Periodontal Regeneration Using Periodontal Ligament Stem Cell-Transferred Amnion

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Periodontal disease is characterized by the destruction of tooth supporting tissues. Regeneration of periodontal tissues using *ex vivo* expanded cells has been introduced and studied, although appropriate methodology has not yet been established. We developed a novel cell transplant method for periodontal regeneration using periodontal ligament stem cell (PDLSC)-transferred amniotic membrane (PDLSC-amnion). The aim of this study was to investigate the regenerative potential of PDLSC-amnion in a rat periodontal defect model. Cultured PDLSCs were transferred onto amniotic membranes using a glass substrate treated with polyethylene glycol and photolithography. The properties of PDLSCs were investigated by flow cytometry and *in vitro* differentiation. PDLSC-amnion was transplanted into surgically created periodontal defects in rat maxillary molars. Periodontal regeneration was evaluated by microcomputed tomography (micro-CT) and histological analysis. PDLSCs showed mesenchymal stem cell-like characteristics such as cell surface marker expression (CD90, CD44, CD73, CD105, CD146, and STRO-1) and trilineage differentiation ability (i.e., into osteoblasts, adipocytes, and chondrocytes). PDLSC-amnion exhibited a single layer of PDLSCs on the amniotic membrane and stability of the sheet even with movement and deformation caused by surgical instruments. We observed that the PDLSC-amnion enhanced periodontal tissue regeneration as determined by micro-CT and histology by 4 weeks after transplantation. These data suggest that PDLSC-amnion has therapeutic potential as a novel cell-based regenerative periodontal therapy.

Introduction

PERIODONTAL DISEASE IS characterized by chronic inflammation of the gums and surrounding tissues because of bacterial infection. The progression of periodontal disease results in destruction of tooth supporting tissues such as bone, periodontal ligament, and cementum as well as eventual tooth loss.^{1,2} The goal of periodontal treatment is to reconstruct the tooth supporting structures lost during progression of the disease.

According to recent progress in tissue engineering, regeneration of lost tissue is now possible using *ex vivo* expanded

cells. Mesenchymal stem cells (MSC) are useful for cell-based regenerative medicine because of their potential to differentiate into multiple lineages.^{3–5} MSC from various tissues have been investigated for cell-based regeneration of periodontal tissues, including the periodontal ligament,^{6–8} bone marrow,^{9–11} adipose tissue,¹² and periosteum.^{13–15} Cultured stem cells from the periodontal ligament (PDLSCs) possess characteristics of MSC such as cell surface MSC marker expression (CD105+, CD90+, CD44+, CD73+, CD45–, CD31–, and CD34–) and multilineage differentiation ability (i.e., into osteoblasts, chondrocytes, and adipocytes).^{16–18} In addition to MSC characteristics, PDLSCs have unique properties to form a cementum/

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periodontal ligament complex-like structure when ectopically transplanted in animals.^{16,19} These data suggest that PDLSCs are ideal cell sources for cell-based periodontal therapy.

The amniotic membrane is the membranous tissue that surrounds the amniotic sac, which contains amniotic fluid that protects the embryo, and it develops from extraembryonic tissue.^{20,21} Amnion has long been clinically used for coverage over dermal burns and ulcers as well as for treatment of necrosis and severe inflammation of the eyes, because of its antimicrobial and antifibrotic properties as well as its mechanical strength, which is sufficient for surgical manipulation.^{22,23} Moreover, amnion can efficiently remain in close contact with the transplanted surface because of its pliable nature. Recently, we developed a novel cell transplantation material that comprises cultured cells transferred onto the amniotic membrane.²⁴ Cell transfer technology makes it possible to control the strength of adhesion between the cells and glass substrate by altering polyethylene glycol degradation using photolithography. Further, monolayer cells can be transferred from a glass substrate surface to scaffold materials under suitable glass substrate conditions. We have reported that transplantation of amniotic membrane with transferred endothelial cells, by using cell transfer technology improved blood perfusion in a mouse ischemia model, which suggests the therapeutic potential of this technology for cell transplantation.^{24,25} Additionally, we have shown that transplantation of osteoblast-transferred amnion, showed increased bone healing in a mouse calvaria bone defect model, which suggests that this technology could be used for hard tissue regeneration.²⁶

