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Original

Effects of Fibrillin Application on Periodontal Ligament Regeneration in Mouse Model of Tooth Replantation

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Abstract: The periodontal ligament (PDL) is fibrous tissue that maintains the connective space between tooth root and alveolar bone while avoiding the root resorption and the ankylosis. Fibrillin is one of the components of the elastic system fibers which exist in PDL as oxytalan fibers, and it plays an important role in organizing PDL with collagen fibers. The aim of the present study was to evaluate the effect of applying fibrillin protein for PDL regeneration using a mouse tooth replantation model. The ratio of root resorption and ankylosis was not improved in a replanted tooth soaked in fibrillin/PBS solution as compared to being soaked in PBS only. Interestingly, replantation with fibrillin and propylene glycol alginate (fibrillin/PGA) induced less root resorption and ankylosis than were observed with teeth replanted with PGA only. The PDL space between tooth root and alveolar bone in the fibrillin/PGA was clearly wider than that with PGA only. This regenerated PDL with Azan staining showed the meshwork structures including rich fibers in the horizontal direction. Furthermore, immunohistochemical expression of periostin was strongly detected in the entire regenerated PDL in the fibrillin/PGA. These results suggest that fibrillin promotes the formation of fibrous connective tissue in a PDL replantation and might be a good candidate as regenerative material for the PDL.

Key words: Periodontal ligament, Replantation, Avulsion, Fibrillin, Regeneration

Introduction

Avulsion is the severest type of dental trauma[1]. The prognosis depends on the success of periodontal ligament (PDL) regeneration after replantation. In replantation of an avulsed tooth, it has been thought that the viability of PDL cells on the root surface is a critical factor for PDL regeneration. However, PDL cell viability depends on post-traumatic factors, such as the extra-oral time, storage medium, and infection risk[2,3]. For the clinical success of tooth replantation for such cases, a scaffold material that promotes PDL regeneration has to be considered for such cases.

As materials for PDL regeneration, fluoride, sodium alendronate, and dexamethasone have been applied on the root surface of avulsed teeth that were replanted into the alveolar socket[4-9]. However, root resorption and ankylosis were could not be inhibited completely. Recently, attempts have been made to use a commercially available biomaterial, Emdogain® (EMD), a protein mixture of porcine amelogenin in a propylene glycol alginate (PGA) gel, for PDL regeneration[10,11]. The effect of EMD on periodontal healing and root resorption after replantation has been investigated in both animal and clinical studies. Some studies have shown that applying EMD promoted periodontal healing, whereas in other studies, EMD did not prevent ankylosis-induced resorption of the replacement root[12,13].

Regarding the PDL components, not only collagen fibers but also oxytalan fibers are very important for maintaining the PDL. Oxytalan fibers are elastic system fibers that do not contain elastin and are characteristically found in the PDL[14]. Oxytalan fibers exhibit unique features, such as vertically oriented enclosure of the root axis, perpendicular orientation of collagen fibers, closer proximity to the cementum than to the alveolar bone, and frequent insertion into the root cementum[15,16]. We have previously shown that fibrillin 1 and 2 are initially expressed at the surface of the developing root and widely observed in mature human PDL tissue[17]. Furthermore, a mutation in the Fibrillin-1 gene causes Marfan syndrome, which displays orofacial manifestations and increases susceptibility to periodontal disease[18,19]. It has also been shown that fibrillin plays an important role in maintaining the PDL through interactions with periostin and collagen[20]. However, little is known about the potential of fibrillin as a material to induce PDL regeneration.

In the present study, we investigated the effects of fibrillin application on PDL regeneration in a mouse tooth replantation

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model, and evaluated the regenerated PDL by fibrillin application, histochemicaly.

Materials and Methods

The protocol for these experiments was approved by the Ethics Committee of Fukuoka Dental College and carried out in accordance with the Institutional Animal Research Guidelines (No. 11019).

Replantation procedure

The upper-left first molar (M1) of 4-week-old mice was extracted under deep anesthesia induced by intraperitoneal injection of pentobarbital (45 μg/g). Each extracted tooth was rinsed with PBS and then replanted in the original alveolar socket. To avoid occlusal force, the lower-left first molar was also extracted. Four weeks after replantation, the maxillary bone was sampled for histological analysis.

Allogenic transplantation procedure

Wnt1-Cre and *R26R* transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The *Wnt1-Cre* transgenic line was crossed with the *R26R* conditional reporter allele, which has been described previously[21], to produce genetically labeled neural crest cells that were identifiable by their expression of β-galactosidase[22]. Allogenic transplantation was performed between *Wnt1-Cre*;*R26R* and control mice. Four weeks after transplantation, the maxillary bone was extracted. Frozen sections of the bone were prepared and then stained with 5-bromo-4-chloro-3-indolyl β-d-galactosidase (X-gal).

