

Histochemical and immunocytochemical study on hard tissue formation in dental pulp during the healing process after tooth replantation in rat molars

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Abstract It has been assumed that dental pulp has the capacity to elaborate both bone and dentin matrix under the pathological conditions following tooth injury. This study aims to clarify the mechanism to induce bone formation in the dental pulp by investigating the pulpal healing process after tooth replantation using micro-computed tomography (μ -CT), immunocytochemistry for heat-shock protein (HSP)-25 and cathepsin K (CK), and histochemistry for both alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP). Under deep anesthesia, the upper right first molar of 4-week-old Wistar rats was extracted and then immediately repositioned in the original socket. In the control teeth at postnatal 4 weeks, the periphery of the coronal dental pulp showed intense ALP- and HSP-25-positive reactions, whereas there were no TRAP- and CK-positive cells. Tooth replantation weakened or terminated ALP- and HSP-25-positive reactions in the pulp tissue at the initial stages. Three to seven days after operation, the ALP-positive region recovered from the root apex to the coronal pulp followed by HSP-25-positive reactions in the successful case that resulted in tertiary dentin formation. In another case, TRAP- and CK-positive cells appeared in the pulp tissue of the replanted tooth at postoperative days 5–10, and remained associated with the bone tissue after 12–60 days. Immunoelectron microscopy clearly demonstrated that CK-positive osteoclast-lineage cells made contact with the mesenchymal cells with prominent nucleoli and developed cell organelles. These data suggest that the appearance of TRAP- and CK-positive cells may be involved in the induction of bone tissue formation in dental pulp.

Keywords Bone development, Dental pulp, Osteoclast, Rats (Wistar), Tooth Replantation

Introduction

Bone and teeth are specialized hard tissues that closely resemble each other. A unique feature of dentin is that it is a mineralized tissue that surrounds the pulp, a non-mineralized tissue. Furthermore, dentin does not remodel, but responds to injury by forming tertiary dentin to protect the dental pulp ([Smith 2002](#)), in contrast to bone suffering remodeling. Odontoblasts produce most of the extracellular matrix components found in dentin and are implicated in dentin mineralization. Consequently, they remain in the periphery of the pulp tissue and are responsible for further dentin formation throughout life ([Smith 2002](#)). Dental pulp not only provides nutritional and sensory properties to dentin, but also has its own reparative capacity. This potential has important implications for dental therapy. Tooth replantation, defined as a therapeutic method in which dropped or dislocated tooth is replaced in its original alveolar socket, is now utilized to treat the complete luxation of teeth whether intentional or accidental. This procedure interrupts the nerve and vascular supply to the dental pulp and the subsequent degeneration of the pulpal cells. In successful cases, pulpal regeneration, re-innervation, and re-vascularization have been shown to occur in human ([Andreasen et al. 1995](#)) and experimental animal studies ([Kvinnsland et al. 1991](#); [Byers et al. 1992](#); [Rungvechvuttivittaya et al. 1998](#); [Shimizu et al. 2000](#); [Ohshima et al. 2001](#); [Nakakura-Ohshima et al. 2003](#)).

The biological properties of the dentin-pulp complex are still unknown especially concerning its capability to form hard tissue. Following tooth replantation, pulpal responses can be divided into at least two types: tertiary dentin and bone tissue formation in the regenerated pulp tissue ([Kvinnsland et al. 1991](#); [Byers et al. 1992](#); [Rungvechvuttivittaya et al. 1998](#); [Shimizu et al. 2000](#); [Ohshima et al. 2001](#)). To date,

the mechanism to determine the divergent healing process after tooth replantation remains unclear. It has recently been assumed that dental pulp contains a different derivation of cell populations: resident mesenchymal cells, already present at the site of tooth development, together with para-axial mesenchyme- and cranial neural crest (CNC)-derived cells ([Goldberg and Smith 2004](#)). This idea is supported by evidence that dental pulp has the capacity to elaborate both bone and dentin matrix under the pathological conditions following tooth injury ([Shimizu et al. 2000](#)). These findings suggest the occurrence of pluripotent stem cells in the dental pulp ([Gronthos et al. 2002](#)).

