Histological analysis of dental pulp response in immature or mature teeth after extra-oral subcutaneous transplantation into mice dorsum

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Abstract

Purpose: The aim of this study was to assess the response of dental pulp associated with donor or host cells in the pulp chamber and root canal after extra-oral transplantation.

Methods: Wild type or green fluorescent protein (GFP) transgenic first molars from 3-week, 6-week, and 12-week mice were transplanted into the subcutaneous layer of GFP mice or wild type mice. The teeth were histologically and immunohistochemically examined at 5 weeks after transplantation.

Results: Blood vessels present in the original coronal pulp had anastomosed with those from the recipient tissue that had invaded the root canal. Two distinct eosin-stained extracellular matrices were observed in the pulp chamber and root canal. Acellular matrix composed of nestin-positive, odontoblast-like cells invaded from the outside and was seen in the root canal of 3-week teeth. Cellular matrix comprising alkaline phosphatase (ALP)-positive fibroblast-like cells appeared in the original coronal pulp. In the root canal of the 6-week and 12-week teeth, cellular extracellular matrix consisting of ALP-positive fibroblast-like cells had invaded the recipient tissue.

Conclusion: Dental pulp from immature teeth might be able to regenerate dentin-like tissue. This model could be useful in the development of an optimized vitalization treatment.

Keywords: dental pulp, extra-oral transplantation, GFP transgenic mice, immature teeth, mature teeth, regeneration

Introduction

Root canal treatment (RCT) is required when the dental pulp becomes partially or completely necrotic because of dental trauma or pulp infection. Conventional RCT is routinely performed for permanent teeth with high tooth survival rates [1]. However, teeth without vital pulp lose their defensive ability and become increasingly vulnerable to external forces. Furthermore, through RCT, complete cleaning and shaping as well as appropriate obturation cannot be achieved for immature roots, resulting in open apical foramen, large root canal, and thin canal wall [2,3]. This decreased protection is followed by tooth fracture and ultimately tooth extraction [2,4].

Traditionally, apexification has been used to treat permanent teeth with immature roots with necrotic pulp. This procedure promotes the formation of a calcified barrier that closes the apex before root canal filling [2,4,5]. However, it does not allow for any further root development in width and length of the dentin walls and a healthy dental pulp is important for the completion of root development in permanent teeth with immature roots [5-7]. Therefore, for permanent teeth with immature roots, pulp vitality must be maintained for continuous root development and apical closure [5-7].

Research has been increasingly focused on the development of biology-based alternative treatment approaches such as revitalization, which is aimed at regeneration of the dental-pulp complex [7-10]. Initially, the term “revascularization” was used for the process [11]; however, later the term “revitalization” was proposed as the more appropriate term, as the canal space contains not only blood vessels but also hard and soft tissues [9,12]. Revitalization treatment is based on the premise that in the absence of bacteria and the presence of a suitable scaffold and stem/progenitor cells in the root canal, a bacterial-tight seal may be created [8-11]. The protocol involves disinfection of the root canal followed by establishing bleeding into the root canal through over-instrumentation [8-11]. After vitalization treatment, continued root development and hard tissue deposition on the dentinal wall occur under ideal circumstances [8-12]. Several studies and case reports demonstrated clinically successful revitalization in vivo [12-14]. Periapical tissues around immature roots have a rich blood supply and contain stem cells that can regenerate hard and soft tissues [15-18]. Compared with necrotic immature roots, revitalization treatment of permanent teeth with mature roots proves a challenge because of the scarcity or absence of apical papilla stem cells in mature roots, which are thought to play an important role in dentin/pulp regeneration [12,14,17,18]. Recently, several histological studies in animals reported that the tissues formed in the canals of revitalized teeth are, in fact, cementum, bone, and periodontium rather than dentin-pulp complex [10,18-20]. Therefore, since the currently available information does not establish evidence-based methods [19], further animal experimental studies are required to elucidate the revitalized dentin-pulp complex and optimize revitalization treatment because histological analyses cannot show the regenerated pulp and dentin when revitalization treatment is clinically practiced. Experimental revitalization treatments have often been performed on standard laboratory animals such as rats and ferrets [19,21-24]. However, it is difficult to perform pulpectomies and revitalization treatment on these animals because of insularity and lack of instrumentation flexibility within the oral cavity in animals. These limitations may delay progress in revitalization treatment research.