On the basis of this background, we hypothesized that PDLSC-transferred amnion (PDLSC-amnion) may have regenerative potential for periodontal tissues, particularly, given the pliable property of the membrane, which makes it possible to cover the denuded root surface directly using the engrafted stem cell surface. We tested our hypothesis by the transplantation of *ex vivo* expanded PDLSCs engrafted on an amniotic membrane into a surgically created periodontal defect model in the rat maxilla.

Materials and Methods

Cell culture

Periodontal ligament tissues were obtained from premolars extracted from healthy subjects (age, 14–28 years, seven donors). The study protocol was approved by the ethics committee for clinical research at the Tokyo Medical and Dental University. Periodontal ligament tissues were minced using a surgical knife (Feather Safety Razor, Osaka, Japan) for 15 min in 500 μ L of α -MEM (Invitrogen, Carlsbad, CA) containing collagenase type I (3 mg/mL; Wako Pure Chemicals, Osaka, Japan) and dispase (4 mg/mL; Invitrogen) (digestion solution). The Digestion solution (4.5 mL) was added to the solution containing minced periodontal ligament and incubated in a water bath at 37°C for 60 min with continuous agitation. After enzymatic digestion, the reaction solution was centrifuged, and 10 mL of mesenchymal stem cell growth medium (MSCGM; Lonza, Walkersville, MD) was added. The cell suspension was passed through a cell strainer (pore size, 70- μ m) (BD Falcon, Bedford, MA) to remove debris. The culture medium was changed every 3 days, and colony-forming PDLSCs were harvested and passaged.

PDLSCs with a passage number less than seven were used for all experiments. Green fluorescence protein (GFP)-expressing PDLSCs (PDLSC-GFP) were established and cultured as previously described.²⁶ Briefly, *Aequorea coerulescens* green fluorescent protein (AcGFP; Takara Bio, Shiga, Japan) coding sequence was cloned downstream of the cytomegalovirus promoter in a retroviral pQCXIP vector (Takara Bio). After transfection of pQCXIP-AcGFP and pCL10A1 packaging vectors into HEK 293T cells, PDLSCs were infected by adding the supernatant and selection was performed by puromycin (Cosmo Bio, Tokyo, Japan).

Osteoblast, adipocyte, and chondrocyte differentiation

For osteoblastic differentiation, 10⁵ PDLSCs were seeded in a six-well cell culture plate and the culture medium was changed to the α -MEM containing ascorbic acid (L-ascorbic acid 2-phosphate, 50 μ g/mL; Sigma, St Louis, MO), dexamethasone (10⁻⁸ M; Sigma), and β -glycerophosphate (10 mM; Sigma). The culture medium was changed every 3 days for 14 days, and deposited calcium was stained using a von Kossa staining kit (Polysciences, Warrington, PA). For adipocyte differentiation, 10⁵ PDLSCs were seeded in a 12-well plate and cultured in the MSCGM. Adipocyte differentiation was induced using the adipocyte differentiation medium (Lonza) according to instructions from the manufacturer. After 28 days of differentiation, the cells were fixed in a 4% paraformaldehyde phosphate buffer for 20 min and washed with phosphate-buffered saline (PBS). The Oil Red O solution (Sigma) was added, incubated for 15 min, and washed with PBS. For chondrocyte differentiation, 2.5 $\times$ 10⁵ PDLSCs in the MSCGM were centrifuged at 150 g for 5 min in polypropylene culture tubes. The cell pellet was cultured in 0.5 mL of chondrogenic induction medium (Lonza) supplemented with 10 ng/mL TGF- β 3 (Lonza). Controls were maintained in the same medium without the TGF- β 3. The medium was changed twice a week. After 4 weeks, cell pellets were fixed in the 4% paraformaldehyde phosphate buffer and sectioned for Alcian blue staining (Wako).