Our recent studies have demonstrated that odontoblasts show a stage-specific expression pattern of immunoreactivity for heat-shock protein (HSP)-25, the family of low-molecular-weight HSPs ([Ciocca et al. 1993](#); [Arrigo and Prévile 1999](#)), in intact teeth ([Ohshima et al. 2000, 2002](#)) and under experimental conditions including tooth replantation ([Ohshima et al. 2001](#); [Nakakura-Ohshima et al. 2003](#)). These findings indicate that this protein can be utilized as a useful marker for monitoring the degeneration/regeneration of odontoblasts during the pulpal healing process after tooth injury in experimental models, leading to differentiation of the divergent healing processes after tooth replantation. On the other hand, it is well established that cathepsin K (CK), cysteine proteinase, is highly and selectively expressed within osteoclasts ([Drake et al. 1996](#)), and both enzymes of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) may be used to identify different cell populations such as osteoblasts, osteoclasts and precursors of these cells, because ALP is a typical (non-specific) marker of osteoblasts and TRAP is a typical (non-specific) marker of osteoclasts ([Bonucci and Nanci 2001](#)). This study aims to elucidate the

mechanism regulating the divergent healing processes of pulp tissue after tooth replantation using several cellular markers such as HSP-25, CK, ALP, and TRAP. Furthermore, we utilized the micro-computed tomography (μ -CT) to evaluate different healing patterns in dental pulp following operation before the preparation of decalcified tissue sections.

Materials and methods

All experiments were performed following the Guidelines of the Niigata University Intramural Animal Use and Care Committee.

Wistar rats, 4 weeks old, were used in this study. The upper right first molar (M1) was extracted with a pair of Howe's pliers (YDM Corp, Tokyo, Japan) with modification and then was immediately repositioned in the original socket under anesthesia by an intraperitoneal injection of chloral hydrate (350 mg/kg). The replanted tooth was left without any further treatment. The upper left first molar of the same animal was used as a control.

Materials were collected in groups of four to fifteen animals at intervals of 1, 3, 5, 7, 10, 12, 14, 28, and 60 days after replantation. At each stage, the animals were anesthetized and transcardially perfused with physiological saline followed with 4% paraformaldehyde in a 0.1 M phosphate buffer. The maxillae were removed *en bloc* and immersed in the same fixative for an additional 12 h. Multiple scans were taken from all maxillae using the μ -CT (Elescan; Nittetsu Elex, Osaka, Japan). The CT settings were as follows: pixel matrix, 256 x 256 x 256; slice thickness, 21 μ m; projection number, 600 x 32; magnification, x 3.88; voltage, 41 kV; electrical current, 0.1 mA. The maxillae were reconstructed using a software program (NDTView, Sony Corp, Tokyo, Japan; 3D bone for WinNT, Ratoc System Engineering Co, Ltd, Tokyo, Japan) to evaluate the three-dimensionally reconstructed and sagittal views of the control and replanted teeth. Following decalcification in a 4.13% ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) solution for 4 weeks at 4°C, the tissue blocks were equilibrated in a 30% sucrose solution or a graded series of 10–20% sucrose solution + OCT compound (Tissue-Tek; Sakura Finetechnical Co, Ltd,

Tokyo, Japan) mixture for cryoprotection. The specimens for histochemistry were cut sagittally at a thickness of about 10 μm with a cryotome (Shandon AS620; Life Sciences International Japan Ltd, Tokyo, Japan), and thaw-mounted on poly-L-lysine-coated glass slides. The specimens for immunohistochemistry were cut sagittally at a thickness of about 50 μm with a freezing microtome (FX-801; Yamato Kohki Co, Tokyo, Japan), collected into cold phosphate buffered saline (PBS), and treated as free-floating sections.

For histochemical demonstration of both ALP and TRAP activity, the azo-dye method ([Burstone 1961](#); [Cole and Walters 1987](#)) was utilized with slight modification. Frozen sections were incubated for 5–6 min at room temperature in a medium comprising 0.01% naphthol AS-BI phosphatase (Na salt) (Sigma Chemical Co, St Louis, MO, USA), 0.06% fast Blue RR salt (Sigma Chemical Co) in 0.2 M tris-HCl buffer (pH 8.3–8.5), and subsequently incubated for 6–7 min at room temperature in a medium comprising 0.01% naphthol AS-BI phosphatase (Na salt) (Sigma Chemical Co), 0.06% fast red violet LB salt (Sigma Chemical Co) 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.