Several extra-oral tooth transplantation models involve rats and mice. The abdominal subcutaneous layer of transplantable sockets is germ-free and the transplanted tooth displays no signs of infection. As revitalization can disclose infection-related inflammation, extra-oral transplantation is a useful and reproducible model [4,8,11,12]. Two studies demonstrated that hard tissues formed in the root canal of immature roots after extra-oral transplantation in rat abdominal subcutaneous [22,23]. However, one of the aforementioned studies did not examine the presence of dentin or odontoblasts in the regenerated tissue of root canal [22] while the other did not investigate the origin of odontoblasts or blood vessels in the regenerated tissue of the root canal [23]. To establish the vitalization treatment in permanent teeth with immature roots, understanding dental pulp response in mature roots is vital to the development of regeneration/revitalization and tooth transplantation protocols using animal models. Nevertheless, neither of the preceding studies evaluated dental pulp response in mature roots.

Successful hard and soft tissue regeneration in the root canal system relies heavily on rapid and efficient blood vessel formation after revitalization treatment or tooth transplantation because teeth with large apices involved increased revascularization rates associated with increased

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ingrowth of blood vessels [21,25]. Therefore, apical diameter in immature or mature roots is a significant factor in optimization of revitalization treatment [25,26]. In this study, an extra-oral tooth transplantation model was established to optimize vitalization treatment in immature/mature roots using mice dorsum. The purpose of the study was to determine the contributions of donor and host cells to the regenerated tissue such as blood vessels and hard and soft tissue in the root canal system of immature/mature roots after extra-oral transplantation into mice dorsum. To this end, this study used both wild type and enhanced green fluorescent protein transgenic mice (GFP mice), and investigated donor tissue replacement by host cells in immature/mature roots after tooth transplantation using GFP mice.

**Materials and Methods**

**Animals**

All animal experimental procedures were performed in accordance with institutional animal care guidelines. Ethical approval was secured from the Animal Research Committee of the School of Dentistry of Aichi Gakuin University (approval number AGUD399). Wild type C57BL/6J mice or transgenic C57BL/6 mice expressing GFP (C57BL/6-Tg [CAG-EGFP]; hereafter, GFP mice) were obtained from Chubu Kagaku Shizai Co., Ltd., (Nagoya, Japan) or Japan SLC, Inc. (Hamamatsu, Japan), respectively. Protocols for the recombinant deoxyribonucleic acid (DNA) experiments conducted on the GFP mice were approved by the Safety Committee of Aichi Gakuin University (approval number 18-1). The animals were housed at the Animal Experimental Center of the School of Dentistry, Aichi Gakuin University.

**Measurements of root length and apical constriction diameter**

To determine the time of completion of tooth root development, root length and apical constriction diameter were measured in three dimensions by μ-CT imaging (Cosmo Scan GX, Rigaku Corp., Tokyo, Japan). Three-week male C57BL/6 mice (n = 6) were anesthetized by intraperitoneal injection of a mixture of medetomidine hydrochloride (0.75 mg/kg; Meiji Seika Pharma, Tokyo, Japan), midazolam (4.0 mg/kg; Astellas Pharma Inc., Tokyo, Japan), and butorphanol tartrate (5 mg/kg; Meiji Seika Pharma). The root length and apical constriction diameter of the mandibular first molars were measured by μ-CT imaging (Cosmo Scan GX, Rigaku Corp.). The exposure parameters were 4 min, 90 kV, and 100 mA. The weekly isotropic voxel size from 3-14 weeks was 5 mm. The axes were standardized and the mesial root length and canal apex (apical constriction) were measured.