Fibrillin application

To investigate the effect of fibrillin (Extra Cellular Matrix Laboratories, Mie, Japan) application, 250 μg/ml and 1 mg/ml fibrillin diluted with PBS or propylene glycol alginate (PGA) were prepared. Extracted teeth were soaked with PBS only, 250 μg/ml or 1 mg/ml fibrillin diluted with PBS for 10 min, and then replanted into their sockets. PGA only, 250 μg/ml or 1 mg/ml fibrillin with PGA was applied on the root surface of extracted teeth, which were then replanted into their sockets. HE, immunohistochemical analysis and Azan staining were performed 4 weeks after replantation. The histological area of the regenerated PDL in each samples were measured using the image measurement software (WinROOF, Tokyo, Japan). The number of samples is indicated in Tables 1 and 2.

Section preparation and immunohistochemistry

Following sampling, the maxillary bones were fixed with 10 % neutralized buffered formalin overnight and then de-calcified for three days. The specimens were then dehydrated in graded ethanol solutions, embedded in paraffin and cut into 7-μm sections for hematoxylin and eosin staining and immunostaining. Primary antibodies, a rabbit anti-periostin polyclonal antibody diluted 1:500 (Abcam Japan, Tokyo, Japan), a rabbit anti-BSP polyclonal antibody diluted 1:1000 (Immundiagnostik AG, Bensheim, Germany), and a mouse anti-αSMA polyclonal antibody diluted 1:1000 (Abcam, Cambridge, UK), were used at 4 °C overnight. Secondary antibodies, biotin-conjugated goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG, were used for 1 h at room temperature. The specimens were sensitized using streptavidin peroxidase (Vector Laboratories, Burlingame, CA, USA) and visualized using a DAB kit (Nichirei Biosciences, Inc., Tokyo, Japan).

PDL cell culture and migration assay

PDL fibroblasts were isolated from a donor and cultured, as described previously[23]. Culture medium for PDL fibroblasts was supplemented with 10 % newborn calf serum (NCS; Invitrogen, Carlsbad, CA, USA) and 100 units/ml penicillin and 100 μg/ml streptomycin (Roche Diagnostics, Mannheim, Germany) at 37 °C in humidified air containing 5 % CO_2. When cells reached confluence, they were harvested with 0.025 % trypsin (Invitrogen) in PBS and seeded into the culture-inserts (Ibidi, Martinsried, Germany) on a dish coated with 250 μg/ml fibrillin diluted with PBS (n=4). After 24 h, the cell insert was removed and cell migration was examined after 12, 24, 36 and 48 h.

Statistical analysis

Statistical analyze of the histological area of the regenerated PDL was performed using the Mann-Whitney test in each group. Values are presented as means ± SD. A P-value of <0.05 was considered significant.

Results

Periostin, αSMA, and BSP expression in regenerated PDL after replantation

A front view of the upper first molar with PDL tissue in normal mice is shown in Fig. 1. All panels include a lower-magnification view in the upper-left square. In normal PDL, the periodontal space between the alveolar bone and root was maintained from the crown to the apical axis (Fig. 1a, b), and reticulated periostin expression can be seen in the whole region between the root surface and alveolar bone (Fig. 1e, f). The expression of αSMA was detected in fibroblasts and blood vessels of the PDL. The blood vessels were aligned on the alveolar bone side of the PDL (Fig. 1i, j). The liner acellular cementum layer with BSP expression was clearly visible at the root surface on the lateral side, but BSP expression on the furcation side was rough (Fig. 1m, n, arrows).

At 4 weeks after replantation, periostin expression in the regenerated PDL exhibited a fiber-like appearance, as in normal PDL. However, the width of the PDL was not uniform (Fig. 1c, d,