For the immuno-peroxidase procedure, the frozen sections were processed for the avidin-biotin peroxidase complex (ABC) method using either a polyclonal antibody to HSP-25 (diluted 1:5000; StressGen Biotechnologies Corp, Victoria, BC, Canada) or CK (diluted 1:200; hCathepsin K-monoclonal antibody, Fuji Chemical Industries, Ltd, Toyama, Japan). A solution of 0.01 M phosphate-buffered saline (PBS, pH 7.4) was used both to dilute the antibody and to rinse the sections. Endogenous peroxidase was inactivated by treatment with 0.3% H_2O_2 in absolute methanol for 30 min. Any non-specific immunoreactivity was inhibited by pre-incubation either in 2.5% normal

horse serum (Vector Lab Inc, Burlingame, CA) for monoclonal antibodies or 2.5% normal goat serum (Vector) for polyclonal antibodies. Following incubation with one of the primary antibodies (4°C, 24 h), the sections were consecutively reacted with biotinylated anti-mouse IgG (for monoclonal antibodies; Vector) or biotinylated anti-rabbit IgG (for polyclonal antibody; Vector) for 2 h, and then the ABC complex (Vector) for 1 h at room temperature. For the final visualization of the sections, 0.05 M Tris HCl buffer (pH 7.6) containing 0.04% 3,3'-diaminobenzidine tetrachloride and 0.002% H₂O₂ was used. The immunostained sections were counter-stained with 0.05% methylene blue. Further characterization of the primary antibody (HSP-25) has been reported elsewhere ([Ohshima et al. 2000](#)).

The dental pulp (20 mg) and periodontal tissue including alveolar bone (20 mg) were suspended in 50 µl Laemmli sodium dodecyl sulfate (SDS) sample buffer (Bio-Rad Laboratories, CA, USA). Equal amounts (20 µl) of the samples were subjected to 12% (w/v) SDS Polyacrylamide Gel (Bio-Rad Laboratories), and the separated proteins were electrophoretically transblotted onto PVDF membrane (Bio-Rad Laboratories). Non-specific binding sites were blocked by incubation in 5% skim milk. The membrane was then incubated with anti-CK antibody at a dilution of 1:5000. After being washed, the membrane was incubated with ALP-conjugated anti-mouse IgG at a dilution of 1:4000, and the bands were visualized with the use of BCIP/NBT (Bio-Rad Laboratories).

For immunocytochemistry using anti-CK antibody at the electron-microscopic level, the immunostaining procedure was the same as described above, except for the inhibition of endogenous peroxidase. The immunostained sections were subsequently postfixed in 1% OsO₄ reduced with 1.5% potassium ferrocyanide, dehydrated in an

ascending series of ethanol, and finally embedded in Epon 812 (Taab, Berkshire, UK).

Ultrathin sections (70 nm in thickness) were double-stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7100 transmission electron microscope.

Immunohistochemical controls were performed by: 1) replacing the primary antibodies with non-immune serum or PBS; 2) omitting the anti-mouse or rabbit IgG, or the ABC complex. These immunostained sections contained no specific immunoreaction.

M1 teeth and pulp areas obtained from μ -CT were measured using the bone morphometrical software (Luzex F; Nireco, Tokyo, Japan). The pulp area fractions (%) of each specimen were calculated. All data were presented as the means and standard deviations (SD) of each group. The comparisons among groups were performed by making the graph of scatter plots without statistical analysis. Furthermore, to assess the relationship between the time spent on tooth replantation after extraction and healing patterns, such as bone formation and root resorption, the frequency of occurrence was compared using Fisher's protected least-significant-difference test [one-way analysis of variance (ANOVA)]. The data were obtained from the samples of 70 animals (Day 12: n = 10; Day 14: n = 21, Day 28: n = 20; Day 60: n = 19). The statistical analysis was done by using statistical software (Statview version 5.0; SAS Institute Inc., Hulinks, Inc., Tokyo, Japan) on a personal computer.

