Capacity of dental pulp differentiation after tooth transplantation

Ryoichiro Ogawa[1, 2], Chikara Saito[2], Han-Sung Jung[3, 4], Hayato Ohshima[1]*

[1]Division of Anatomy and Cell Biology of the Hard Tissue and [2]Division of Reconstructive Surgery for Oral and Maxillofacial Region, Department of Tissue Regeneration and Reconstruction, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan; [3]Division of Anatomy and Developmental Biology, Department of Oral Biology, Research Center for Orofacial Hard Tissue Regeneration, Oral Science Research Center, College of Dentistry, Brain Korea 21 project for Medical science, Yonsei University, Seoul, Korea; [4]Department of Pathology, Tokyo Dental College, Chiba, Japan.

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*corresponding author:
Hayato Ohshima, DDS, PhD
Division of Anatomy and Cell Biology of the Hard Tissue, Department of Tissue Regeneration and Reconstruction, Niigata University Graduate School of Medical and Dental Sciences, 2-5274 Gakkocho-dori, Niigata 951-8514, Japan
TEL +81-25-227-2812
FAX +81-25-227-0804
E-mail: histoman@dent.niigata-u.ac.jp

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Abstract Dental pulp elaborates both bone and dentin matrix under pathological conditions, where the contribution of periodontal tissue to this event cannot be excluded. This study aimed to clarify the capability of dental pulp to deposit bone matrix through an auto-graft experiment using immunohistochemistry for 5-bromo-2'-deoxyuridine (BrdU) and nestin, and histochemistry for tartrate-resistant acid phosphatase (TRAP). Following the extraction of the molars of 3-week-old mice, the roots and pulp floor were resected and immediately transplanted into the sublingual region. On Days 5-7, tubular dentin formation commenced next to the preexisting dentin at the pulp horn where nestin-positive odontoblast-like cells were arranged. Until Day 14, bone-like tissue formation occurred in the pulp chamber, where intense TRAP-positive cells appeared. These results suggest that the odontoblast- and osteoblast-lineage cells reside in the dental pulp. Overall, specific dental pulp regeneration will provide fundamental knowledge for the realization of human tooth regeneration in the near future.

Key words auto-graft; bone development; dental pulp; mouse; tooth transplantation

Introduction

The dentin-pulp complex is capable of repair after tooth injuries (Nanci 2003). Tooth injuries such as cavity preparation and tooth replantation induce destructive changes in the odontoblasts at the affected site as well as an acute inflammatory reaction (Ohshima et al. 2003; Nakakura-Ohshima et al. 2003). If the odontoblasts do not survive, pulpal mesenchymal cells replace the degenerated cells to differentiate into odontoblast-like cells (Smith 2002). This phenomenon may indicate that dental pulp stem cells (DPSCs) exist in the pulp tissue (Gronthos et al. 2002), although the existence of DPSCs is still controversial. DPSCs can be induced to differentiate into odontoblasts, adipocytes, and neural-like cells. Recent evidence has also determined that a cell population of human DPSCs is capable of differentiating into osteoblast precursors (Laino et al. 2005).

Pulpal responses to tooth replantation 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; Tsukamoto-Tanaka et al. 2006). Recently, the notion that dental pulp is composed of different cell derivations has been proposed: cranial neural crest (CNC)- and mesoderm-derived cells (Goldberg and Smith 2004). This idea is supported by evidence that the condensed dental mesenchyme consists of CNC- and an increasing number of non-CNC-derived cells during tooth development (Chai et al. 2000). Two possibilities are proposed regarding the derivation of bone-forming cells in the replanted pulp: cells migrating from the periodontal tissue and/or resident pulpal mesenchymal cells (Shimizu et al. 2000). Furthermore, certain pulpal progenitor cells are supposed to have the ability to differentiate into osteoblasts with the assistance of osteoclast-lineage cells (Tsukamoto-Tanaka et al. 2006).

