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ORIGINAL ARTICLE

Root development of rat tooth germs implanted in the tooth socket and in the subcutaneous tissue

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OBJECTIVE: This study was designed to investigate root development of a rat tooth germ implanted in a tooth socket or in a subcutaneous region.
MATERIALS AND METHODS: Tooth germs of the upper left first molars in 2-week-old rats were extracted and implanted in the original tooth socket or in the subcutaneous region of the back. The upper right first molar was used as a control. The rats were fixed in weeks 1, 2, 4, 8 and 12. The root development was examined quantitatively with X-ray radiographic morphometry. The cellular activity of producing matrix proteins was assessed using *in situ* hybridization for type I collagen.
RESULTS: Root development was observed in the implanted teeth in the tooth socket as also in the control teeth. In contrast, roots hardly developed in subcutaneously implanted teeth. Histology showed that periodontal ligaments were arranged around roots of implanted teeth in the tooth socket as around control teeth, but few periodontal ligaments were identified in the subcutaneous implantation. Dentin and cementum formed in both the implanted teeth as also in the control teeth and odontoblasts, cementoblasts and cementocytes expressed type I collagen.
CONCLUSION: Tooth sockets may possess specific environments that allow root development of a tooth germ.
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Keywords: tooth germs; root development; implantation; rats; type I collagen; *in situ* hybridization

Introduction

Implantation of tooth germs or developing teeth provides various prospects for clinical application. The implanted tooth germs are clinically expected to develop roots and alveolar bone to restore occlusion while keeping the periodontal ligaments functional (Hernandez and Cuestas-Carnero, 1988; Lundberg and Isaksson, 1996; Bauss *et al*, 2005). In contrast, biological information about how tooth roots, periodontal ligaments and alveolar bone develop after implantation is limited. Our previous study showed that roots develop when rat tooth germs or immature teeth with undeveloped roots were implanted in tooth sockets (Akiba *et al*, 2006). However, details are not understood about the process of root elongation and formation of periodontal tissues i.e. cementum, periodontal ligaments and alveolar bone of the implanted teeth. Furthermore, it is not known whether the site of implantation affects root development and periodontal tissue formation.

This study was designed to investigate the process of root development and periodontal tissue formation of rat tooth germs implanted in tooth sockets comparing with that of control tooth germs, then with tooth germs implanted in subcutaneous tissue of the back to examine if the difference in implantation sites affects the root development and periodontal tissue formation.

The root elongation was quantified using X-ray radiographic morphometry. The periodontal tissue formation was examined histologically. The cellular activity of producing matrix proteins in odontoblasts, cementoblasts, cementocytes, osteoblasts, osteocytes and periodontal ligament cells was assessed using *in situ* hybridization (ISH) for type I collagen (Sasano *et al*, 2001).

Materials and methods

Animals and implantation

The guidelines for animal users (NIH Animal Research Advisory Committee, 2000) were followed as well as specific national laws. The preparation was performed according to animal protocols that were institutionally approved by Tohoku University. Ninety-six male Wistar rats were used in this study at 2 weeks postnatally, which was designated as week 0. The rats were

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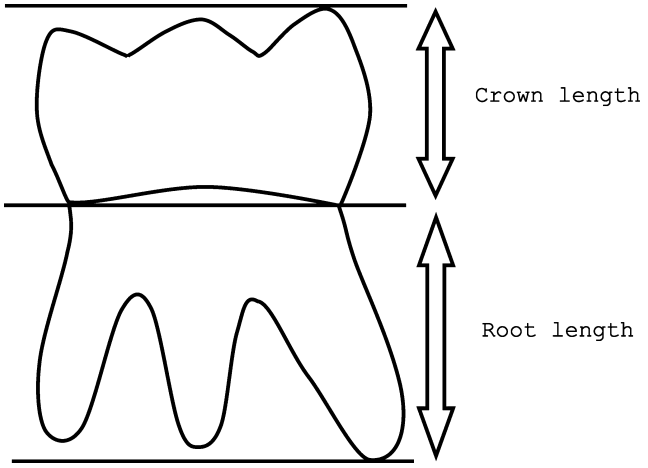

Figure 1 The distance between the cemento-enamel junction and the tip of the highest cusp on the X-ray radiograph is defined as the crown length of a specimen, whereas the distance between the cemento-enamel junction and the apex of the longest root is defined as the root length. The proportion of the root length to the crown length is used as a standard to evaluate root length of different specimens

anesthetized with sodium pentobarbital (12.5 mg kg^{-1}) intraperitoneally with supplemental ether inhalation.