Results

Micro-computed tomography (μ -CT) analysis

Three dimensional-reconstructed, sliced views of the control and affected teeth obtained by μ -CT clearly showed the divergent healing patterns in the dental pulp during tooth development or after replantation (Fig. 1). In the controls during 1 to 60 days (from 4 to 12 weeks after birth), root elongation and dentin and cementum formation progressed leading to the reduction of the pulp space and enlargement of alveolar bone volume (Fig. 1a, b). Compared with the control groups, root elongation was arrested and the root or/and alveolar bone resorption occurred frequently in the experimental animals (Fig. 1c-j). No considerable changes can be seen in the dental pulp except for the spontaneous dentin resorption during 1–10 days after tooth replantation (Fig. 1c, d). After postoperative Day 12, hard tissue formation was detected in the dental pulp. Two healing patterns, *i.e.* tertiary dentin and bone tissue, are clearly differentiated in the pulp tissue from 14–60 days after the operation (Fig. 1e-j), resulting in a considerable reduction in pulp space at Day 60 (Fig. 1i-j). The graph of scatter plots clearly demonstrated that considerable pulpal reductions occurred in the experimental groups compared with the control groups (Fig. 2).

From histological sections, the dental pulp showed three regeneration patterns after 12 days (Table 1).

Alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) analysis

In the control group, the periphery of the dental pulp showed an ALP-positive reaction, and the subodontoblastic layer represented particularly intense reactivity, whereas the pulp tissue contained no TRAP-positive reaction (Fig. 3a, b). On the other hand, ALP-

and TRAP-positive reactions were observed inside the bone marrow and around the bone tissue (Fig. 3a, c). Tooth replantation caused a reduction in ALP-positive reactivity by 3 days after the operation (data not shown), although positive reactions remained in the pulp tissue at Day 1 (Fig. 3d). ALP-positive reactions recovered until 5 days after tooth replantation in the successful case where tertiary dentin was recognized, while TRAP-positive reactions increased in intensity in the periodontal tissue and TRAP-positive multinucleated cells were occasionally situated adjacent to the root dentin (Fig. 3e, g). After 7 days after operation, clear tertiary dentin was recognized at the periphery of the dental pulp where the regenerated odontoblasts showed ALP-positive reactions (Fig. 3j, k, n). In another case where tertiary dentin was not deposited, numerous TRAP-positive cells with mononucleated or multinucleated features appeared in the dental pulp, where developed blood vessels were easily recognizable (Fig. 3h, i, l, m). TRAP-positive cells at the pulp-dentin border elongated their cellular processes into the dentinal tubules (Fig. 3l, m), and remained around the bone matrix after 14 days (Fig. 3o).

Cathepsin K (CK) analysis

In the control group, CK-immunopositive reactions were observed inside the bone marrow and around the alveolar bone, and occasionally recognized on the surface of the root dentin (Fig. 4a, c). The dental pulp contained no intense CK-immunopositive reaction, although certain cells showed a weak immunoreaction even in the control tooth (Fig. 4b), and these immunoreactivities did not change throughout the experimental period of the successful case where tertiary dentin formed (Fig. 4d, e, h). On the other hand, CK-immunoreactivity in the periodontal tissue increased in intensity

over time (Fig. 4d–f), and subsequently remained at the localized area adjacent to the root dentin where considerable root resorption advanced (Fig. 4f, h, i, k). In another case where tertiary dentin was not recognized, numerous CK-immunopositive cells appeared in the dental pulp, where developed blood vessels were easily recognizable, during 5–10 days after tooth replantation (Fig. 4f, g), and some of the cells in the periphery of the pulp tissue gathered along the pulp-dentin border and extended their cellular processes into the dentinal tubules (Fig. 4g). From 12 days after the operation, bone tissue occurred in the dental pulp, and CK-immunopositive cells were associated with these bone matrices, some of them showing a dendritic appearance in addition to oval-shaped features (Fig. 4i–k).

Anti-CK antibody reacted with protein bands of 27 kDa, showing proteolytic activation of the latent, high molecular weight pro form of CK into the corresponding active, low molecular weight form *in vivo* (Kontinen et al. 2002). The band was shown in the sample of the periodontal tissue including alveolar bone, but not in dental pulp (Fig. 5).

At the ultrastructural level, the CK-immunopositive cells along the pulp-dentin border showed a multinucleated appearance with developed Golgi apparatus and numerous mitochondria in their cytoplasm. Mesenchymal cells with prominent nucleoli and developed cell organelles made contact with CK-immunopositive mono- and multi-nucleated cells in the pulp tissue (Fig. 6a–c). The close relationship between CK-immunopositive cells and mesenchymal cells continued throughout the experimental stages (Fig. 6d, e).