It is conceivable that there is no unique phenotypic marker of secretory odontoblasts, and the combination of expression of dentin phosphoprotein (DPP), dentin sialoprotein (DSP),

dentin matrix protein-1 (DMP-1), and nestin may be valuable (Goldberg and Smith 2004). Heat-shock protein (HSP)-25-immunoreactivity is also available as a marker for differentiated odontoblasts during tooth development and under pathological conditions (Ohshima et al. 2000, 2002, 2003; Nakakura-Ohshima et al. 2003; Nakasone et al. 2006). Among them, the intermediate filament nestin could be used as a specific marker for odontoblasts (Terling et al. 1995), because nestin is exclusively expressed in matured odontoblasts in the erupted tooth and its surrounding tissue. Furthermore, odontoblast processes also show nestin-immunoreactivity in addition to their cell bodies (About et al. 2000). This study aimed to clarify the capability of dental pulp to elaborate bone tissue in addition to tubular dentin by the auto-graft tooth transplantation of the coronal portion into the sublingual region using immunohistochemistry for 5-bromo-2'-deoxyuridine (BrdU) as a cell proliferation assay and nestin as an odontoblastic marker, and histochemistry for tartrate-resistant acid phosphatase (TRAP) as a marker for osteoclast-lineage cells (Bonucci and Nanci 2001). Our study provides a comprehensive understanding of dentin-pulp regeneration as well as the possible roles of the deposition of bone matrix after tooth transplantation.

Materials and methods

Auto-graft Tooth Transplantation into the Sublingual Region

All experiments were performed following the Guidelines of the Niigata University Intramural Animal Use and Care Committee. Crlj:CD1 (ICR) mice, 3 weeks old, were used in this study. The upper-right first molar was extracted with a pair of dental tweezers with modification under anesthesia by an intraperitoneal injection of chloral hydrate (350 mg/kg) and the roots and pulp floor were resected with a razor blade. The coronal portion of the resected samples without the periodontal tissue was immediately transplanted into the sublingual region after cutting the ventral side of the tongue, and the section was sutured with a nylon suture. The resected tooth without transplantation was used as a control that was immediately immersed in the following fixatives. To investigate the contribution of the surrounding lingual tissue to hard tissue formation, the teeth that underwent extreme heat (in boiling water for 1 or 2 min) or cold (repetition of freezing with CO_2 spray and thawing), where the complete death of the original pulpal cells was expected, were transplanted into the sublingual region. Furthermore, the auto-graft of the isolated dental pulp and the allo-graft of the incisor teeth without pulp of the littermates were also performed to evaluate the influences of the extreme heat or cold on the transplanted dentin and the contribution of the dentin matrix to hard tissue formation.

Histological Procedure

Materials were collected in groups of five animals at intervals of 1, 3, 5, 7, 10, 14, and 28 days after transplantation (n=35) in addition to the control (n=5), tooth samples that suffered damage (n=10), auto-graft of isolated dental pulp (n=3), and allo-graft of teeth without pulp (n=3). At each stage, the animals were intraperitoneally injected with BrdU (150 mg/kg), and subsequently perfused with physiological saline transcardially followed with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) 2 h after a BrdU injection under deep

anesthesia. The tongues including the transplanted teeth were removed *en bloc* and immersed in the same fixative for an additional 6 h. Following decalcification in a 10% ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) solution for 2 weeks at 4°C, the specimens were embedded in paraffin, and sagittal sections of transplants with surrounding lingual tissues were cut at 5 μm. The paraffin sections were mounted on MAS-coated glass (Matsunami Glass Ind, Osaka, Japan) slides and stained with hematoxylin and eosin (H&E).

Immunohistochemical Analysis

For the immuno-peroxidase procedure, the sections were processed for the Calbiochem BrdU immunohistochemistry system (EMD Biosciences Inc, Darmstadt, Germany) and Nichirei Histofine Simple Stain Mouse MAX-PO (Nichirei Biosciences Inc, Tokyo, Japan) using a mouse anti-nestin monoclonal antibody diluted to 1:500 (Chemicon International Inc, Temecula, CA, USA). Immunohistochemical controls were performed by: 1) replacing the primary antibodies with non-immune serum or PBS; 2) omitting the streptavidin-peroxidase or the MAX-PO solution. These immunostained sections contained no specific immunoreaction.

Histochemical Analysis

For the histochemical demonstration of TRAP activity, the azo-dye method was utilized with slight modification (Tsukamoto-Tanaka et al. 2006). The frozen sections were incubated for 15 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 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.

