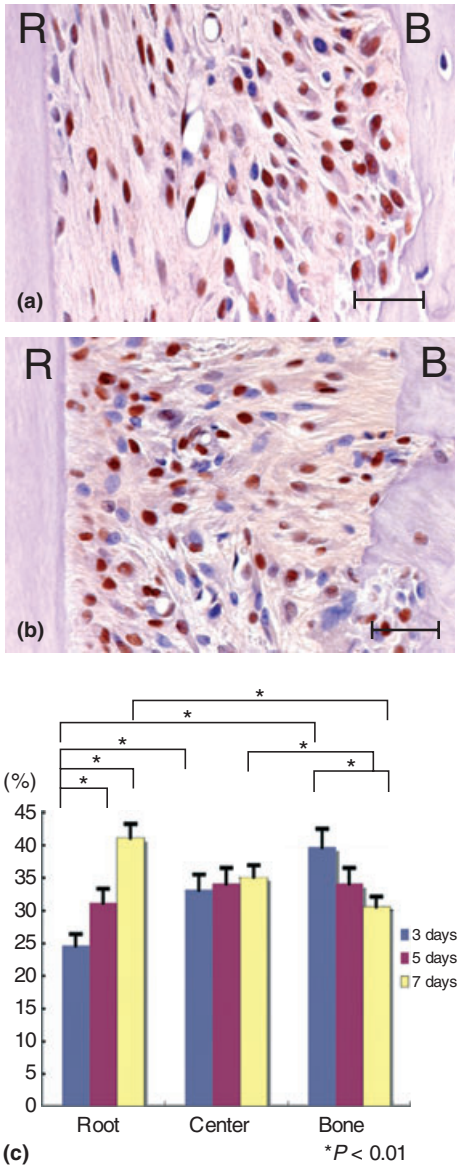

Figure 2 Immunohistochemistry of proliferative cell nuclear antigen (PCNA). (**a**) Nuclei of periodontal ligament cells after tooth replantation are stained with anti-PCNA antibody immunohistochemically at 3 days (B, alveolar bone; R, root; bar = 50 μm). (**b**) At 7 days after tooth replantation, PCNA-positive cells increases on root sides, but decreases on bone sides at 7 days after tooth replantation (B, alveolar bone; R, root; bar = 50 μm). (**c**) PCNA-positive ratio on bone side is higher than that on root side at 3 days after tooth replantation. Ratio on bone side decreases gradually until 7 days after replantation. Ratio on root side is higher than that on alveolar bone side ($P < 0.01$) at 7 days after tooth replantation. Statistically significant differences are observed between 3 and 7 days after tooth replantation on bone side ($P < 0.01$)

Tracking of proliferating cells (PKH26 labeling)
Periodontal ligament cells attached to the roots were labeled with PKH26 red fluorescence to track proliferating and migrating cells. PKH26-labeled cells were observed in the superficial layer of the PDL that was attached to the root immediately after tooth replantation (Figure 3a). Number of labeled cells increased day by day, as cells that divided from the labeled cells were also labeled with PKH26 red. At 3 weeks after tooth replantation, the labeled cells were located in the middle of the

PDL space (Figure 3b). However, the labeled cells did not spread to the surface of the cementum or alveolar bone (Figure 3b). As a negative control, when PDL cells were stained without PKH26, no labeled cells were seen in the PDL (data not shown).

Cell death (TUNEL staining)

Apoptotic cells were detected as dark brown in color using TUNEL staining. Apoptotic cells were observed in all areas of the PDL, and TUNEL-positive cells were detected on both the bone and root sides at 3, 5 and 7 days after replantation (Figures 4a–d), and ranged from 2.5% to 4.0% of all cells. TUNEL positive cells were detected on root side at 3 days after replantation (Fig 4a,b). Number of TUNEL-positive cells decreased gradually on the root sides after 3 days, with the ratio reaching $<2.5\%$. On the other hand, number of apoptotic cells increased at 5 and 7 days on the bone side (Figure 4c,d). Data summarizing these results are shown in Figure 4e.