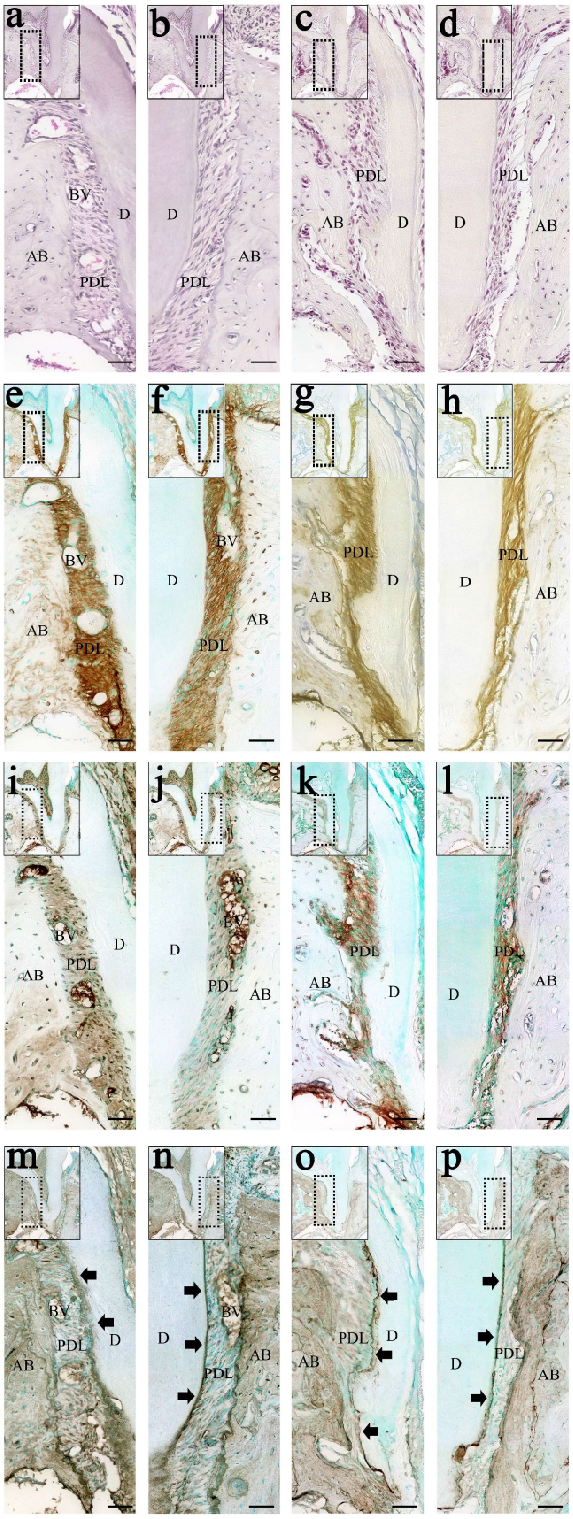

Figure 1.Periostin, alpha-smooth muscle actin (αSMA) and bone sialoprotein (BSP) expression in normal PDL tissue. Lower-magnification views are in the upper-left corner in all panels. Hematoxylin and eosin staining of sections on furcation and lateral sides of the upper molar in normal mice (a, b) and in mice at 4 weeks after replantation (c, d). Immunohistochemical staining of periostin (e, f), αSMA (i, j) and BSP (m, n) in normal mice. Immunohistochemical staining of periostin (g, h), αSMA (k, l) and BSP (o, p) in mice with replanted teeth. Scale bar: 100 μm; D: Dentin; AB: Alveolar Bone; PDL: Periodontal Ligament; C: Cementum; BV: Blood Vessel.

g, f). A few round blood vessels with αSMA were observed in the PDL, but fibroblastic cells with αSMA were observed in the regenerating PDL compared to the normal PDL (Fig. 1k, l). BSP expression was detected in the acellular cementum on the root surface at 4 weeks after replantation (Fig. 1o, p, arrows). According to the αSMA expression, blood vessels were not completely organized at 4 weeks after replantation. However, periostin was expressed throughout the entire regenerated PDL, and acellular cementum with BSP remained in the replanted root in both the lateral and furcation sides of the PDL at 4 weeks after replantation.

Cells derived from both the root surface and alveolar socket regenerate new PDL

To evaluate the cell source for PDL regeneration, *Wnt1-Cre*;*R26R* mice were used as host and donor for tooth transplantation. X-gal-stained teeth and PDL of *Wnt1-Cre*;*R26R* mice without transplantation are shown in Fig. 2a. Blue-stained cells were present in the dental pulp and PDL tissue (Fig. 2b, c, d). Four weeks after tooth transplantation from control into *Wnt1-Cre*;*R26R* mice, blue-stained cells were not detected in the pulp of the transplanted first molar (Fig. 2f). Blue-stained cells derived from host *Wnt1-Cre*;*R26R* mice were partially observed in the regenerated PDL; the lateral side had a greater proportion of blue cells than the furcation side (Fig. 2g, h). Extracted teeth from *Wnt1-Cre*;*R26R* mice were subsequently transplanted into control mice (Fig. 2i). As in the previous experiment, the lateral side of the PDL contained mostly non-blue-stained cells derived from host control mice, and more blue-stained cells from the transplanted *Wnt1-Cre*;*R26R* teeth were observed on the furcation side (Fig. 2k, l). These results suggested that the regenerated PDL comprised cells derived from both the PDL of the replanted tooth and its alveolar socket in our replantation mice model.