Statistical Analysis of Cell Proliferation

The number of BrdU-positive cells in the pulp areas including the pulpal horn and chamber and the surrounding lingual tissue of each specimen was calculated. Quantitative analysis was performed in 3 sections for each sample. The data were obtained from the samples of 35 animals (the number in each group was five; the final number of samples was 105 sections), and the grid (100 x 100 μm^2) was selected at random in each area (pulp horn, pulp chamber, and lingual muscle in Fig. 2G). All data were presented as the means and standard deviations (SD) of each group. Furthermore, the number of cells in the pulp chamber among different times after transplantation (1-28 d) was compared using Bonferroni's test [one-way analysis of variance (ANOVA)].

Results

Histological Analysis

The H&E stained paraffin sections clearly demonstrated the two types of hard tissue formation, *i.e.* tubular dentin and bone-like tissue, in the dental pulp chamber of the transplant during the healing process (Fig. 1). The original pulpal tissue remained only in the pulp horn of the coronal portion of the tooth in the control (Fig. 1A). On Day 1, the pulp chamber was mainly occupied by inflammatory lesions including numerous neutrophils and fibrin networks. Neutrophils migrated into the pulp horn and replaced the degenerated odontoblast layer (Fig. 1B, G). On Days 3-28, the pulp tissue was gradually increased in cell density and volume, in concomitant with the reduced number of inflammatory cells (Fig. 1C-F). On Day 3, the different type of cells appeared along the pulp-dentin border in addition to the neutrophils (Fig. 1H). The odontoblast-like cells came to be arranged along the pulp-dentin border and deposited tubular dentin next to the preexisting dentin at the pulp horn on Days 5-7 (Fig. 1I, J). On Days 14-28, bone-like tissue formation occurred in the pulp chamber independently of the tubular dentin (Fig. 1F, K, L). The distinction between dentin and bone-like tissue was easily determined by the existence of dentinal tubules in the case of dentin and cell inclusion in the case of bone-like tissue (Fig. 1J-L).

Cell Proliferation Assay by BrdU Labeling

In the control, the dental pulp hardly contained BrdU-positive cells (data not shown). On Day 1, a small number of BrdU-positive cells was recognized in the pulp chamber, whereas the surrounding lingual muscle tissue contained numerous BrdU-reactive cells (Fig. 2A, B), which were considerably reduced in number after Day 3. On Days 3-28, the number of BrdU-positive cells was consistently very large in the pulp chamber (Fig. 2C–F). The number of BrdU-labeled cells was statistically analyzed separately in three areas such as the

pulp horn, the pulp chamber except for the horn, and the surrounding lingual muscle tissue (Fig. 2G).

Nestin Analysis

In the control group, nestin-immunoreactivity was exclusively expressed in the odontoblasts (data not shown). On Days 1-3, although a nestin-positive reaction was not recognizable in the pulp chamber (data not shown), the newly differentiated odontoblast-like cells in the pulp horn came to reveal intense immunoreactivity for nestin in their cytoplasm after Day 5 (Fig. 3A). On the other hand, the osteoblast-like cells beneath the bone-like tissue matrix did not show any nestin-immunoreactivity (Fig. 3B).

TRAP Analysis

In the control group, the pulp tissue contained no TRAP-positive reaction (data not shown). Tooth transplantation caused the appearance of intense TRAP-positive reactions in the pulp chamber, where developed blood vessels were easily recognizable on Day 5 (Fig. 3C). TRAP-positive cells were occasionally situated at the pulp-dentin border and elongated their cellular processes into the dentinal tubules (Fig. 3C, D), and remained around the bone matrix until Day 28 (Fig. 3E, F).

No bone formation occurred in the transplant of the teeth that underwent extreme heat or cold on Day 14 (Fig. 3G). The volume of the pulp chamber in such a transplant was considerably smaller than untreated transplants, and lingual muscle cells migrated into the pulp horn (Fig. 3H). The tubular dentin formation was also disturbed in the pulp horn where a bone-like tissue phenotype was occasionally recognizable (6 of 10 samples; 2 of 3 in heat for 1 min, 3 of 4 in heat for 2 min, and 1 of 3 in cold). No hard tissue formation was also observed in the transplants 14 days after the auto-graft of the isolated dental pulp and the allo-graft of the incisor teeth without pulp (Fig. 4). Intense TRAP-positive cells appeared in

the mesenchymal tissues near the transplanted dentin in the allo-graft experiment, where they were situated both on the exposed dentin and apart from the dentin matrix (Fig. 4A-D). The transplantation of isolated dental pulp also induced the migration of TRAP-positive cells into the transplant (Fig. 4E, F).