Discussion

The PDL plays an important role in maintaining tooth position and distributing masticatory force to the alveolar bone (Schroeder, 1986). It has been thought that both cell proliferation and cell death participate in maintaining the homeostasis of the PDL, which involves, for example, preserving regular spacing following PDL regeneration after tooth replantation. Therefore, in this study, we investigated cell proliferation and cell death after tooth replantation.

Proliferative cell nuclear antigen is a 36-kDa polypeptide expressed in the late G1 to S (DNA synthesis) phases of the cell cycle, and is used as a marker of cell proliferation. The previous studies using labeling of DNA-synthesis showed that proliferative activity ranged from 1% to 4% in normally functioning mouse and rat molar PDL (Weiss *et al*, 1968; Baumrind and Buck, 1970). Mabuchi *et al* (2002) reported that the average PCNA-positive ratio was 27% (from 17.9% to 36.1%) at 3 days after orthodontic forces. Furthermore, studies by Gould *et al* (1977, 1980) revealed that stimulation of mouse molar PDL by traumatic wounding increased the labeling index approximately 6-fold. In this study, PCNA-labeled cells ranged from 17% to 37% (average 28%) of all PDL cells at 3 days after tooth replantation. Taken together with the results of earlier studies, our

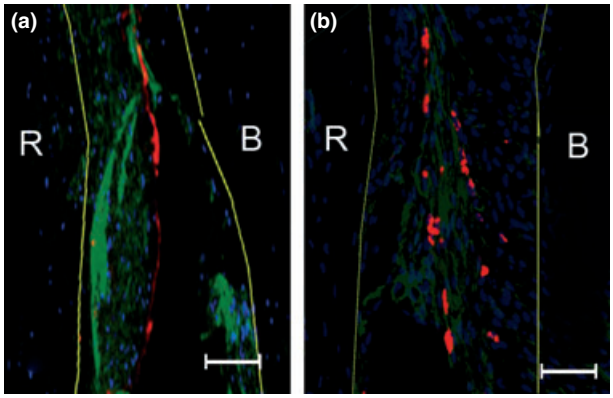

Figure 3 Tracking of superficial cells in periodontal ligament (PDL) attached to root (PKH26 labeling). (**a**) PKH26-labeled cells are observed at superficial layer of PDL attached to root immediately after tooth replantation (B, alveolar bone; PDL, periodontal ligament; R, root; bar = 150 μm). (**b**) At 3 weeks after tooth replantation, labeled cells are located in middle of PDL space. However, labeled cells did not spread to surface of cementum or alveolar bone (B, alveolar bone; R, root; bar = 150 μm)