**Tooth transplantation into mouse dorsal subcutaneous pockets**

Three-week, 6-week, and 12-week male C57BL mice (n = 12 each) or GFP mice (n = 13 each) were used to evaluate dental pulp response after transplantation into the dorsal subcutaneous pockets of 3-week male GFP mice (n = 36) or C57BL mice (n = 39), respectively. Deep anesthesia with a mixture of medetomidine hydrochloride (0.75 mg/kg; Meiji Seika Pharma), midazolam (4.0 mg/kg; Astellas Pharma Inc.), and butorphanol tartrate (5 mg/kg; Meiji Seika Pharma) was induced and the mandibular first molars were extracted with modified dental forceps. Blood clots and bone particles were removed and teeth with root fractures were discarded. Dorsal subcutaneous pockets were prepared by blunt dissection during the extra-oral period of the extracted teeth. Extracted teeth from either GFP or wild type mice were inserted through the skin at a certain distance (10 mm) from the incision in either 3-week GFP or 3-week wild type mice. The incision was then closed with nylon suture. All extracted teeth were transplanted subcutaneously for 5 weeks.

**Histology**

Transplanted teeth were harvested with the surrounding skin from wild type and GFP mice at 5 weeks after transplantation. The transplants were fixed in 4% (v/v) paraformaldehyde for 1 day and decalcified in 10% (w/v) EDTA-2Na (Muto Pure Chemicals, Tokyo, Japan) for 3 weeks. The specimens were embedded in paraffin and sagittal sections were cut to 5-μm thickness. The sections were then stained with hematoxylin and eosin (H&E).

**Immunofluorescence and Immunohistochemistry (IHC)**

For immunofluorescence, the tissue sections were blocked with 2.5% (v/v) normal horse serum (Vector Laboratories, Burlingame, CA, USA) for 60 min at 25°C and incubated overnight at 4°C with the following primary antibodies: chicken polyclonal anti-nestin antibody (NB100-1604, 1:10,000; Novus Biologicals, Centennial, CO, USA) to detect odontoblasts and rabbit polyclonal anti-GFP antibody (ab290, 1:1,000; Abcam, Cambridge, UK) to detect GFP mouse cells. The cells were then stained with the following fluorescent dye-labeled secondary antibodies: Alexa Fluor 488-labeled anti-rabbit (ab150073, 1:200; Abcam) and Alexa Fluor 594-labeled anti-chicken secondary antibodies (A11042, 1:200; Thermo Fisher Scientific, Waltham, MA, USA) for 60 min at 25°C. They were then counterstained with 1 μg/mL of 4',6-diamidino-2-phenylindole (DAPI; Dojindo, Kamimashiki-gun, Japan). Images were captured with a BZ-X710 fluorescence microscope (Keyence, Osaka, Japan).

For the IHC, endogenous peroxidase activity was quenched with 3% (v/v) hydrogen peroxide in methanol for 30 min and the sections were blocked with 2.5% (v/v) normal horse serum (Vector Laboratories) for 60 min at 25°C. The sections were then incubated overnight at 4°C with rabbit polyclonal anti-periostin antibody (ab14041, 1:800; Abcam) to detect periodontal ligament and cementoblasts. They were then incubated with an ImmPRESS HRP anti-rabbit IgG (peroxidase) polymer detection kit (Vector Laboratories) at room temperature for 60 min at 25°C. They were then counterstained with 1 μg/mL of 4',6-diamidino-2-phenylindole (DAPI; Dojindo, Kamimashiki-gun, Japan). Images were captured with a BZ-X710 fluorescence microscope (Keyence, Osaka, Japan).

To observe the osteoblasts and osteoclasts, the sections were stained with alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP; Sept. Sapie Co., Ltd, Tokyo, Japan).