Figure 5 summarizes the chronological changes in the distribution patterns of BrdU-, nestin-, and TRAP-positive cells in the regenerative process of the transplant.

Discussion

The present observation provides direct evidence that the dental pulp contains two types of competent progenitor cells capable of differentiating into either odontoblast- or osteoblast-like cells. The ability of the pulp tissue to induce both tubular dentin and bone-like tissue was reported in a previous study using an isolated dental pulp transplant under the kidney capsule, where osteotypic matrix deposition precedes tubular dentin formation (Braut et al. 2003). The inductive signals from the dental epithelium and the presence of basement membrane are necessary for the differentiation of odontoblasts from CNC-derived dental papilla (Ruch et al. 1995). On the other hand, nestin-positive odontoblast-like cells became to be arranged at the pulp-dentin border to deposit tubular dentin until postoperative Day 7 independently of bone-like tissue formation in this study. Taken together, the existence of scaffolds such as preexisting dentin or osteotypic matrix could be necessary for the differentiation of odontoblast-like cells. This notion is supported by the result that the isolated dental pulp induce no hard tissue formation in the transplant in the current study and the evidence that signals similar to those involved in physiological dentinogenesis are thought to be sequestered within the dentin matrix and released under pathological conditions (Tziafas et al. 2000; Smith and Lesot 2001; Goldberg and Smith 2004). However, the presence of scaffolds and/or signals is not sufficient for the differentiation of odontoblasts in the transplanted tooth.

In the case of tooth replantation, the appearance of osteoclast-lineage cells is associated with the induction of bone-like tissue formation in dental pulp. Once these cells appear at the pulp-dentin border, bone-like matrix deposition can be induced even beneath the preexisting dentin (Tsukamoto-Tanaka et al. 2006), whereas the temporal appearance of dendritic cells there induces the tubular dentin formation (Shimizu et al. 2000; 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 the stimuli received from the external environment (Matsuo and Ray 2004). Therefore, one can speculate that the microenvironment after tooth injury determines the types of cells migrating along the pulp-dentin border. In fact, there is the close relationship between the appearance of intense TRAP-positive cells and the induction of bone-like tissue formation in the current study. These positive cells could be categorized as osteoclast-lineage cells judging from their morphological and histochemical features. Osteoclast-lineage cells are always associated with rich blood vessels. The appearance of osteoclast-lineage cells via the blood vessels may be involved in the induction of bone-like tissue formation in the transplanted tooth.

It cannot be excluded that the host tissue contributed to the bone-tissue formation in the transplants in the previous studies (Zussman 1966; Luostatinen and Ronning 1977; Yamamura 1985; Takei et al. 1988; Inoue and Shimono 1992). Isolated pulp tissue implanted in a variety of sites gives rise to an osteotypic matrix but not to tubular dentin, although we fail to induce the hard tissue formation by the auto-graft of isolated dental pulp in this study. Postnatal stem cells have been isolated from various tissues, including bone marrow, neural tissue, skin, retina, and tooth (Harada et al. 1999; Fuchs and Segre 2000; Bianco et al. 2001; Blau et al. 2001; Gronthos et al. 2002), and bone marrow stromal stem cells have been defined as multipotential adult stem cells (Prockop 1997; Bianco et al. 2001) capable of differentiating into different kinds of cells. In this study to verify the contribution of mesenchymal stem cells in lingual muscle tissue to bone-tissue formation, the complete death of pulp tissue by extreme heat or cold resulted in the lack of bone-like tissue formation. Thus, the presence of surviving pulp tissue is suggested to be necessary for the induction of bone-like tissue formation in transplants with the assistance of osteoclast-lineage cells. This notion is supported by the findings that the allo-graft of the teeth without pulp fail to induce the hard tissue formation in the transplants. However, we can not still exclude the possibility that the progenitor cells from the surrounding lingual tissue migrate into the

pulp chamber and proliferate to give rise to hard tissue-forming cells. Actually, numerous TRAP-positive cells appear from the circulatory system of the host tissue in both the auto- and allo-graft transplants. The allo-graft transplantation experiments using green fluorescent protein (GFP) or ROSA26 reporter mice are necessary to certainly exclude this possibility. The exact control of cell proliferation and their differentiation in pulp tissue is necessary for biological regenerative endodontic therapy in the future, because teeth containing bone-like tissue deposition in the dental pulp could easily suffer root resorption and/or ankylosis (Shimizu et al. 2000; Tsukamoto-Tanaka et al. 2006; Tate et al. 2006). There are still numerous fundamental questions to be addressed for our understanding of the biological properties of dental pulp. Further studies using various approaches will provide useful information on the mechanism of regulating the dentin-pulp and bone matrix regeneration.