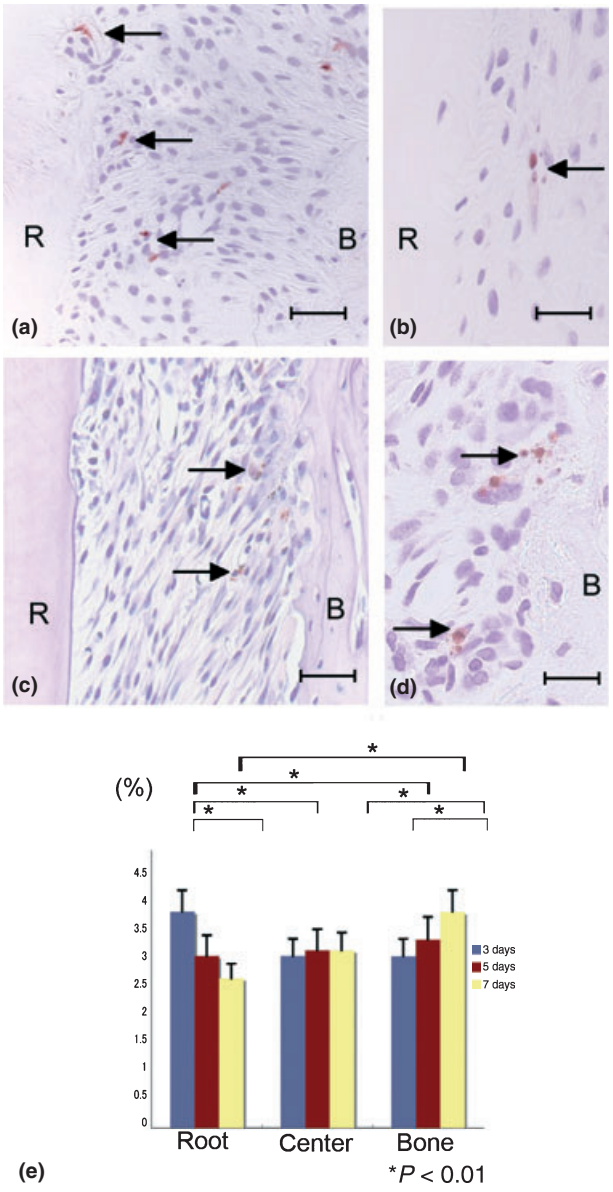

Figure 4 Cell death detected by TUNEL staining. (**a**, **b**) Nuclei of periodontal ligament (PDL) cells after tooth replantation are stained with TUNEL at 3 days (arrows) [B, alveolar bone; R, root; bar = 50 μm (**a**), =25 μm (**b**)]. (**c**, **d**) Number of apoptotic cells (arrows) increases on the bone side at 7 days. (**d**) Morphologically apoptotic cells (arrows) show nuclear fragmentation, and are detected as dark brown in color using TUNEL staining. (**e**) Number of TUNEL-positive cells decrease gradually on the root sides, while the ratio increase on bone side gradually. Statistically significant differences are observed between 3 and 7 days after tooth replantation on bone and root sides ($P < 0.01$)

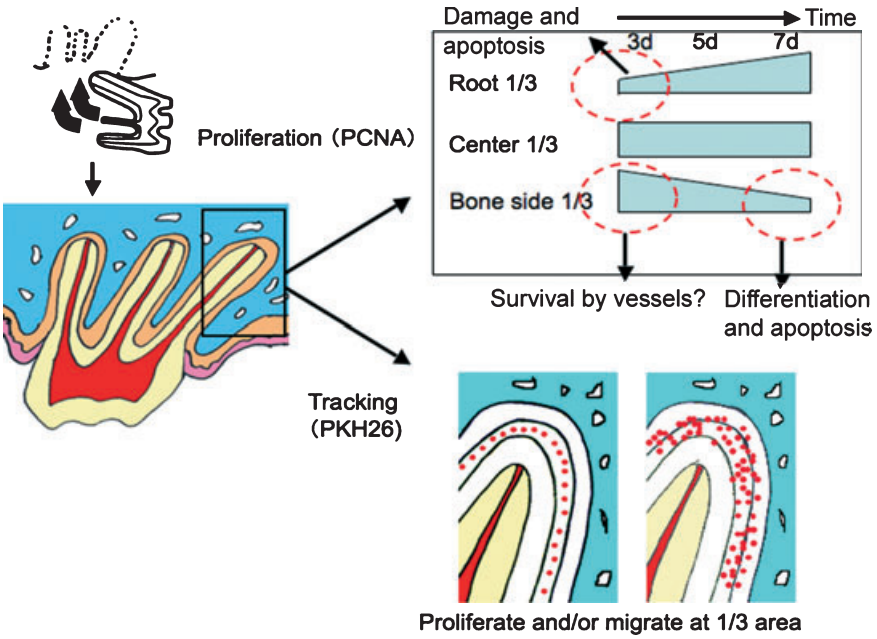

Figure 5 Summary of cell proliferation, migration and cell death after tooth replantation. Cell proliferation migration and apoptosis occur in different patterns and at different times to maintain regular spacing of the periodontal ligament after tooth replantation

findings here indicate that mechanical stimulation of the PDL such as through orthodontic treatment or trauma increases PCNA-labeling approximately 6-fold in comparison with under normal conditions.