**Results**

**Measurements of root length and apical constriction diameter**

The mandibular first molars had two slightly divergent roots and had reached maturity. The mean mesial root lengths were 0.94 ± 0.02 mm at 3 weeks, 1.42 ± 0.02 mm at 6 weeks, 1.58 ± 0.01 mm at 8 weeks, and 1.67 ± 0.02 mm at 12 weeks. The mean apical foramen diameters were 201 ± 3 μm at 12 weeks (Fig. 1). Hence, tooth root development was completed by 8 weeks. Based on the foregoing results, this study selected three types of teeth, namely, those from 3-week and 6-week mice as immature roots (hereafter, 3 wk teeth and 6-wk teeth) and those from 12-week mice as mature roots (hereafter, 12-wk teeth).

**Histology of the three types of teeth**

All three types of teeth exhibited normal histology before transplantation. The root canal system in the space within the tooth contains pulp tissue. The root canal system is divided into two portions: the pulp chamber, which
is located in the anatomic crown of the tooth, and the root canal, encased in the root [27]. The apical region of the 3-wk teeth showed that the apex aperture was very wide. The root canal system in 6-wk teeth was smaller than that in the 3-wk teeth (Fig. 2). Root formation was more complete and dentin walls and cementum were thicker in the 12-wk than the 6-wk teeth.

High magnification disclosed that the pulps comprised the odontoblast layer in the root canal system (Fig. 3a, b) and nestin immunoreactivity was exclusively expressed in the odontoblasts (Fig. 3c, d). For teeth of all ages, all other cells in the dental pulp were negative for nestin (Data for 6-wk and 12-wk teeth are not shown).

Macroscopic observation
None of the mice lost body weight throughout the experimental period. No visible complications were observed in any of the mice. Immediately after transplantation of the extracted teeth on the muscle under the subcutaneous layer, no blood vessels formation was observed. However, each tooth was surrounded by granulation tissue and many newly formed blood vessels by 5 weeks after transplantation (Fig. 4).

Presence of vital tissue in the root canal system of transplanted tooth
Histological sections revealed that after transplantation, vital tissues with blood vessels were present and eosin-stained extracellular matrix was deposited in the root canal system from all three groups. Matrix formation and pulp necrosis data are summarized in Table 1. Vital tissues were found in 68.0% of the 3-wk- and 6-wk teeth and their abundance differed from that observed in the 12-wk teeth.

Histological structures revealed eosin-stained cellular and acellular extracellular matrices in the root canal system, respectively, for all groups (Fig. 5). Hence, there were three distinct dental pulp responses. In all groups, several ectopic cellular matrix islands were observed in the pulp chamber. The cellular matrix filled the half pulp chamber from the 6-wk teeth and the entire pulp chamber from the 12-wk teeth. The root canal displayed a continuous acellular matrix on the dentinal walls of the 3-wk teeth. In contrast, the root canal exhibited a continuous cellular matrix on the dentinal walls of the 6-wk- and 12-wk teeth. The canal walls were covered by thick cellular matrix at the root apex and the latter was open. There were no signs of internal or external root resorption in any of the transplanted teeth.

Fig. 2 Representative histological images in 3-week, 6-week, and 12-week teeth in mandible before transplantation. (a) Histological sections reveal that the mesial and distal root apices were open in 3-week tooth. (b) Relative root elongation in 6-week tooth. Root canal was narrower in 6-week than 3-week tooth. (c) Root development was completed in 12-week tooth. Scale bars: 1 mm

Fig. 3 High-magnification images of normal dental pulp from mandibular first molar in 3-week teeth stained with H&E (a, b) and nestin (c, d). (a) Normal appearance of coronal pulp with odontoblastic layer. (b) Normal appearance of pulp with large central blood vessels (arrow) and intact odontoblastic layer in root canal. (c, d) Nestin immunoreactivity (red) exclusively expressed in odontoblastic layer of pulp in pulp chamber and root canal in 3-week teeth (red). Scale bars: 100 μm