The upper left first molar was extracted with a special-attention not-to-touch dental papilla, dental sac and Hertwig's epithelial root sheath by holding a crown using fine forceps, and then immediately returned into the original tooth socket. Otherwise, the extracted upper left first molar was implanted into the subcutaneous region of the back. The upper right first molar was used as a control. All the experiments were carried out under aseptic conditions. The rats were kept with a soft diet.

Table 1 The number of rat first molars used for the experiment

Weeks	*Implantation Tooth socket*	*Implantation Subcutaneous region*	*Control*
0			2
1	5	4	5
2	6	4	6
4	6	4	8
8	4	4	6
12	4	4	6
Total	25	20	33

Tissue preparation

The rats were fixed with 4% paraformaldehyde – 0.5% glutaraldehyde by perfusion through the aorta at 1, 2, 4, 8 and 12 weeks after implantation. The maxillae and subcutaneous tissues including implanted and control teeth were resected and kept in the same fixative overnight at 4°C.

The specimens examined with X-ray radiographic morphometry were decalcified and embedded in paraffin, serial sections were cut and adjacent sections were stained with hematoxylin–eosin or processed for ISH for type I collagen. Some teeth implanted in the

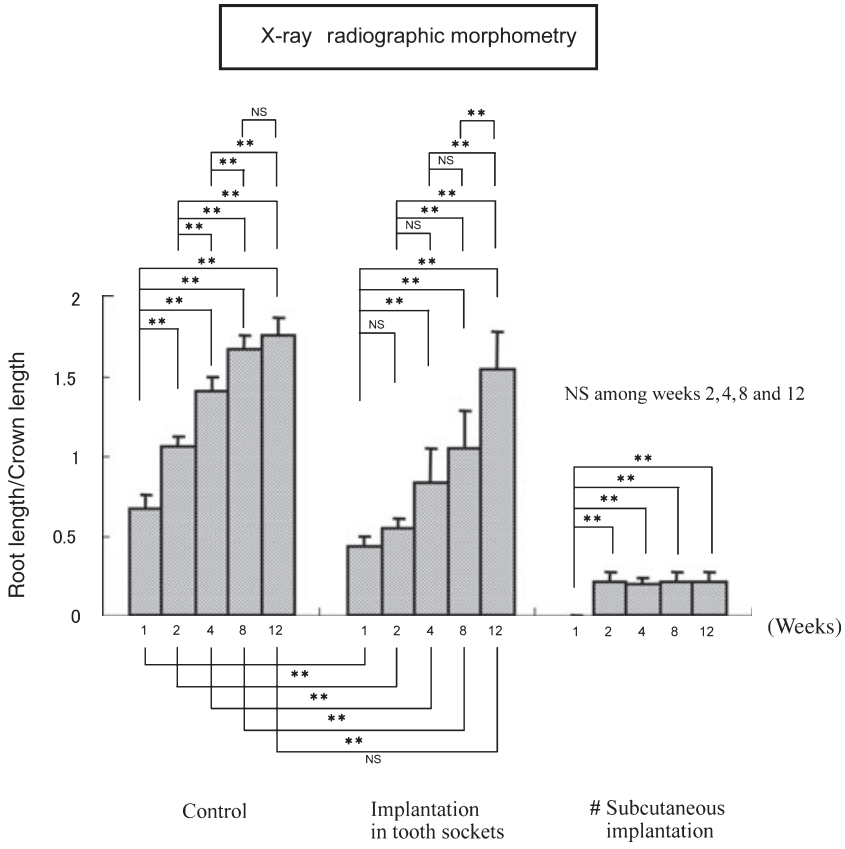

Figure 2 The root length of subcutaneously implanted teeth was significantly smaller than that of corresponding implanted teeth in tooth sockets and control teeth. $^{**}P < 0.01$ and NS, not significant

Figure 3 X-ray radiograph in week 0. The crown shape formation is completed but the root is not identifiable. Arrowheads: a cemento-enamel junction, double arrowheads: the tip of the highest cusp