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References

About I, Laurent-Maquin D, Lendahl U, Mitsiadis TA (2000) Nestin expression in embryonic and adult human teeth under normal and pathological conditions. Am J Pathol 157:287-295

Bianco P, Riminucci M, Gronthos S, Robey PG (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 19:180-192

Blau HM, Brazelton TR, Weimann JM (2001) The evolving concept of a stem cell: entity or function? Cell 105:829-841

Bonucci E, Nanci A (2001) Alkaline phosphatase and tartrate-resistant acid phosphatase in osteoblasts of normal and pathologic bone. Ital J Anat Embryol 106:129-133

Braut A, Kollar EJ, Mina M (2003) Analysis of the odontogenic and osteogenic potentials of dental pulp in vivo using a Col1a1-2.3-GFP transgene. Int J Dev Biol 47:281-292

Byers MR, Kvinnsland I, Bothwell M (1992) Analysis of low affinity nerve growth factor receptor during pulpal healing and regeneration of myelinated and unmyelinated axons in replanted teeth. J Comp Neurol 326:470-484

Chai Y, Jiang X, Ito Y, Bringas P Jr, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM (2000) Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 127:1671-1679

Fuchs E, Segre JA (2000) Stem cells: a new lease on life. Cell 100:143-155

Goldberg M, Smith AJ (2004) Cells and extracellular matrices of dentin and pulp: a biological basis for repair and tissue engineering. Crit Rev Oral Biol Med 15:13-27

Gronthos S, Brahim J, Li W, Fisher L W, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S (2002) Stem cell properties of human dental pulp stem cells. J Dent Res 81:531-535

Harada H, Kettunen P, Jung HS, Mustonen T, Wang YA, Thesleff I (1999) Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. J Cell Biol 147:105-120

Inoue T, Shimono M (1992) Repair dentinogenesis following transplantation into normal and germ- free animals. Proc Finn Dent Soc 88:183-194

Kvinnsland I, Heyeraas KJ, Byers MR (1991) Regeneration of calcitonin gene-related peptide immunoreactive nerves in replanted rat molars and their supporting tissues. Arch Oral Biol 36:815-826

Laino G, d'Aquino R, Graziano A, Lanza V, Carinci F, Naro F, Pirozzi G, Papaccio G (2005) A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). J Bone Miner Res 20:1394-1402

Luostarinen V, Ronning O (1977) Differences in the osteoinductive potentialof transplanted isogeneic dental structures of the rat. Acta Anat 99:76-83

Matsuo K, Ray N (2004) Osteoclasts, mononuclear phagocytes, and c-Fos: new insight into osteoimmunology. Keio J Med 53:78-84

Nakakura-Ohshima K, Watanabe J, Kenmotsu S, Ohshima H (2003) Possible role of immunocompetent cells and the expression of heat shock protein-25 in the process of pulpal regeneration after tooth injury in rat molars. J Electron Microsc 52:581-591

Nakasone N, Yoshie H, Ohshima H (2006) An immunohistochemical study of the expression of heat-shock protein-25 and cell proliferation in the dental pulp and enamel organ during odontogenesis in rat molars. Arch Oral Biol 51:378-386

Nanci A (2003) Ten Cate’s Oral Histology: development, structure, and formation, 6th ed. Mosby, St Louis, pp 397-416

Ohshima H, Ajima H, Kawano Y, Nozawa-Inoue K, Wakisaka S, Maeda T (2000) Transient expression of heat shock protein (HSP)-25 in the dental pulp and enamel organ during odontogenesis in the rat incisor. Arch Histol Cytol 63:381-395

Ohshima H, Nakakura-Ohshima K, Yamamoto H, Maeda T (2001) Alteration in the expression of heat shock protein (HSP)-25-immunoreactivity in the dental pulp of rat molars following tooth replantation. Arch Histol Cytol 64:425-437