Flow cytometry

PDLSCs were harvested and fixed in the 4% paraformaldehyde phosphate buffer for 20 min. Blocking was performed in PBS containing 2% bovine serum albumin and 0.02% Triton-X 100. After washing with 2% fetal bovine serum PBS, 2 $\times$ 10⁵ cells were stained with the following antibodies and normal sera for 30 min. Mouse anti-human PE-conjugated CD146 and 140b, CD166, CD73, CD31, and CD34 antibodies (1:10; BD Bioscience, San Jose, CA); mouse anti-human FITC-conjugated CD44, CD90, and CD45 antibodies (1:10; BD Bioscience); mouse anti-human STRO-1 antibody (1:10; R&D systems, Minneapolis, MN); rabbit PE-conjugated anti-mouse IgM (1:10; Santa Cruz, California, CA); normal mouse IgM (1:10; Santa Cruz); normal mouse IgG-PE (1:10; Santa Cruz); and normal mouse FITC-conjugated IgG (1:10; Santa Cruz). All flow cytometry tests were performed on a FACSaria (BD Bioscience).

Amniotic membrane preparation

Human fetal membranes were harvested after obtaining informed consent from patients undergoing cesarean

delivery at the Tokyo Medical and Dental University Hospital. This research protocol was approved by the ethics committee for clinical research at the Tokyo Medical and Dental University. The amniotic membrane was washed and cut into small pieces and incubated for 1 h at 37°C in 0.02% EDTA. After incubation, the chorion was mechanically removed from the amnion using a cell scraper. Cell components in the amnion were removed by high hydrostatic pressure treatment as reported previously.^{27,28} The resulting decellularized amnion was stored in the Dulbecco's modified Eagle's medium containing 50% glycerol (Invitrogen) at -80°C until use.

Transplantation of PDLSC-transferred amniotic membrane in periodontal defects

All study protocols and procedures were approved by the Animal Care Ethics Committee of Tokyo Medical and Dental University. Six male nude rats (F344/N-Jcl-rnu/rnu; age 7 weeks, $n=6$) were anesthetized with isoflurane. The gingival flap of the palatal side was elevated at the maxillary first molar. A periodontal defect mimicking class II furcation involvement (bucco-palatal depth, 2 mm; horizontal depth, 1.5 mm) was created by removing the alveolar bone, periodontal ligament, and root cementum using a dental burr and stereoscopic microscope. After creation of the defect, 3×2 mm of PDLSC-amnion (experimental) or amnion (control) was placed to fully cover the defect. In the experimental group, the cell-transferred surface of PDLSC-amnion faced the defect. In each rat, maxillary first molar in the right and left side were assigned to PDLSC-amnion and amnion site, respectively. The gingival flap was sutured with a 10-0 nylon suture (Mani, Tochigi, Japan). The transplanted PDLSC-GFP were visualized using an *in vivo* imaging system (photon imager; Biospace Measures, Paris, France).

Histological analysis

Four weeks after transplantation, the maxilla block of the rats was removed following perfusion fixation with a 4% paraformaldehyde phosphate buffer. The maxilla blocks were decalcified in 10% EDTA at 4°C for 4 weeks followed by dehydration, paraffin embedding, and sectioning. Tissue sections of 8 µm thickness were prepared. The sections were stained with hematoxylin-eosin and azan for microscopic observation. Images of histological sections were obtained using a Leica 5000B microscope (Leica Microsystems, Wetzlar, Germany) and Keyence BZ800 (Keyence, Osaka, Japan). For cross-sectional observation of PDLSC-amnion, PDLSC-GFP were transferred onto the amnion as described above, and 5-µm frozen sections were obtained. After nuclear staining with TO-PRO3 (Invitrogen), cross-sectioned PDLSC-amnion was observed under a confocal microscope (LSM 510; Zeiss, Oberkochen, Germany).