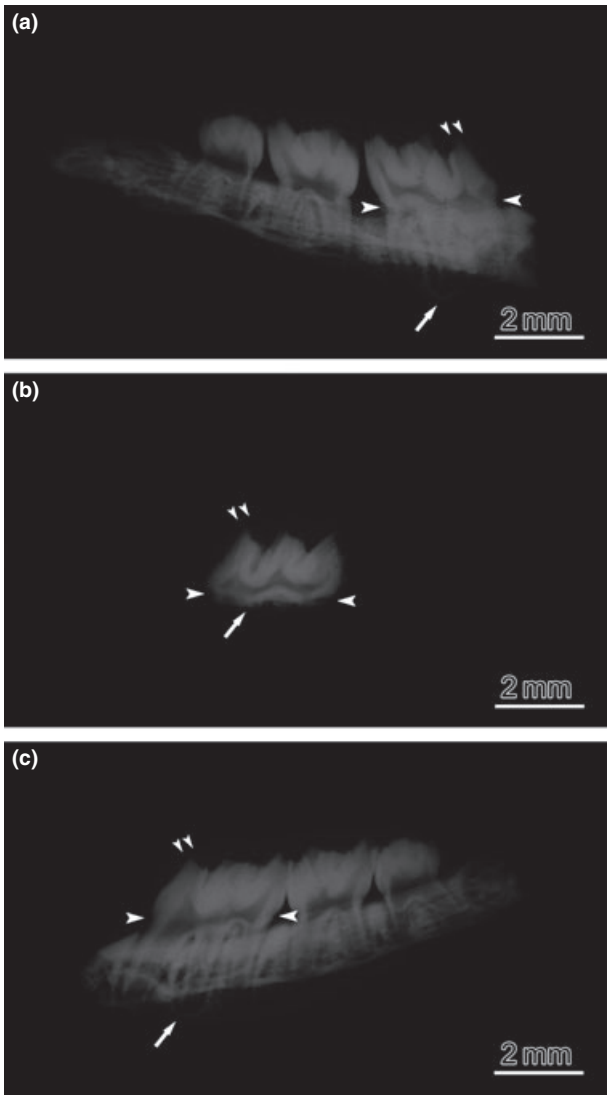

Figure 4 X-ray radiograph in week 4. Roots elongate in teeth implanted in the tooth socket (**a**) as well as in control teeth (**c**), whereas root elongation is hardly identified in teeth implanted subcutaneously (**b**). Arrowheads: a cemento-enamel junction, double arrowheads: the tip of the highest cusp, arrow: the apex of the longest root

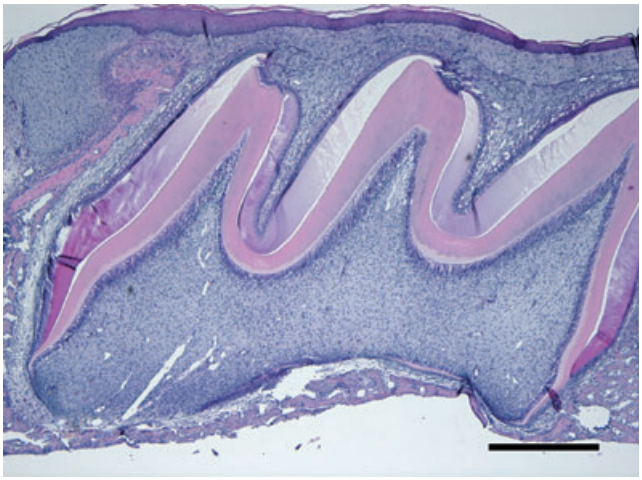

Figure 5 Histology in week 0. Formation of the crown shape is almost completed, whereas root formation is hardly identified. Scale bar = 500 μm

subcutaneous region were processed specifically for radiographic morphometry after surrounding connective tissues were removed without any fixation.

X-ray radiographic morphometry

The teeth in maxillae or the teeth surrounded by subcutaneous tissues were radiographed by means of a microradiography unit (Softex CMR Unit; Softex, Tokyo, Japan) with X-ray film (FR; Fuji photo film, Tokyo, Japan) under standardized conditions (20 kV, 5 mA, 2 min). The X-ray radiographed films were scanned at 300 dots per inch in a flatbed scanner (FS-8000, Seiko Epson Corp., Suwa, Japan) and the root development was examined quantitatively.

The cemento-enamel junction was defined as a boundary between the crown and the root on the X-ray radiograph. The distance between the cemento-enamel junction and the tip of the highest cusp was defined as the crown length of a specimen, whereas the distance between the cemento-enamel junction and the apex of the longest root was defined as the root length. The longest root was considered to represent roots of a tooth. The proportion of the root length to the crown length was used as a standard to evaluate the root length among different specimens (Figure 1).

Fifty-five 2-week-old rats were used. Twenty-five upper left first molars were used for implantation into the tooth socket, 20 were for implantation in the subcutaneous region and 33 upper right first molars were for the control (Table 1).

One-way analysis of variance (ANOVA) was used to compare the mean values between the groups of both implanted teeth and control teeth. *Post hoc* analyses were performed using Tukey's test, with the level of statistical significance taken as $P < 0.05$(Stat flex 5.0; Artech, Osaka, Japan).