Fig. 4 Macroscopic observations of subcutaneously transplanted teeth. (a) Three-week teeth placed under subcutaneous layer in 3-week mice. No blood vessels around transplanted teeth immediately after transplantation. (b-d) Numerous newly-formed blood vessels observed in the periphery of the transplanted teeth at 3-week (b), 6-week (c) and 12-week (d) teeth after transplantation. Scale bar: 1 mm

Origins of the cell populations in the root canal system after 5 weeks transplantation
Vital tissues were observed in all groups but most odontoblasts had degenerated and the cuboidal cells had disappeared. The origin of the tissue-forming cells was examined using GFP mice as the donors or hosts. When the GFP mice were the donors and the wild type mice were the hosts, green-stained cells were detected in the endothelial and other cell types in the coronal pulp from all transplanted teeth (Figs. 6-11). In contrast, cells from the GFP mice did not express green color in the root canals from all groups. However, GFP mouse fibroblasts and endothelial cells expressed green color in the root canals from all groups (Figs. 6-11). Thus, vital tissues such as blood vessels and the eosin-stained extracellular matrix in the root canal had been replaced by host cells.

Characterization of the hard tissue in the pulp chamber at 5 weeks after transplantation
Eosin-stained extracellular matrix containing numerous cells was observed after transplantation in the ectopic space independently of tubular dentin and the continuous tubular dentin layer of all groups (Figs. 6-11). However, neither matrix contained tubular structures. The cellular matrix was stained with ALP, nestin, perioistin, or TRAP and examined to distinguish
among dentin, bone, and cementum. The flattened cells appressed to the cellular matrix showed no nestin reaction (Fig. 12) but did present with an ALP-positive reaction (Fig. 13; data not shown for 3-wk- or 6-wk teeth).

The periodontal ligament intensely expressed periostin whereas the dental pulp did not. After transplantation, none of the cells showed periostin reactions in any of the groups (Fig. 14). TRAP-positive multinucleated cells were detected on the alveolar bone surface in the dental bifurcation area. However, none of the cells showed TRAP reactions on the surfaces of the cellular matrices of all groups (Fig. 15).

Characterization of the hard tissue in the root canal at 5 weeks after transplantation

Two different eosin-stained extracellular matrices were observed in the root canals from all groups (Figs. 6-11). An acellular matrix was deposited at the root dentinal wall in 3-wk teeth and cuboidal odontoblast-like cells were aligned with the matrix surface. A cellular matrix was appressed to the root dentinal walls in the 6-wk- and 12-wk teeth. The dentin and hard tissues were distinguished by the presence of nestin-positive cells. The cuboidal cells displayed nestin immunoreactivity (Fig. 12). However, in the 6-wk- and 12-wk teeth, the flat cells adjacent to the cellular matrix showed no nestin immunoreactivity but positively reacted to ALP staining (Fig. 13). However, the flat cells did not react with anti-periostin or TRAP stain (Figs. 14, 15).

Characterization of the hard tissue in the apical portion of root canal at 5 weeks after transplantation

After transplantation, the apical portion of root canal was open in all groups and pulp-like tissue in the root canal connected with the periodontium outside the apex. The canal walls had thickened and there was ingrowth of the cellular cementum-like matrix. The flat cells were aligned on the matrix surface (Fig. 5). They showed periostin immunoreactivity (Fig. 14) and a positive reaction to ALP (Fig. 13).

Discussion

The aim of this study was to evaluate dental pulp response after extra-oral tooth transplantation into the dorsal subcutaneous layer of mice. A reliable extra-oral tooth transplantation model is required to explore various aspects of endodontic regeneration and revitalization treatment and tooth replantation. Teeth with large apical constrictions present with increased revascularization rates [25,26]. Hence, the diameter of the apical constriction is a significant factor in dental pulp response [25,26]. There have been
no reports on dental root development in mice. Therefore, root length and apical constriction diameter were measured in healthy mice teeth. The images disclosed no major changes in root length over 8-10 weeks. Nevertheless, the apical root length gradually increased after 8 weeks. Histological images demonstrated that root length increased via continuous cementum deposition even after radicular dentin growth was complete.