Effect of soaking teeth in fibrillin with PBS before replantation

Using the tooth replantation model, we evaluated root absorption and ankylosis as indicators of the condition of the regenerated PDL on the furcation and lateral sides. The rate of incidence of root absorption and ankylosis on the furcation and lateral sides was shown in Table 1. Teeth soaked in PBS with/without fibrillin exhibited a high rate of root absorption. Histological views with periostin, αSMA and BSP expression on the furcation side of the PDL without root resorption and ankylosis are shown in Fig. 3. All panels include a lower-magnification view in the upper-left square. Teeth soaked in PBS with/without fibrillin exhibited faint periostin expression in the PDL as low-density fiber-like structures (Fig. 3b, f, j). There were no differences in expression of αSMA and BSP among each group (Fig. 3c, d, g, h, k, l). According to these results, even if root absorption or ankylosis is not observed, periostin expression is decreased by soaking teeth in PBS. Moreover, applying fibrillin with PBS does not reduce the rate of root absorption.

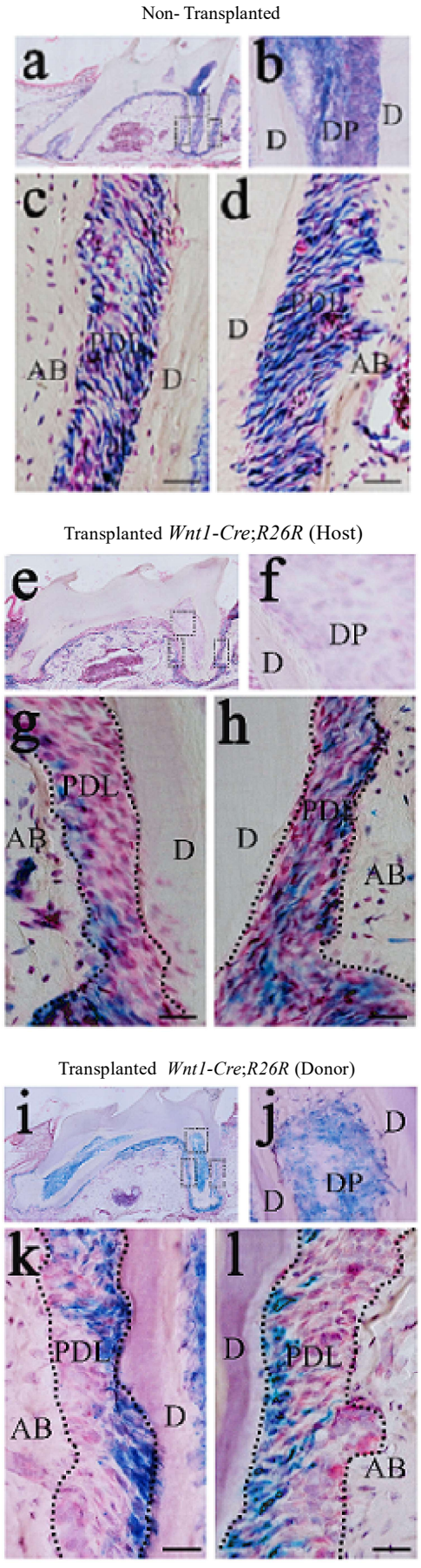

Figure 2. X-gal staining of non-transplanted and transplanted upper first molars. X-gal staining of non-transplanted upper molar in *Wnt1-Cre*;*R26R* mouse (a). Higher-magnification views of regions outlined in panel a (b, c, d). X-gal staining of transplanted molar of control into littermate *Wnt1-Cre*;*R26R* at 4 weeks after transplantation (e). Higher-magnification views of regions outlined in panel e (f, g, h). X-gal staining of transplanted molar of *Wnt1-Cre*;*R26R* into littermate control at 4 weeks after transplantation (i). Higher-magnification views of regions outlined in panel i (j, k, l). Scale bar: 100 μm; D: Dentin; DP: Dental Pulp; AB: Alveolar Bone; PDL: Periodontal Ligament.

Effect of applying fibrillin with PGA to teeth before replantation

Next, fibrillin with PGA was applied like a gel on the root surface of extracted teeth, which were then replanted. The rate of incidence of root absorption and ankylosis on the furcation and lateral sides was evaluated (Table 2). Application of PGA reduced the rate of root absorption. However, ankylosis of the root and alveolar bone was observed on the lateral side of the PDL in two cases when only PGA was applied. Interestingly, fibrillin with PGA also reduced the rate of not only root resorption but also ankylosis. Histological views with periostin, αSMA and BSP expression on the lateral side of the PDL are shown in Fig. 4. The regenerated PDL in PGA was very thin, and periostin expression was detected but did not appear as fiber-like structures (Fig. 4a, b). Blood vessels with αSMA expression were not clearly visible (Fig. 4c), but BSP expression was detected on the root surface (Fig. 4d). For 250 μg/ml fibrillin with PGA, the regenerated PDL was also very thin, and periostin expression did not appear as fiber-like structures (Fig. 4e, f). On the other hand, a PDL with sufficient thickness was regenerated by application of 1 mg/ml fibrillin with PGA (Fig. 4i, j). Moreover, reticulated PDL with periostin and αSMA expression was widely seen (Fig. 4j, k), and continuous BSP expression throughout the acellular cementum was also detected (Fig. 4l).