Heat-shock protein (HSP)-25 analysis

In the control group at 1 day (4 weeks after birth), an intense immunoreaction for HSP-25 was found exclusively in the coronal odontoblasts, whereas the odontoblasts in the root and floor pulp showed little or no immunoreactivity (Fig. 7a). The odontoblasts in the coronal pulp, appearing in a pseudo-stratified arrangement, revealed intense immunoreactivity for HSP-25 in their cytoplasm, but their cytoplasmic processes entering the dentin and predentin were devoid of any immunoreactivity except for their bottoms in the predentin (Fig. 7b). Tooth replantation caused the complete disappearance of HSP-25-immunoreactivity in the odontoblasts throughout the pulp at postoperative Day 1 (data not shown). In the successful case where tertiary dentin began, a certain area of the pulp-dentin border in the coronal pulp began to show HSP-25-immunoreactivity, and the regenerating nervous elements became HSP-25-immunopositive, reaching the pulp horn after 5 days (data not shown). Presumably newly differentiated odontoblasts aligned along the pulp-dentin border and showed intense HSP-immunoreactivity (Fig. 7c). On the other hand, the other case where bone tissue would be induced in the dental pulp lacked HSP-25-immunoreactive cells along the pulp-dentin border (data not shown). Twelve days after replantation, two healing patterns, *i.e.* tertiary dentin or bone tissue, were clearly recognized in the dental pulp. With tertiary dentin formation, most odontoblasts in the coronal pulp became immunoreactive for HSP-25, although the odontoblasts in the root and floor pulp showed weak immunoreactivity even in the replanted teeth (Fig. 7d, f, g). The cells associated with the bone matrix, presumably certain osteoblasts, showed HSP-25-immunoreactivity, and the teeth including the bone tissue occasionally suffered considerable root resorption (Fig. 7e, h, i).

Figure 8 summarizes the hypothesis on the mechanism to determine the divergent healing processes after tooth replantation. As for the relationship between the time spent on tooth replantation after extraction and the healing pattern, there was significant relationship between the time for operation and root resorption ($P < 0.01$: Fig. 9).

Discussion

In the present histochemical and immunocytochemical study, tooth replantation caused drastic time-related alterations in HSP-25- and CK-expression as well as ALP- and TRAP-reactions in the dental pulp. The chronological changes in HSP-25-immunoreaction indicated the degeneration/regeneration process of the odontoblasts under the pathological conditions shown by the present and previous studies (Ohshima et al. 2001; Nakakura-Ohshima et al. 2003). This expression pattern was almost identical to that of ALP-reactions, although intense HSP-25-immunoreactive areas were restricted in the differentiated odontoblasts in the pulp tissue indicating that HSP-25-labeled cells showed no TRAP-reactivity. ALP activity is associated with hard tissue formation, including bone and teeth (Hoshi et al. 1997; Goseki-Sone et al. 1999; Hotton et al. 1999). Its overall distribution indicates that this enzyme performs important biological functions, particularly in mineralized tissues, although the physiological role of this enzyme is poorly understood. Thus, the chronological changes in these two markers are essential in evaluating the pulpal healing patterns, especially tertiary dentin formation. Furthermore, the TRAP- and CK-positive cells dramatically changed their locations and populations depending on the healing pattern after tooth replantation. It is noteworthy that developed blood vessels were easily recognizable in the area where numerous TRAP- and CK-positive cells accumulated in the pulp chamber.

Our most notable finding was the close relationship between the appearance of TRAP- and CK-positive cells during the 5–10 days after the operation, and the induction of bone tissue formation in the dental pulp. These positive cells could be categorized as osteoclast-lineage cells judging from their ultrastructural features

including developed Golgi apparatus and numerous mitochondria, in addition to histochemical and immunohistochemical features such as TRAP and CK being highly and selectively expressed within these cells. Interestingly, osteoclast-lineage cells made contact with pulpal mesenchymal cells with prominent nucleoli and developed cell organelles. These interactions between two types of cell remained in the pulp chamber after the bone matrix was deposited there. In bone, osteoblasts/stromal cells regulate osteoclast formation and bone resorption by producing factors like osteoprotegerin (OPG), osteoclast-differentiating factor/receptor activator of NFκB ligand (ODF/RANKL), and macrophage colony-stimulating factor (M-CSF) that interact with hematopoietic osteoclast precursor cells ([Roodman 1996](#); [Suda et al. 1997](#)). Since physical contact between osteoblast/stromal cells and osteoclast progenitors is essential for osteoclast formation ([Takahashi et al. 1999](#)), the similar physical contact between dental pulp mesenchymal cells and osteoclast-lineage cells in this study might play a role in ectopic bone metabolism in the dental pulp. In conclusion, the appearance of TRAP- and CK-positive cells may be involved in the induction of bone tissue formation in dental pulp.