Three tooth types (3, 6, and 12 weeks) with different apical diameters were transplanted to establish the influence of the preoperative apical constriction diameter. Despite a temporary interruption in the blood supply and root maturation, all transplanted teeth regained circulation. This phenomenon was declared in reports of successful clinical cases in mature teeth on regenerative endodontics [28,29]. In contrast, successful calcification occurred in the root canal system at a higher rate in the 3-week and 6-week mice than the 12-week mice (Table 1). A previous study showed that immature roots are more amenable to dental pulp regeneration than mature roots as the former have wider apical constrictions [26]. Revascularization occurs earlier in immature roots than mature roots [26-29]. When teeth with different apical constriction diameters were transplanted into subcutaneous layer, the obtained outcomes were in accordance with those of earlier studies [30-32]. The results suggested that tooth transplantation into dorsal subcutaneous layer of mouse is a promising and reliable model to evaluate dental pulp response.

Highly vascularized connective tissue ingrowth occurs in pulp revascularization [25,33]. Blood vessels already present in the pulp chamber after transplantation. Then, GFP mice have been used to study targeted cell lineages. When teeth from wild type mice were transplanted into the dorsal subcutaneous layer of GFP mice, the vascular endothelial cells were immunopositive for GFP in the root canal but immunonegative in the pulp chamber. This finding shows that in revascularization, blood vessels present in the original pulp anastomose with those that have invaded from the periodontium.

Recent case reports strongly suggested that the regenerated tissue within the root canal system is not functional pulp consisting of vascular nonspecific connective tissue and hard tissue [8,12,13,16-20,25]. Histological examination here disclosed that two types of eosin-stained extracellular matrices were generated in the root canal system. This discovery was consistent with previous reports [22-24]. An acellular matrix
was appressed to the dentinal wall in the root canals in 3-wk teeth. The matrices were assessed by nestin, ALP, periostin, and TRAP staining which identified odontoblasts, osteoblasts, osteoclasts, and cementoblasts, respectively [34-36]. The specific marker nestin is expressed only by odontoblasts [22,23,35]. Periostin was applied to rule out any possibility that periodontal tissue contributed to the regenerated tissue in the root canal system [35]. The appearance of osteoclasts is associated with induction of bone formation in dental pulp after tooth replantation. The TRAP reaction distinguishes bone [34]. Nestin-positive cells were localized to the acellular matrix in 3-wk teeth but not in 6-wk or 12-wk teeth. This finding was consistent with an earlier report wherein teeth were transplanted into the sublingual region of the tongue [24]. Other markers such as TRAP and periostin showed a negative reaction. The foregoing results suggest that the acellular extracellular matrix is dentin. When wild type teeth were transplanted into GFP mice, the nestin-positive cells lining the matrix were immunopositive for GFP. Therefore, nestin-positive cells could originate from recipient cells arriving via the bloodstream. Peripheral blood contains totipotent stem cells such as bone marrow cells [8,10,11,31,33,35]. However, the exact source of nestin-positive cells remains to be determined. A possible source is systemic blood but not local tissues such as dental pulp, apical papilla, or periodontium [9,10,17,23,30,31,35]. Further experimentation is required to elucidate the mechanism by which totipotent stem cells differentiate into odontoblasts.