In situ *hybridization*

The protocol has been reported elsewhere (Nakamura *et al*, 2005) and is briefly described as follows:

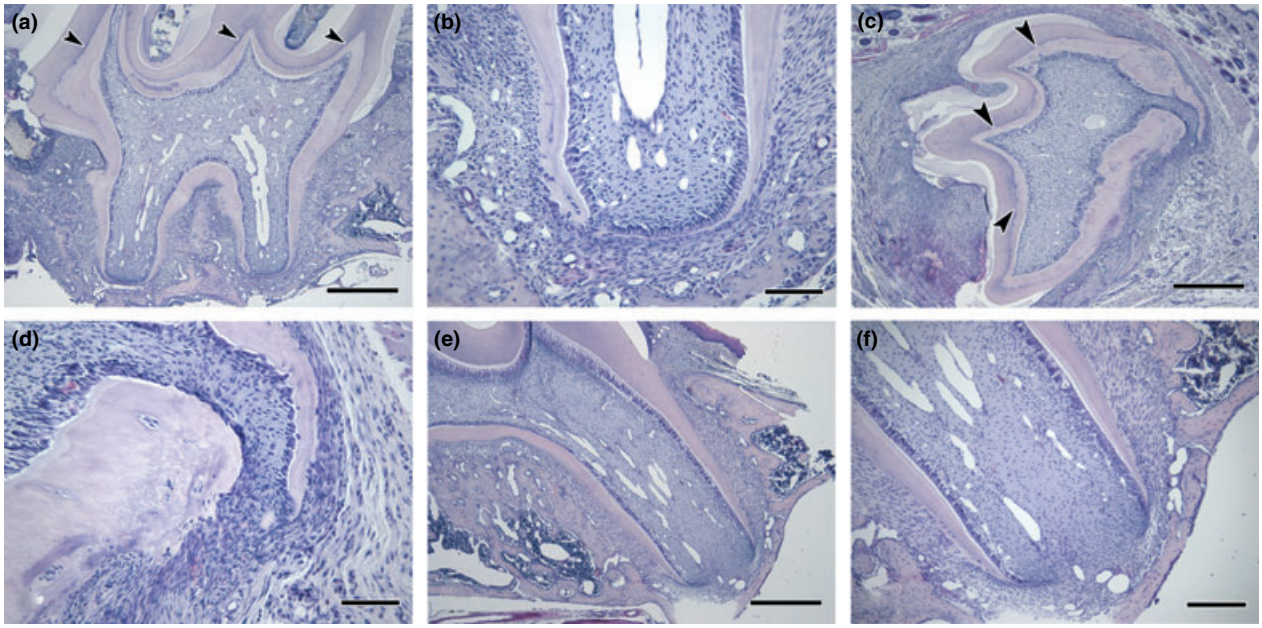

Figure 6 Histology in week 2. Crown dentin becomes thicker after implantation both in the tooth socket (**a**) and in the subcutaneous region (**c**) in week 2. The dentin matrix in the implanted teeth is divided into two layers by a line deeply stained with hematoxylin (arrowheads). Odontoblasts and dental pulp cells in both implanted teeth (**a, b, c, d**) look as structurally sound as those in the control teeth (**e, f**). Roots elongate in teeth implanted in the tooth socket (**a, b**) as well as control teeth (**e, f**) whereas little root elongation is identified in teeth implanted subcutaneously (**c, d**). Scale bars = 500 μm (**a, c, e**), 100 μm (**b, d**) and 200 μm (**f**)

The sections were deparaffinized and washed in phosphate-buffered saline (PBS), pH 7.4, and then immersed in 0.2 N HCl for 20 min. After being washed in PBS, the sections were incubated in proteinase K (20 g ml^{-1}; Roche; Mannheim, Germany) in PBS for 30 min at 37°C. After washing, the sections were dipped in 100% ethanol, dried in air and incubated with the antisense probe or the sense control probe (400 ng ml^{-1}) in a hybridization mixture for 16 h at 45°C. Digoxigenin-labeled, single-strand riboprobes for rat pro-alpha 1(I) collagen (Sasano *et al*, 2002) were used.

The sections were washed and treated with RNase (Type 1a, 20 μg ml^{-1}; Sigma, St Louis, MO, USA) for 30 min at 37°C. After washing, the hybridized probes were detected immunologically by using the Nucleic Acid Detection Kit (Roche), counterstained with methyl green, and mounted with a mounting medium.

At least two sections from each of the three specimens at each stage were examined using the same probe. The intensity of hybridization signals was evaluated by observing at least three fields of every section.