Internal resorption on the coronal and root dentin surrounding the pulp tissue is a rare pathological process in permanent human teeth ([Trope et al. 1994](#)). In contrast, physiological root resorption commonly occurs in human deciduous teeth where numerous odontoclasts, the same cell type as osteoclasts in their ultrastructural features and functions ([Sasaki 2003](#)), gather along the pulp-dentin border and extend their cellular processes into the dentinal tubules ([Sasaki et al. 1988](#); [Sahara et al. 1996](#); [Kannari et al. 1998](#); [Linsuwanont et al. 2002](#)). Similar findings are observed in the different pathological condition in the present study, and in the previous report, where

numerous TRAP-positive osteoclast-lineage cells accumulate along the pulp-dentin border after tooth replantation (Shimazu et al 2002). On the other hand, the temporal appearance of dendritic cells along the pulp-dentin border has been also reported after tooth injury such as tooth replantation and cavity preparation (Shimizu et al. 2000; Ohshima et al. 2003; Nakakura-Ohshima et al. 2003). The common myeloid precursors commit to either the osteoclast or the mononucleated phagocyte-lineage that further differentiate into dendritic cells or macrophages, depending on stimuli received from the external environment (Matsuo and Ray 2004). The microenvironment after tooth injury may determine what cell type appears along the pulp-dentin border to induce the divergent healing patterns: the dendritic cells appear in tertiary dentin formation (Shimizu et al. 2000; Ohshima et al. 2003; Nakakura-Ohshima et al. 2004), whereas the osteoclast-lineage cells appear in bone tissue formation in this study. Furthermore, since there is a significant relationship between the time spent on the operation and root resorption ($P < 0.01$; Fig. 9), a prolonged tooth replantation procedure may induce the same conditions as physiological root resorption in the human deciduous tooth.

The dentin-pulp complex can respond to a variety of pathological conditions by localized deposition of a tertiary dentin matrix. In pathological conditions that cause the death and destruction of odontoblasts, the damaged dentin is repaired by reparative dentin secreted by a new generation of odontoblast-like cells (Smith 2002). Our previous results provide evidence that the dental pulp cells give rise to new generations of odontoblast-like cells secreting reparative dentin in an experimental model of tooth replantation by monitoring the degeneration/regeneration process of HSP-25-positive cells (Ohshima et al. 2001; Nakakura-Ohshima et al. 2003). When odontoblasts

degenerate after tooth injury, mesenchymal cells of the pulp tissue differentiate into odontoblast-like cells that will form tertiary dentin (Tziafa et al. 2000; Smith 2002). Concerning the biological properties of the dentin-pulp complex, pulpal mesenchymal cells could elaborate bone tissue under pathological conditions judging from the present results and the recent notion that dental pulp is composed of different cell populations including resident mesoderm- and cranial neural crest (CNC)-derived cells (Goldberg and Smith, 2004). This idea is based on the evidence that condensed dental mesenchyme consists of CNC-derived cells together with an increasing number of non-CNC-derived cells during tooth development (Chai et al., 2000). Recently, it has been reported that the dental pulp cells express factors such as stimulatory (RANKL, M-CSF) and inhibitory (OPG) factors, which are important regulating in osteoclastogenesis and bone resorption (Sheela-Rani and MacDougall 2000; Ogasawara et al. 2003). Certain cell populations in the pulp tissue might have the ability to differentiate into bone-forming cells, and their differentiation might take place under pathological conditions where certain regulatory mechanisms for suppressing bone formation are disturbed (Fig. 8). This pathological condition may be the same as seen in the dental pulp of human deciduous teeth prior to shedding where RANKL-immunoreactivity is detected in the odontoblasts and pulp fibroblasts (Lossdörfer et al 2002). Further studies are needed to clarify the biological properties of the dental pulp, especially the derivation of bone tissue-forming cells.