![Fig. 12 Identification and derivation of columnar cells in root canal of 3-week teeth at 5 weeks after transplantation. (a, b) Odontoblast-like columnar cells on surface of predentin-like tissue. (c) Columnar cells facing predentin-like tissue expressed nestin and GFP immunoreactivity when wild type teeth were transplanted in GFP mice (host GFP). (d) Columnar cells facing predentin-like tissue expressed only nestin immunoreactivity when GFP teeth were transplanted into wild type mice (donor GFP). (e, f) No nestin-positive cells in pulp chamber. Scale bars: 100 μm](image1)

![Fig. 13 Immunolocalization of tissue-nonspecific alkaline phosphatase (ALP) in 12-week teeth at 5 weeks after transplantation. (a) H&E staining of pulp chamber. (b) H&E staining of root canal. (c) Intense ALP immunoreactivity detected in periphery of eosin-stained extracellular matrix (asterisks). (d) Intense ALP immunoreactivity in periphery of eosin-stained extracellular matrix tissue (asterisks). Weak ALP immunoreactivity in cellular matrix (asterisks). Scale bars: 200 μm](image2)

![Fig. 14 Periostin immunolocalization. (a) Intense periostin immunoreactivity detected in periodontal ligament and cementum. Dental pulp lacked periostin immunoreactivity in normal 3-week teeth. (b) No periostin immunoreactivity in pulp chamber from 3-week teeth after transplantation. (c) Inner space of root apex showed intense periostin immunoreactivity in mesial root of 6-week teeth (arrow). (d) Inner space of root apex showed intense periostin immunoreactivity in distal root of 12-week teeth (arrow). Hard tissues showed negative periostin reaction (asterisk). White scale bar: 200 μm. Black scale bars: 100 μm](image3)

![Fig. 15 Tartrate-resistant acid phosphatase (TRAP) staining. (a) TRAP-positive reactions in red (arrow) in cells. (b) No TRAP-positive cells were localized to the pulp chamber or root canals in 3-week teeth after transplantation. (c) No TRAP-positive cells were localized to the pulp chamber or root canal in 6-week teeth. (d) No TRAP-positive cells were localized to the pulp chamber or root canals in 12-week teeth. Scale bars: 200 μm](image4)
periotin-expressing cells appeared in dental pulp [37]. Ambiguity over the presence or absence of periotin-expressing cells in the root canal system may be attributed to the differences between subcutaneous layer and alveolar sockets as transplantation sites. ALP-positive cells were localized to the pulp chamber and root canals. Calcific metamorphosis is a dental pulp response to trauma characterized by the deposition of hard tissue in the root canal system [38]. Studies have shown that pulpal changes after blood vessel and nerve fiber rupture, impaired odontoblast function, and damage to the neurovascular supply can induce pulp-dentin complex responses and accelerate hard tissue deposition [39]. Eosin-stained cellular matrix might actually be undergoing calcific metamorphosis and eventually become mineralized hard tissue such as cementum or bone. As the observation period in this study was short, long-term observations are required to clarify the nature of the matrix and characterize the matrix-forming cells. Previous studies indicated that these might in fact be undifferentiated periosteal cells, fibroblasts, or odontoblasts [38,39]. The origin of the constituted cells differed between the pulp chamber and root canals. When GFP-expressing teeth were transplanted into the dorsal subcutaneous layer of wild type mice, the cells were GFP-immunopositive in the pulp chamber but not the root canal. This finding indicates that the matrix might have been formed by surviving donor pulp cells differentiating into matrix-forming cells in the pulp chamber. However, the formative cells invaded from the recipient cells arriving via circulation in the root canal. Further studies are required to determine the origin of the recipient cells. It is known that they do not derive from local tissues such as dental pulp, apical papilla, or periodontal tissue.

Two distinct matrices are formed in the root canal system of subcutaneous transplanted teeth. The two hard tissues are distinguished by the presence or absence of nestin-positive cells. Relative differences in apical constriction diameter might account for their different appearances. In fact, apical constriction diameter might affect the characterization of newly formed hard tissue [25]. Calcific metamorphosis may be the mechanism by which dental pulp heals after replantation of avulsed immature permanent teeth. The mouse model presented here could help clarify the mechanism by which calcific metamorphosis occurs after dental trauma.

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Conflict of interest
None.

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By which calcific metamorphosis occurs after dental trauma.

Which dental pulp heals after replantation of avulsed immature permanent teeth?

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