Results

X-ray radiographic morphometry

The result of the X-ray radiographic morphometry was summarized in Figure 2. The length of the crown was constant with about 0.15 mm on the X-ray radiograph between the implanted teeth and the control teeth from weeks 0 (Figure 3) through 12. The proportion of the root length to the crown length increased in teeth implanted in the tooth socket (Figures 2 and 4a) as well as control teeth (Figures 2 and 4c) from weeks 1 through 12, whereas the root length proportion showed no increase in subcutaneously implanted teeth (Figures 2 and 4b). The proportion of the root length in teeth implanted in the tooth socket was significantly smaller than that of control teeth except in week 12, but significantly larger than that of teeth implanted in the subcutaneous region (Figure 2).

Histology

Formation of the crown shape was almost completed in week 0, whereas the root formation was hardly identified (Figure 5). Crown dentin became thicker after implantation both in the tooth socket (Figure 6a) and in the subcutaneous region (Figure 6c) in week 2. The dentin matrix in the implanted teeth was divided into 2 layers by a line deeply stained with hematoxylin (Figure 6a,c). The line indicated the boundary between dentins formed before implantation and after implantation (Akiba *et al*, 2006). Odontoblasts and dental pulp cells in both implanted teeth (Figure 6a–d) looked as structurally sound as those in the control teeth (Figure 6e,f). Roots elongated in teeth implanted in the tooth socket (Figure 6a,b) as well as control teeth (Figure 6e,f) whereas little root elongation was identified in teeth implanted in the subcutaneous region (Figure 6c,d).

Cementum was formed in teeth, which had been implanted both in tooth sockets as well as subcutaneous regions, by week 2 (Figure 6) and became thicker by week 4 (Figure 7), whereas the roots hardly elongated in the teeth implanted in the subcutaneous

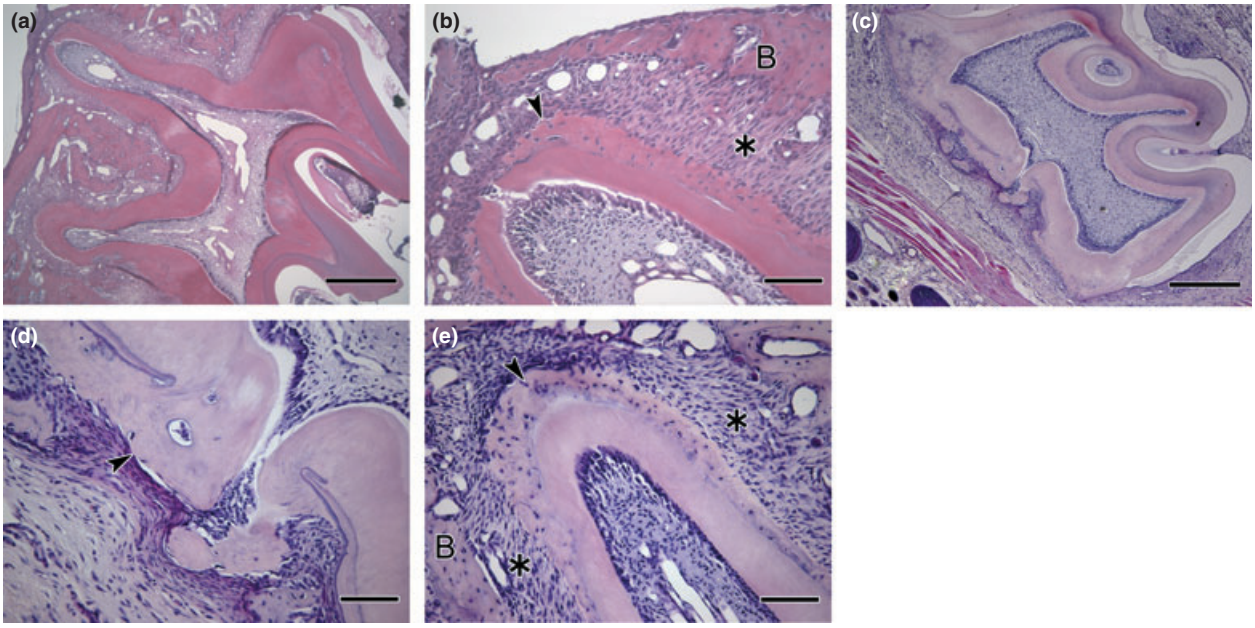

Figure 7 Histology in week 4. Cementum (arrowheads) is formed in both implanted teeth in the tooth socket (**a, b**) and the subcutaneous region (**c, d**), whereas roots hardly elongate in teeth implanted subcutaneously. Periodontal ligaments (asterisks) are formed around roots of teeth implanted in the tooth socket (**b**) as around roots of control teeth (**e**). Alvelolar bone (B) is formed around roots of teeth implanted in the tooth socket (**b**). Scale bars = 500 μm (**a, c**), 100 μm (**b, d, e**)