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Figure legends

Fig. 1 Three-dimensional reconstructed and sliced views of the control at 1 (a) and 60 (b) days (4 and 12 weeks after birth, respectively), and replanted teeth at 1 (c), 7 (d), 14 (e, f), 28 (g, h), and 60 (i, j) days after operation obtained by μ -CT. (a, b) In the control groups during 1 to 60 days (from 4 to 12 weeks after birth), root elongation and dentin and cementum formation progress resulting in the reduction of the pulp space and enlargement of alveolar bone volume. (c-j) In the experimental groups, root elongation is arrested and root or/and alveolar bone resorption is observed. Two types of hard tissue formation, *i.e.* tertiary dentin (e, g, i) and bone tissue (f, h, j), are differentiated in the dental pulp after 12 days.

Fig. 2 Comparison of the pulpal reductions with the advance of root formation in the control groups during 1 (C1) to 60 (C60) days (4 to 12 weeks after birth) and those at 60 days after tooth replantation (Mix mixed form of dentin and bone, D tertiary dentin, B bone). Pulpal reductions occur in the experimental groups compared with the control groups.

Fig. 3 Alkaline phosphatase (ALP)- (colored blue) and tartrate-resistant acid phosphatase (TRAP)-reactions (colored red) in sagittal sections of the control at 1 day (4 weeks after birth) (a-c) and replanted teeth at 1 (d), 5 (e-i), 7 (j-m), and 14 (n, o) days after operation. (a) The periphery of the dental pulp shows an ALP-positive reaction, whereas the pulp tissue contains no TRAP-positive reaction. (b) Higher magnification of the area indicated by the *arrow* in a. The odontoblasts and subodontoblastic layer represent an intense ALP-reactivity. (c)

TRAP-positive cells are observed on the surface of the alveolar bone. **(d)** ALP-positive reaction in the pulp tissue is almost identical to that in the control group. **(e)** ALP-positive reaction is observed in the dental pulp. **(f)** Higher magnification of the area indicated by the *arrow* in **e**. The odontoblasts and subodontoblastic layer represent intense ALP-reactivity. Note the tertiary dentin matrix deposition. **(g)** Higher magnification of the area indicated by the *arrowhead* in **e**. TRAP-positive reactions increase in intensity in the periodontal tissue and TRAP-positive multinucleated cells are situated adjacent to the root dentin. **(h)** Numerous TRAP-positive cells appear in the dental pulp. Note the cell aggregation (*) in the coronal pulp. **(i)** Higher magnification of the area indicated by the *arrow* in **h**. Mononucleated and multinucleated TRAP-positive cells occur in the pulp tissue. **(j, n)** The periphery of the pulp tissue shows an ALP-positive reaction. **(k)** Higher magnification of the area indicated by the *arrow* in **j**. ALP-positive odontoblasts align beneath the tertiary dentin. **(l)** TRAP-positive cells are observed in the pulp tissue. **(m)** Higher magnification of the area indicated by the *arrow* in **l**. TRAP-positive cells at the pulp-dentin border extend their cellular processes into the dentinal tubules (*arrowheads*). **(o)** TRAP-positive cells are located around the bone matrix in the pulp chamber. *AB* alveolar bone, *B* bone, *D* dentin, *DP* dental pulp, *OB* odontoblasts, *PDL* periodontal ligament, *TD* tertiary dentin, *Bars* 500 μm (**a, d, e, h, j, l, n, o**), 50 μm (**b, c, f, g, i, k, m**)

Fig. 4 Cathepsin K (CK)-immunoreactivities in the control at 1 day (4 weeks after birth) (**a–c**) and replanted teeth at 1 (**d**), 5 (**e**), 7 (**f, g**), 12 (**h**), 14 (**i, j**), 28 (**k**) days after

operation. (a) The dental pulp contains no intense CK-immunopositive reaction. (b) Higher magnification of the area indicated by the *arrow* in a. Certain cells show a weak immunoreaction (*open arrowheads*). (c) Higher magnification of the area indicated by the *arrowhead* in a. CK-immunoreactive cells are observed on the surface of the alveolar bone, and recognizable on the surface of root dentin (*open arrowheads*). (d, e, h) CK-immunoreactivity in the dental pulp is almost identical to that in the control group, whereas immunoreactivity in the periodontal tissue increases in intensity. (f) Numerous CK-immunopositive cells appear in the dental pulp (*). (g) Higher magnification of the area indicated by the *arrow* in f. CK-immunopositive cells gather along the pulp-dentin border and extend their cellular processes into the dentinal tubules (*arrows*). (i, k) CK-immunopositive cells are associated with the bone tissue occurring in the dental pulp. (j) Higher magnification of the area indicated by the *arrow* in i. Certain CK-immunopositive cells show a dendritic appearance (*open arrowheads*). *AB* alveolar bone, *B* bone, *D* dentin, *DP* dental pulp, *OB* odontoblasts, *PDL* periodontal ligament, *arrowheads* considerable root resorption, *bars* 500 μm (a, d, e, f, h, i, k), 100 μm (j), 50 μm (b, c, g)