Applying fibrillin induced fiber formation in regenerated PDL

Since sufficiently thick PDL was regenerated in teeth replanted with fibrillin/PGA, we performed Azan staining to observe the fiber structure. In teeth replanted with PGA only, the fibers were mostly parallel to the tooth root axis (Fig. 5a, b). In teeth replanted with 250 μg/ml fibrillin/PGA, the area of the regenerated PDL was larger than that of the PDL regenerated in the presence of PGA only. Azan staining revealed a few fibers perpendicular to the root surface; however, these fibers were not connected to alveolar bone (Fig. 5c, d). On the other hand, a high density of fiber-like structures parallel to the tooth root axis that reached and connected with alveolar bone was observed in teeth replanted with 1 mg/ml fibrillin/PGA (Fig. 5e, f). The area of the regenerated PDL was significantly larger than the area of the other specimens (Fig. 6). Taken together, there were no clear differences in the incidence of root resorption and ankylosis by fibrillin application, but regenerated PDL in 1 mg/ml fibrillin/PGA had a better quality

Table 1. Rate of Incidence of Root Absorption and Ankylosis

	PBS		Fibrillin 250μg/ml		Fibrillin 1mg/ml	
Number of samples	8		6		6	
Side	Furcation	Lateral	Furcation	Lateral	Furcation	Lateral
Root absorption	3 (37.5)	4 (50.0)	3 (50.0)	2 (33.3)	3 (50.0)	3 (50.0)
Ankylosis	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)

Note: Number of samples with root condition (percentage of samples)

Table 2. Rate of Incidence of Root Absorption and Ankylosis

	PGA		PGA+Fibrillin 250μg/ml		PGA+Fibrillin 1mg/ml	
Number of samples	5		4		6	
Side	Furcation	Lateral	Furcation	Lateral	Furcation	Lateral
Root absorption	1 (20.0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)
Ankylosis	0 (0.0)	2 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)

Note: Number of samples with root condition (percentage of samples)

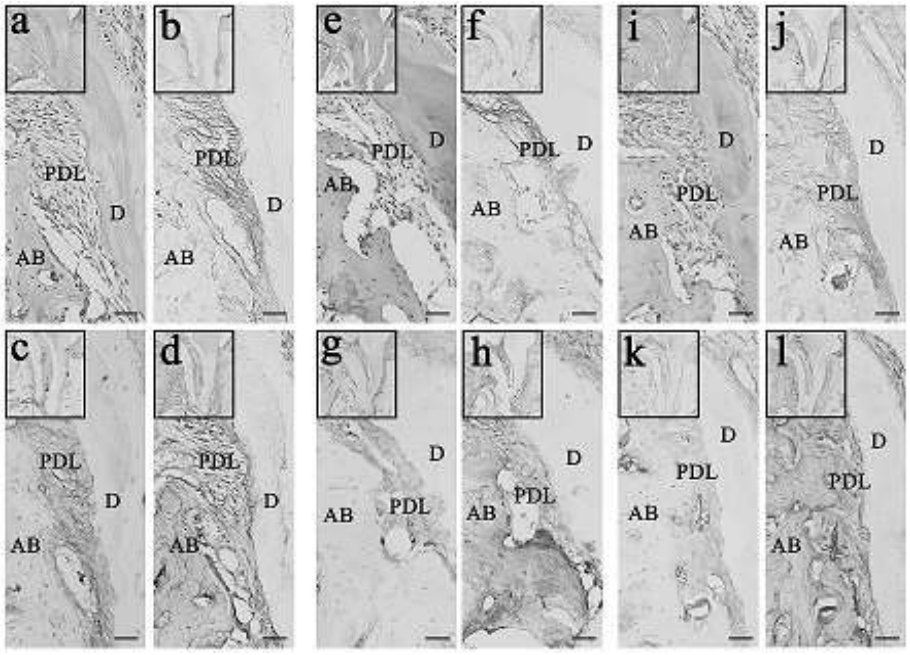

Figure 3. Histological view of replanted teeth after soaking in fibrillin. Lower-magnification views are in the upper-left corner in all panels. Hematoxylin and eosin staining of the replanted upper molar after soaking in PBS (a), 250 μg/ml fibrillin (e) and 1 mg/ml fibrillin (i). Immunohistochemical staining of periostin (b, f, j), αSMA (c, g, k) and BSP (d, h, l) in PBS, 250 μg/ml fibrillin and 1 mg/ml fibrillin. Scale bar: 100 μm; D: Dentin; AB: Alveolar Bone; PDL: Periodontal Ligament.

than the others, histologically.