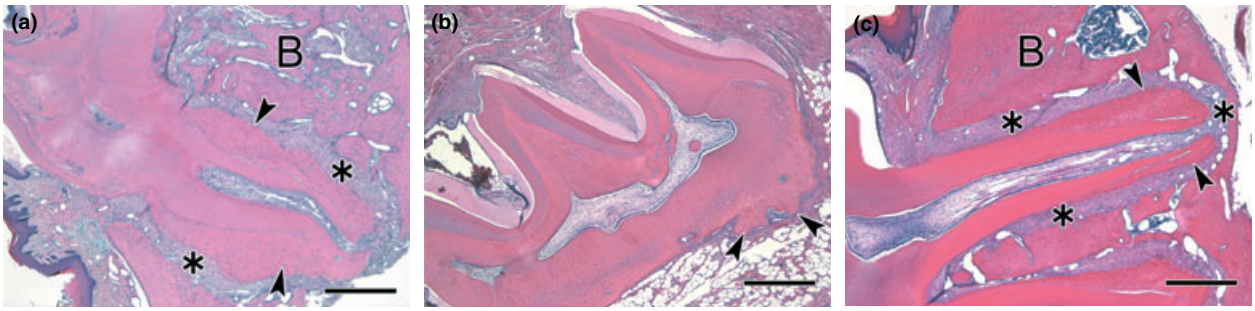

Figure 8 Histology in week 12. Cementum (arrowheads), periodontal ligaments (asterisks) and alveolar bone (B) develop in week 12 in teeth implanted in the tooth socket (**a**) as well as in control teeth (**c**), whereas only cementum developed in the subcutaneous implantation (**b**). Scale bars = 500 μm

region (Figure 7c,d). Periodontal ligaments were formed around the roots of teeth implanted in the tooth socket (Figure 7b) in week 4 as also around roots of control teeth (Figure 7e), whereas few periodontal ligaments were seen around teeth implanted in the subcutaneous region (Figure 7d). The epithelial root sheath was identified in the apical region of developing roots of control teeth and implanted teeth in the tooth socket as well as around an opening at the base of crowns of subcutaneously implanted teeth (Figure 6). Alvelolar bone was formed around roots of teeth implanted in the tooth socket (Figure 7b) and control teeth (Figure 7e). Alveolar bone was hardly seen around teeth implanted in the subcutaneous region (Figure 7c,d).

Cementum, periodontal ligaments and alveolar bone developed in weeks 8 and 12 in teeth implanted in the tooth sockets (Figure 8a) as well as in control teeth (Figure 8c). In contrast, cementum developed but periodontal ligaments and alveolar bone were hardly observed in subcutaneous implantation (Figure 8b).

In situ *hybridization*

Odontoblasts expressed type I collagen actively in weeks 2 (Figure 9) and 4 (Figure 10) in teeth implanted both in the tooth socket (Figures 9a,b and 10a,b) and in the subcutaneous region (Figures 9c,d and 10c,d) as well as control teeth (Figures 9e,f and 10e). Cementoblasts and cementocytes also expressed type I collagen actively in weeks 2 and 4 in both implanted teeth as well as control teeth. Periodontal ligament cells and osteoblasts and osteocytes expressed type I collagen around roots of teeth implanted in the tooth socket as well as control teeth. In contrast, weaker expression of type I collagen was identified around teeth implanted in the subcutaneous region.