Ohshima H, Nakakura-Ohshima K, Maeda T (2002) Expression of heat-shock protein 25 immunoreactivity in the dental pulp and enamel organ during odontogenesis in the rat molar. Connect Tissue Res 43:220-223

Ohshima H, Nakakura-Ohshima K, Takeuchi K, Hoshino M, Takano Y, Maeda T (2003) Pulpal regeneration after cavity preparation, with special reference to close spatio-relationships between odontoblasts and immunocompetent cells. Microsc Res Tech 60:483-490

Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71-74.

Rungvechvuttivittaya S, Okiji T, Suda H (1998) Responses of macrophage-associated antigen-expressing cells in the dental pulp of rat molars to experimental tooth replantation. Arch Oral Biol 43:701-710

Ruch JV, Lesot H, Begue-Kirn C (1995) Odontoblast differentiation. Int J Dev Biol 39:51-68

Shimizu A, Nakakura-Ohshima K, Noda T, Maeda T, Ohshima H (2000) Responses of immunocompetent cells in the dental pulp to replantation during the regeneration process in rat molars. Cell Tissue Res 302:221-233

Smith AJ (2002) Dentin formation and repair. In: Hargreaves KM and Goodis HE (ed) Seltzer and Bender's dental pulp. Quintessence Publishing Co, Chicago, pp 41-62

Smith AJ, Lesot H (2001) Induction and regulation of crown dentinogenesis: embryonic events as a template for dental tissue repair? Crit Rev Oral Biol Med 12:425-437

Takei K, Inoue T, Shimono M, Yamamura T (1988) An experimentalstudy of dentinogenesis in autografted dental pulp in rats. Bull Tokyo Dent Coll 29:9-19

Tate Y, Yoshiba K, Yoshiba N, Iwaku M, Okiji T, Ohshima H (2006) Odontoblast responses to GaAlAs laser irradiation in rat molars: an experimental study using heat-shock protein-25-immunohistochemistry. Eur J Oral Sci 114:50-57

Terling C, Rass A, Mitsiadis TA, Fried K, Lendahl U, Wroblewski J (1995) Expression of the intermediate filament nestin during rodent tooth development. Int J Dev Biol 39:947-956

Tsukamoto-Tanaka H, Ikegame M, Takagi R, Harada H, Ohshima H (2006) Histochemical and immunocytochemical study on hard tissue formation in dental pulp during the healing process after tooth replantation in rat molars. Cell Tissue Res in press

Tziafas D, Smith AJ, Lesot H (2000) Designing new treatment strategies in vital pulp therapy. J Dent 28:77-92

Yamamura T (1985) Differentiation of pulpal cells and inductive influences of various matrices with reference to pulpal wound healing. J Dent Res 64:530-540

Zussman WV (1966) Osteogenic activity of odontoblasts in transplanted tooth pulps. J Dent Res 45:144-151

Figure legends

Fig. 1. H&E-stained sections of the control (A) and the transplanted teeth at 1 (B, G), 3 (C, H), 5 (D, I), 7 (E, J), and 14 (F, K, L) days after operation. A. The original pulpal tissue remains only in the pulp horn of the coronal portion of the tooth. B. The pulp chamber is mainly occupied by inflammatory lesions including numerous neutrophils and fibrin networks. C-F. The pulp tissue is gradually increased in cell density and volume, in concomitant with the reduced number of inflammatory cells. G. Higher magnification of the boxed area labeled by *G* in B. Neutrophils migrate into the pulp horn and replace the degenerated odontoblast layer (*arrows*). H. Higher magnification of the boxed area labeled by *H* in C. Cells with an irregular shape (*arrowheads*) appear along the pulp-dentin border in addition to the neutrophils. I. Higher magnification of the boxed area labeled by *I* in D. The odontoblast-like cells are arranged along the pulp-dentin border. Note the regenerated blood vessels (*). J, K. Higher magnification of the boxed area labeled by *J* in E and *K* in F. Odontoblast-like cells with columnar-shaped deposit tubular dentin (*TD*) next to the preexisting dentin at the cusped areas. L. Higher magnification of the boxed area labeled by *L* in F. Bone-like tissue (*B*) formation occurs in the pulp chamber. The blue dotted lines show the boundary between the pulp chamber and the surrounding lingual muscle tissue. *D* dentin, *OB* odontoblast-like cells, *PC* pulp chamber, *PH* pulp horn, *bars* 200 μm (A-F), 50 μm (L), 25 μm (G-K).