Effect of fibrillin on PDL cell migration

For the cell migration assay, the area of cells that migrated within a specified region was measured at each time period (Fig. 7, inside region marked by dotted lines). No significant differences were found between control cells and those cultured on fibrillin-coated dishes, indicating that fibrillin does not induce cell migration (Fig. 8).

Discussion

The viability of PDL cells of extracted teeth is critical for the success of tooth replantation. However, the conditions of extracted teeth are unique, and PDL cells of extracted teeth become necrotic

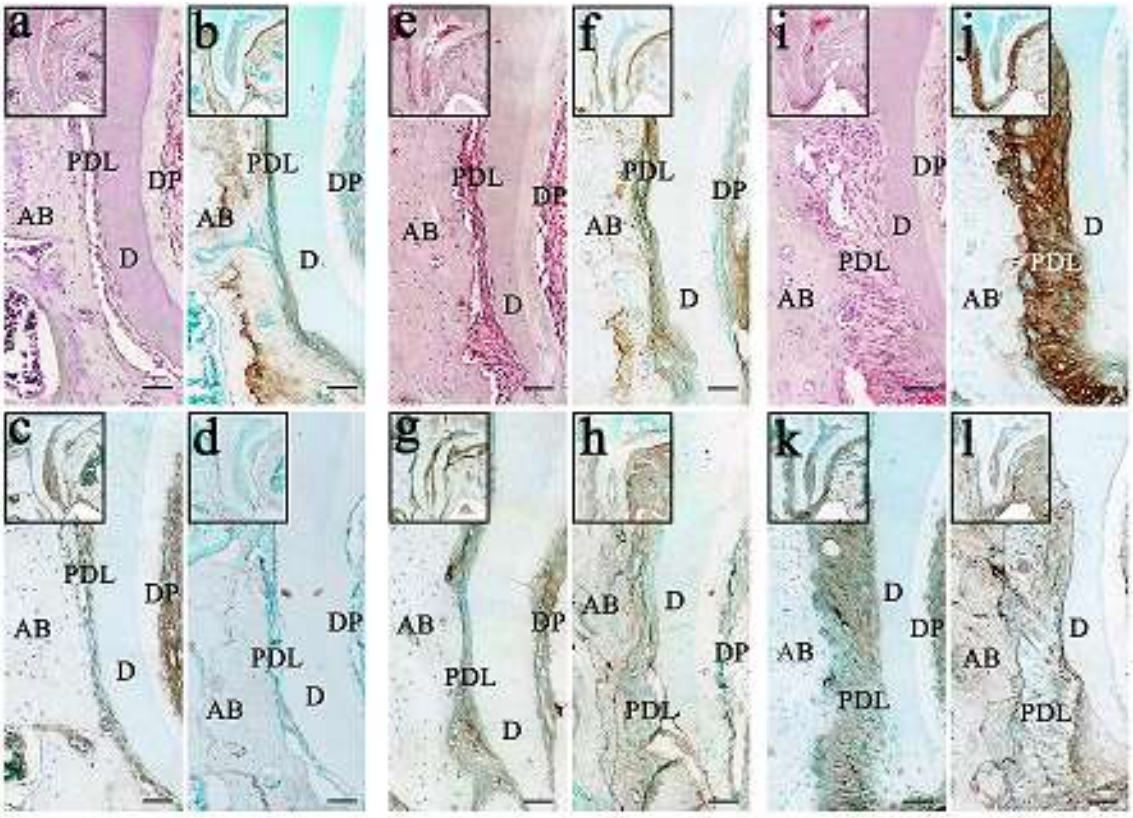

Figure 4. Histological view of replanted teeth with fibrillin/PGA. Lower-magnification views are in the upper-left corner in all panels. Hematoxylin and eosin staining of the replanted molar with PGA (a), PGA with 250 μg/ml fibrillin (e) and PGA with 1 mg/ml fibrillin (i). Immunohistochemical staining of periostin (b, f, j), αSMA (c, g, k) and BSP (d, h, l) in PBS, 250 μg/ml fibrillin and 1 mg/ml fibrillin. Scale bar: 100 μm; D: Dentin; AB: Alveolar Bone; PDL: Periodontal Ligament.