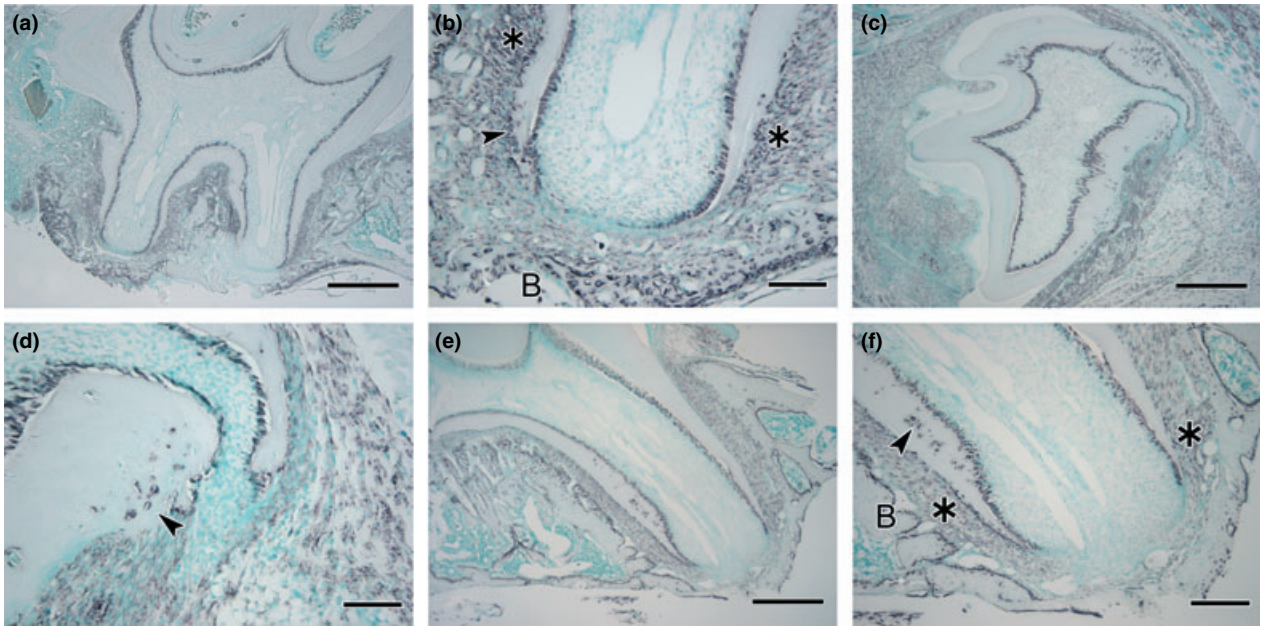

Figure 9 *In situ* hybridization in week 2. Odontoblasts express type I collagen actively in teeth implanted both in the tooth socket (**a, b**) and in the subcutaneous region (**c, d**) as well as control teeth (**e, f**). Cementoblasts and cementocytes (arrowheads) also express type I collagen actively in both implanted teeth as well as control teeth. Periodontal ligament cells (asterisks) and osteoblasts and osteocytes of alveolar bone (B) express type I collagen around roots of teeth implanted in the tooth socket as well as control teeth. In contrast, weaker expression of type I collagen is identified around teeth implanted sucutaneously. Scale bars = 500 μm (**a, c, e**), 100 μm (b,d) and 200 μm (**f**)

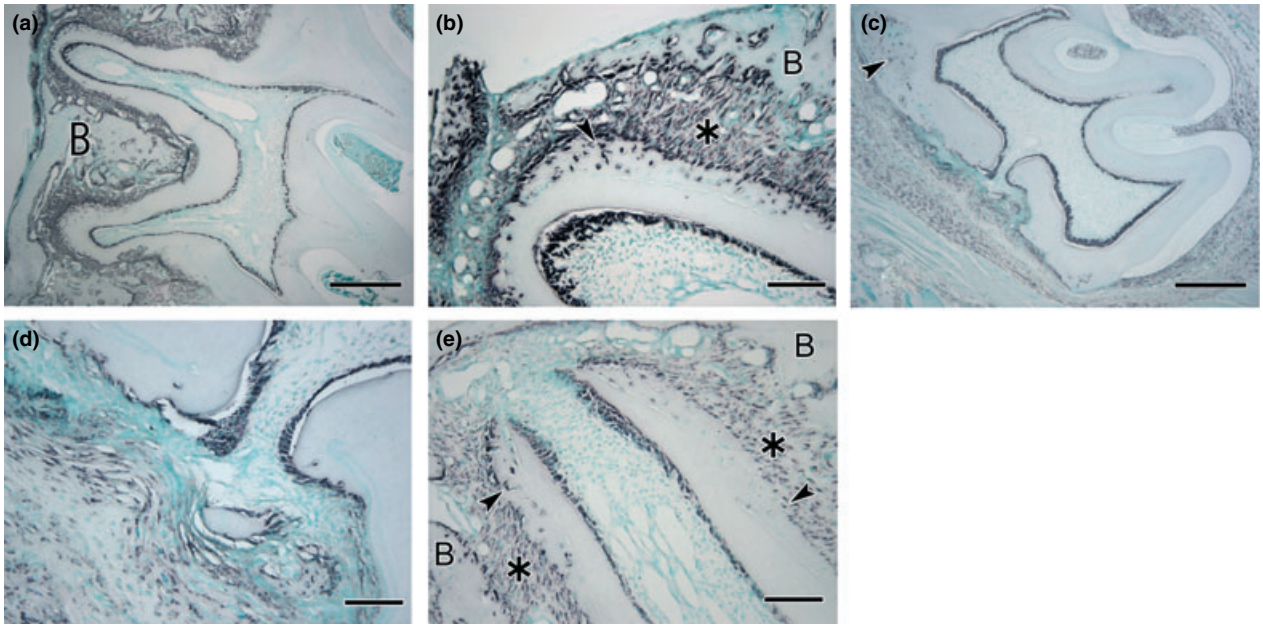

Figure 10 *In situ* hybridization in week 4. Odontoblasts express type I collagen actively in teeth implanted both in the tooth socket (**a, b**) and in the subcutaneous region (**c, d**) as well as control teeth (**e**). Cementoblasts and cementocytes (arrowheads) also express type I collagen actively in both implanted teeth as well as control teeth. Periodontal ligament cells (asterisks) and osteoblasts and osteocytes of alveolar bone (B) express type I collagen around roots of teeth implanted in the tooth socket as well as control teeth. In contrast, weaker expression of type I collagen is identified around teeth implanted sucutaneously. Scale bars = 500 μm (**a, c**), 100 μm (**b, d, e**)