The ratios of PCNA-positive cells on the alveolar bone side were higher than those on the root side at 3 and 5 days after replantation. These data suggest that the PDL cells on the bone side were less injured than those on the root side. This may be explained by the number and distribution of vessels that are located near the alveolar bone and which supply nourishment to cells in the PDL (Murrell *et al*, 1996). Furthermore, the PDL is exposed to hypoxic conditions after tooth extraction and during tooth replantation. Hypoxia is known to induce cell-cycle arrest in the G1 phase, and cell death induced by hypoxia is recognized as apoptosis (Gardner *et al*, 2001). Numerous molecules, including p53, retinoblastoma protein (Rb), cyclins, cyclin-dependent kinases (CDK), CDK inhibitors and hypoxia-inducible factor 1, play an essential role in deciding the fate of a cell, determining whether cell proliferation or apoptosis will result from G1 arrest caused by hypoxia (Goda *et al*, 2003). In this study, the PCNA-positive ratio was lower on the root side than on the alveolar bone side at 3 days after tooth replantation, and apoptotic cells were observed on the root side at 3 days. These results imply that hypoxic conditions caused by extraction may induce cell cycle arrest and cell death in PDL cells near the root at 3 and 5 days after replantation.

Our results showed that the PCNA-positive ratio on the bone side was lower than that on the root side at 7 days. There are two possible explanations for this. One possibility is differentiation of PDL cells into osteogenic cells. In general, cell differentiation and change in function occur after cell proliferation. MC3T3-E1 cells (osteoblastic cells) do not express non-collagenous proteins such as osteopontin and osteocalcin when they are in the proliferating phase. The PDL cells located on the alveolar bone side at 7 days after tooth replantation in this study may have been in the functional phase. Another possible explanation involves cell death: Mabuchi *et al* (2002) showed that cell death occurred after orthodontic treatment in the PDL, and concluded that this was to maintain the spacing of the PDL. The decrease in the PCNA-positive ratio seen at 7 days after tooth replantation in this study may, therefore, have been as a result of maintenance of PDL spacing.

Recently, several studies have tracked transplanted periodontal cells in transgenic mice (Kim *et al*, 2006; Hosoya *et al*, 2008). Kim *et al* (2006), using lacZ transgenic ROSA26 mice, showed that donor PDL cells were replaced by host cells, and that the PDL regenerated after tooth transplantation (Kim *et al*, 2006). However, tracking of replanted PDL cells is not well understood. PKH26 labeling, which is fluorescent dye into plasma membrane, is used to determine migration and proliferation of transplanted cells *in vitro* and *in vivo*. In this study, to investigate proliferative PDL cells after tooth replantation, we used the fluorescent dye PKH26, which stains the lipid bi-layer of the plasma membrane. At 3 weeks after tooth replantation, labeled cells were located in the middle of the PDL space, but no labeled cells were observed on either the root or alveolar bone sides. This suggests that the superficial layer of PDL cells that attaches to the surface of extracted teeth proliferates only at the middle, and does not spread widely during PDL healing after replantation. The proliferation may protect over growth of the cells from bone side or root side.

The TUNEL method, which is based on the addition of labeled UTP to the 3′ ends of fragmented DNA by Terminal deoxynucleotidyl Transferase, has been reported as an *in situ* labeling method for detecting

apoptotic cells (Gavrieli *et al*, 1992). It has been suggested that homeostasis in renewing tissues is maintained by a regulated balance between cell proliferation, cell differentiation, and cell death (Haake and Polakowska, 1993). The previous reports on cell death in the PDL have been restricted to descriptions of necrosis after mechanical injury (Roberts and Chase, 1981). Mabuchi *et al* (2002) reported that apoptosis occurred following cell proliferation when orthodontic forces acted on the PDL to maintain regular spacing. The results of this study, taken together with those of these earlier studies, suggest that both cell proliferation and cell death occur in the PDL after tooth replantation to maintain regular spacing.

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Author contributions

K Sato and T Muramatsu contributed equally to this work.

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