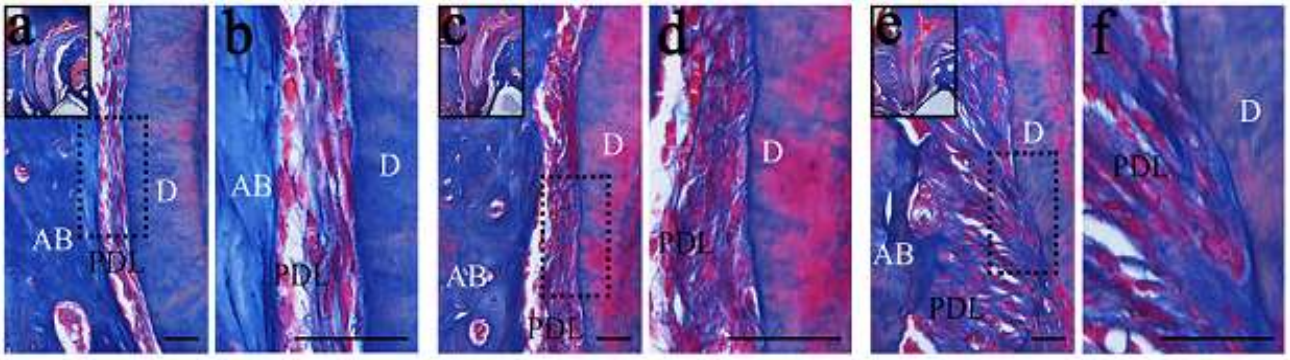

Figure 5. Histological view of Azan staining. Lower-magnification views are in the upper-left corner in a, c and e panels. Azan staining of replanted molar with PGA (a), PGA with 250 μg/ml fibrillin (c) and PGA with 1 mg/ml fibrillin (e). Higher-magnification views of regions outlined in panel a (b), c (d) and e (f). Scale bar: 100 μm; D: Dentin; AB: Alveolar Bone; PDL: Periodontal Ligament.

in some cases. Therefore, an approach for regeneration of the PDL that uses cells derived from alveolar sockets is needed. Previously, Kim et al. and Saito et al. demonstrated that host cells replaced PDL tissue in transplanted teeth[24,25]. Our present transplantation experiment using *Wnt1-Cre*;*R26R* mice also showed that the PDL tissue contained cells derived from alveolar sockets. Therefore, our investigations using the present tooth replantation model should take into consideration the effect of cells derived from both the PDL of the extracted tooth and its socket.

The discomfort associated with tooth replantation is often caused by root resorption. Root resorption after replantation is divided into two types: inflammatory resorption and replacement resorption following ankylosis[3,26]. Clinically, early endodontic treatment of transplanted teeth is recommended to avoid the inflammatory resorption due to inflammation of the root canal. However, in our mouse replantation model, endodontic treatment was technically difficult. Teeth with immature roots are more likely to have sufficient blood supply to avoid necrosis of the pulp tissue

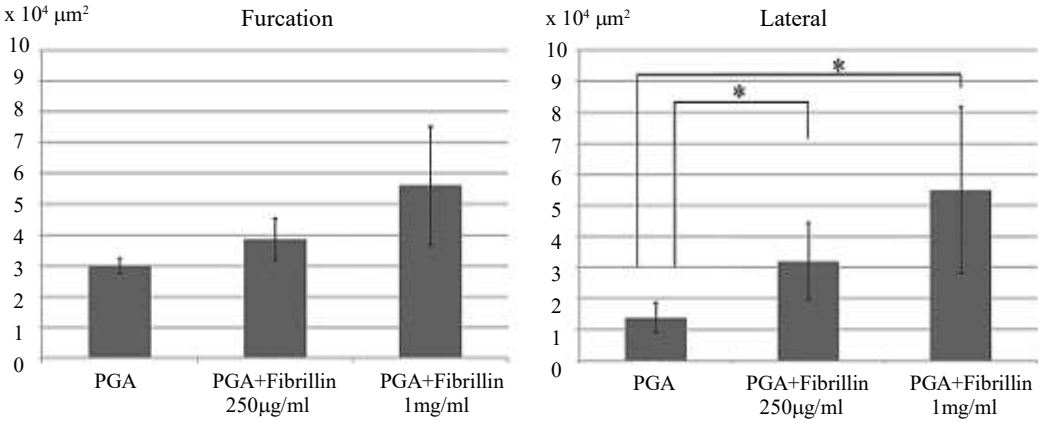

Figure 6. Area of the regenerated PDL.The histological area was measured in furcation and lateral side of replanted tooth. The bar graph indicates the average of PGA only, fibrillin 250 μg/ml and 1 mg/ml with PGA. Error bars represent the standard deviation. *P<0.05