Fig. 5 Immunoblot analysis. Anti-CK antibody reacts with protein bands of 27 kDa, showing proteolytic activation of the latent, high molecular weight pro form of CK into the corresponding active, low molecular weight form *in vivo*. The band is shown in the sample of the periodontal tissue including alveolar bone, but not in dental pulp. *Lane 1* Precision Protein Standard (from Bio-Rad Laboratories), *Lane 2* rat dental pulp, *Lane 3* periodontal tissue including alveolar bone

Fig. 6 Semithin sections (**a, b, d**) and electron micrographs (**c, e**) of CK-immunoreactivities in the pulp tissue of replanted teeth at 7 (**a–c**) and 14 (**d, e**) days after operation. (**a, b, d**) Mononucleated or multinucleated CK-immunopositive cells (*arrows*) make contact with mesenchymal cells with prominent nucleoli (*arrowheads*), some of them showing weak immunoreactivity (*open arrows*). (**c, e**) Mesenchymal cells with developed cell organelles (*) make contact with CK-immunopositive osteoclasts (*OC*) that possess developed Golgi apparatus and numerous mitochondria (*open arrowheads*). *B* bone, *BV* blood vessel, *D* dentin, *PD* predentin, *bars* 25 μm (**a, b, d**), 5 μm (**c, e**)

Fig. 7 HSP-25-immunoreactivities in the control at 4 weeks after birth (**a, b**) and replanted teeth at 5 (**c**), 12 (**d**), 14 (**e**), and 28 (**f–i**) days after operation. (**a**) Intense immunoreaction for HSP-25 is observed in the coronal odontoblasts, whereas the odontoblasts in the root and floor pulp show little or no immunoreactivity. (**b**) Higher magnification of the area indicated by the *arrow* in **a**. The odontoblasts reveal intense immunoreactivity for HSP-25, and cytoplasmic processes are devoid of immunoreactivity except for their bottoms in the predentin. (**c**) Presumably newly differentiated odontoblasts at the pulp-dentin border are immunopositive for HSP-25. (**d, f**) Most odontoblasts in the coronal pulp except for the root and floor pulp become immunoreactive for HSP-25. (**e, h**) Bone tissue occurs in the dental pulp. (**g**) Higher magnification of the area indicated by the *arrow* in **f**. HSP-25-immunoreactive odontoblast-like cells with several processes are arranged in the proper odontoblast layer. (**i**)

Higher magnification of the area indicated by the *arrow* in **h**. The cells associated with the bone matrix, presumably certain osteoblasts, show HSP-25-immunoreactivity. *AB* alveolar bone, *B* bone, *D* dentin, *DP* dental pulp, *OB* odontoblasts or odontoblast-like cells, *TD* tertiary dentin, *arrowheads* considerable root resorption, *bars* 500 μm (**a, d, e, f, h**), 100 μm (**i**), 50 μm (**b, c, g**)

Fig. 8 A schematic diagram summarizing the divergent regeneration processes after tooth replantation. Tooth replantation may induce odontoblast cell death, leading to differentiation of pulpal mesenchymal cells into HSP-25-positive new odontoblasts responsible for reparative dentin formation. With bone formation, numerous osteoclast-lineage cells appear in the pulp chamber and make contact with mesenchymal cells that may differentiate into osteoblasts (*OB*) under the pathological condition where negative regulation may be disturbed due to the absence of odontoblasts and/or cranial neural crest-derived cells. *BV* blood vessels, *D* primary dentin, *OC* osteocyte, *MC* mesenchymal cells *PD* predentin, *RD* reparative dentin

Fig. 9 Relationship between the time spent on tooth replantation after extraction and the frequency of root resorption compared using analysis of variance (ANOVA). Replanted teeth with root resorption are valued at “2”, and teeth without resorption at “1”. The standard errors of the left and right groups are 0.078 and 0.085, respectively.