Discussion

This study has shown that the root elongation of rat maxillary first molars starts in week 2 as we previously reported (Maruya *et al*, 2003). The rat maxillary first molar in week 2 was, therefore, considered an appropriate starting material for the implantation experiment to examine root formation. Roots of teeth implanted in

the tooth socket elongate in week 1 and cementum is formed on the root surface in week 2 and becomes thicker in week 4. Periodontal ligaments are formed around roots of the teeth. The root length of the implanted tooth almost catches up with that of the control tooth in week 12. The present report has described for the first time the detailed process of root elongation and periodontal tissue formation of tooth germs implanted in the tooth socket, i.e. replanted tooth germs, and has shown that the process is comparable to that of the control, although their morphology, such as a shape of roots, is somewhat altered.

The length of the crown on the X-ray radiograph was constant from week 0 through week 12 between both implanted teeth and control teeth. It suggests that formation of the crown of a rat first molar is completed in week 2. Therefore, the length of the crown on the X-ray radiograph can be a reliable standard to evaluate the root length that further increases in week 2 and thereafter.

In teeth implanted both in the tooth socket and in the subcutaneous region, it is assumed that new dentin formed after implantation as a boundary line appeared, which distinguishes dentin that formed before implantation from the one that formed after implantation (Akiba *et al*, 2006). Furthermore, odontoblasts looked sound histologically and ISH showed that odontoblasts in the implanted teeth expressed type I collagen actively in weeks 2 and 4, which also indicates that odontoblasts revive and synthesize dentin matrix after implantation. It suggests that the vasculature that had been interrupted by implantation was reorganized and the circulation restarted to supply oxygen and nutrients to odontoblasts and allow them to synthesize dentin as per this study.

Root dentin and cementum formed in both implanted teeth, however, roots did not elongate in teeth implanted in the subcutaneous region. Periodontal ligaments and alveolar bone developed and constituted a periodontal tissue complex with cementum formed on the root dentin in teeth implanted in the tooth socket, whereas periodontal ligaments and alveolar bone were hardly observed around cementum in teeth implanted in the subcutaneous region. Progenitors of alveolar bone osteoblasts and periodontal ligament cells may have originated from the tooth socket but not from the tooth germ, whereas those of cementoblasts may have resided in tooth germs. Development of surrounding periodontal ligaments and alveolar bone may be involved in elongation of roots. Meanwhile, the productivity of matrices for dentin and cementum may be equivalent between teeth implanted in the tooth socket and in the subcutaneous region as odontoblasts, cementoblasts and cementocytes in both implanted teeth similarly expressed type I collagen, which is the most abundant matrix proteins in dentin and cementum (Wiesmann *et al*, 2005). The direction of matrix formation of dentin and cementum in subcutaneously implanted teeth may be different from that in teeth implanted in the tooth socket. More dentin and cementum may be formed in the lateral direction to the crown in the subcutaneously implanted teeth.

Information about molecular mechanisms that regulate root development is very limited (Yamashiro *et al*, 2003; Yokohama-Tamaki *et al*, 2006). The mutation in the *Nfic* gene was reported to display a phenotype with molars lacking roots in mice (Steele-Perkins *et al*, 2003). Odontoblasts were reported to express this gene actively during dentin formation. The transcription-replication protein encoded by the gene may be involved in the difference in root development on account of different implantation sites observed in this study. A recent study (Yokohama-Tamaki *et al*, 2006) suggested that disappearance of FGF10 signaling leads to the transition from crown to root formation. Involvement of FGF10 may be different between teeth implanted in the tooth socket and the subcutaneous region.

Further investigation using the experimental model may provide a better understanding of biology of root development for clinical dentistry of tooth germ implantation.

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Author contributions

Yoshinori Ina primarily performed the experiment. Yasuyuki Sasano, the corresponding author, designed the research and analyzed the data. Nami Akiba instructed Ina to perform the experiment. Kouki Hatori and Takahiro Honma helped Ina with his experiment. Keiichi Sasaki made substantial contributions to statistical analysis of the data.

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