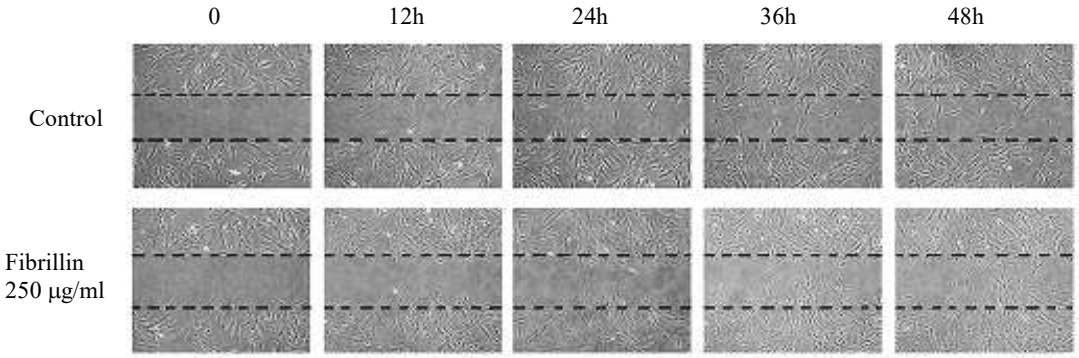

Figure 7. Cell migration assay using PDL cells. Images of cells at 0, 12, 24, 36 and 48 h. Dotted lines indicate the margin of the cell insert at the 0 point.

during root development[25,27]. Therefore, we used the first immature upper molar from 4-week-old mice for replantation in the present study.

In our study, inflammatory root resorption occurred in some cases for replanted teeth that had been soaked in PBS for 10 min, and inflammatory tissue was observed in PDL and pulp. Extra-oral time directly affected not only the viability of PDL cells but also the survival of pulp cells in extracted teeth, and fibrillin application did not reduce the rate of root resorption. Consequently, liquid forms of storage media may not be suitable for PDL regeneration in tooth replantation.

Although fiber formation with periostin expression, blood vessels, and undisrupted acellular cementum in the regenerated PDL without inflammatory resorption was observed, most of the regenerated PDL in our soaking experiments was thinner than the normal PDL. The appropriate thickness should be maintained using supplemental materials for renewing the PDL, so we used PGA materials as the scaffold for replantation. Applying only PGA clearly inhibited the inflammatory resorption of the root compared to soaking in PBS. On the other hand, the rate of ankylosis increased with PGA application.

Fibrillins, which are large glycoproteins, are major components of microfibrils in oxytalan fibers. Oxytalan fibers are unique in that they do not contain elastin and thus differ from other elastic fibers expressed in the skin, lungs or arteries[28]. Oxytalan fibers are only found in the PDL and the ciliary zonula of the eyes[29]. Fibrillin can form a structural network with collagen, periostin or other extracellular matrix proteins in PDL and maintain the elasticity required for mastication[30].

Therefore, we hypothesized that exogenous fibrillin may support periostin expression or deposition in PDL regeneration. According to the results of the present study, tooth replantation with 1 mg/ml fibrillin/PGA significantly accelerated thickening of the PDL. Furthermore, the regenerated PDL exhibited a high density of fiber structures. Interestingly, the fibers were mostly connected between the root and alveolar bone in a direction perpendicular to the tooth root axis. It has been reported that periostin expression in PDL is significantly decreased in fibrillin-deficient mice, which exhibit loose connective tissue in the PDL[19,20]. In the present study, periostin was highly expressed in the PDL regenerated following application of 1 mg/ml fibrillin/PGA. It was suggested that fibrillin, in association with periostin,

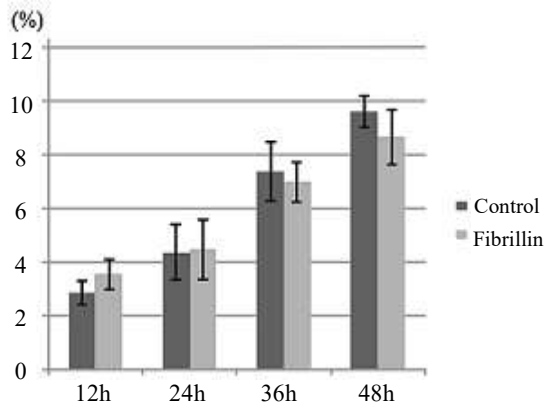

Figure 8. The ratio of the area that cells migrated within the region marked by the dotted lines for each group. Error bars represent the standard deviation.

played an important role in the formation of fibers in the PDL. Results of an in vitro cell migration assay showed that fibrillin does not promote migration of cultured PDL cells. Therefore, it is still unclear how fibrillin affects PDL regeneration. The effect of fibrillin on cells derived from both the PDL of a tooth and its socket need to be further clarified.

In conclusion, fibrillin may be a good candidate as regenerative material for fiber formation during PDL regeneration in tooth replantation. Further investigation of the molecular mechanisms of fibrillin for PDL regeneration is warranted.

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Conflict of Interest

The authors have declared that no COI exists.

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