Fig. 2. BrdU-labeled sections of the transplanted teeth at 1 (A, B), 3 (C), 5 (D), 7 (E) and 14 (F) days after operation, and a bar graph (G) indicating the chronological changes in the number of BrdU-labeled cells per unit area at the different areas. A. No BrdU-positive cells are recognized in the pulp horn. B. The surrounding lingual muscle tissue (LM) contains numerous BrdU-reactive cells. C-F. Numerous BrdU-positive cells are

observed in the pulp chamber. G. High proliferative activities are recognized in the pulp chamber on Days 3-28, whereas the BrdU-labeled cells in the lingual muscle tissue were considerably reduced in number after Day 3. The red dotted lines show the boundary between the pulp chamber and the surrounding lingual muscle tissue. *D* dentin, *PC* pulp chamber, *PH* pulp horn, *TD* tubular dentin, *bars* 100 μm (D, E), 50 μm (A-C, F).

Fig. 3. Nestin-immunoreactivities (A, B), tartrate-resistant acid phosphatase (TRAP)-reactions (C-F), and H&E staining (G, H) in the sections of the transplanted teeth at 5 (C), 14 (A, B, D, G, H), and 28 (E, F) days after operation. A. The newly differentiated odontoblast-like cells (*OB*) in the pulp horn reveal intense immunoreactivity for nestin in their cytoplasm. B. The osteoblast-like cells (OsB) beneath the bone-like tissue matrix do not show any nestin-immunoreactivity. C, D. Intense TRAP-positive cells appear in the pulp chamber, where they are occasionally situated at the pulp-dentin border and elongate their cellular processes into the dentinal tubules (*arrowheads*). E. TRAP-positive reactions are observed in the pulp chamber. F. Higher magnification of the boxed area labeled by *F* in E. TRAP-positive cells remain around the bone matrix. G. No bone formation occurs in the pulp chamber of the tooth that underwent extreme heat. H. Higher magnification of the boxed area labeled by *H* in G. The volume of pulp tissue is considerably small, and lingual muscle cells migrate into the pulp horn (*white arrows*). The blue dotted line shows the boundary between the pulp chamber and the surrounding lingual muscle tissue. *D* dentin, *PC* pulp chamber, *PH* pulp horn, *B* bone-like tissue, *TD* tubular dentin, *Bars* 500 μm (E, G), 100 μm (C), 50 μm (D, F, H), 25 μm (A, B).

Fig. 4. TRAP-reactions (A-E) and H&E staining (F) in the sections of the transplants at 14 days after the auto-graft of the tooth that underwent extreme heat (A, B) or isolated dental pulp (E, F), and the allo-graft of the incisor tooth without pulp (C, D). A, C. Intense TRAP-positive reactions are observed in the mesenchymal tissues in association with the transplanted dentin matrix without enamel covering. No hard tissue formation occurs in the transplants. B, D. Higher magnification of the boxed area labeled by *B* and *D* in A and C, respectively. Intense TRAP-positive cells appear in the mesenchymal tissues near the dentin, where they are situated both on the exposed dentin (*arrows*) and apart from the dentin matrix (*arrowheads*). E, F. No hard tissue formation occurs in the transplant, where intense TRAP-positive cells are recognized (*arrowheads*). The blue dotted line shows the boundary between the transplant and the surrounding lingual muscle tissue. *D* dentin, *Bars* 200 μm (A, C), 100 μm (E, F), 50 μm (B, D).

Fig. 5. A schematic diagram summarizing the chronological changes in the transplant. TRAP-positive cells appear in the pulp chamber where numerous BrdU-labeled cells are observed on Day 5. Tubular dentin deposition (*colored pink*) is recognizable in the pulp horn where nestin-positive, newly differentiated odontoblast-like cells are arranged at the pulp-dentin border on Day 7. Bone-like tissue (*B*) formation occurs in the pulp chamber where TRAP-positive cells are associated with the matrix, independently of tubular dentin formation. *D* dentin, *DP* dental pulp, *E* enamel.