Microcomputed tomography analysis

Healing of the periodontal defect was examined using microcomputed tomography (micro-CT) scans (SMX-90CT; Shimadzu Co., Kyoto, Japan) 4 weeks after operation. Three-dimensional images were constructed from the scanned data using VG Studio MAX 2.0 (Volume Graphics, Heidelberg, Germany). To quantify bone healing at the furcation area, we

measured the length of the bone in sliced CT images as demonstrated in Figure 5B. We drew two tangent lines at the mesial and distal sides of palatal and mesiobuccal roots. A perpendicular line on the distal tangent line was drawn at the center of the furcation. On the perpendicular line, the amount of horizontal bone filling was calculated as follows using length as shown in Figure 5B; percent bone filled = $(a/a+b) \times 100$.

Statistical analysis

Statistical differences of the experimental groups were determined by using the Student's *t*-test. Differences with $p < 0.05$ were considered significant.

Results

MSC properties of cultured PDLSC

First, we confirmed the MSC properties of PDLSCs such as unique cell surface antigen expression and differentiation into osteoblasts, adipocytes, and chondrocytes. As shown in Figure 1A, PDLSCs expressed MSC markers, including CD90, CD44, CD73, and CD105 and were negative for the endothelial cell marker CD31 and pan-leukocyte marker CD45. STRO-1 and CD146, which have been reported as markers for progenitors of mesenchymal cells, were also expressed in cultured PDLSCs. When PDLSCs were cultured under specific differentiation conditions, they exhibited von Kossa-positive calcium deposition (Fig. 1B), Oil Red O-positive lipid accumulation in the cytoplasm (Fig. 1C) and Alcian blue-positive sulfated proteoglycans in the cell pellet, which represented osteoblast, adipocyte, and chondrocyte differentiation, respectively. The control group in chondrocyte differentiation did not form chondrogenic spheres large enough to make sections.

PDLSC transfer onto amnion

We used a decellularized amniotic membrane (amnion) as a scaffold for PDLSC transplantation (Fig. 2A). Figure 2B shows a schematic drawing of PDLSC transfer onto the amnion. Three-hour intervals of PDLSC incubation on the transfer substrate and a 20-h cell transfer to the amnion were optimized in a pilot study. Figure 2C shows a cross-sectional image of amnion with transferred PDLSC-GFP (PDLSC-GFP-amnion) on the membrane using the cell transfer technique. The cell-transferred membrane showed a monolayer of PDLSCs on the amnion. The PDLSC-amnion is a thin, flexible membrane and can mechanically withstand surgical manipulation by tweezers and probes, while holding transferred cells in place (Fig. 2D-1–D-5). PDLSC-GFP were stably observed on the amnion, despite wrinkling (Fig. 2D-2), folding (Fig. 2D-3), stretching (Fig. 2D-4), and dragging (Fig. 2D-5) of the membrane by surgical forceps and probes.

Surgical creation of periodontal defects in rat maxilla

To test the regenerative ability of PDLSC-amnion, we used a surgically created periodontal defect model in rats. We generated periodontal defects with a bucco-palatal depth of 2 mm and a horizontal depth of 1.5 mm at the mesial furcation; we removed bone, periodontal ligament, and root cementum using a dental burr. Figure 3 shows stereoscopic microscope views and 3D

FIG. 1. MSC-like characteristics of PDLSCs. MSC-like characteristics of cultured human PDLSCs were examined. **(A)** PDLSCs expressed cell surface antigens, including CD90, CD44, CD73, CD105, STRO-1 and CD146, whereas CD31 and CD45 were not detected in flow cytometry. **(B–D)** Triple lineage differentiation ability of PDLSCs. PDLSCs showed von Kossa **(B)**, Oil Red O **(C)**, and Alcian blue **(D)** staining under specific differentiation conditions to osteoblasts **(B)**, adipocytes **(C)**, and chondrocytes **(D)**, respectively. Scale bar = 100 μ m **(B, C)** and 300 μ m **(D)**. Magnification = $\times 4$ **(B)**, $\times 12$ **(C)**, and $\times 2$ **(D)**. PDLSC, periodontal ligament stem cell; MSC, mesenchymal stem cell. Color images available online at www.liebertpub.com/tea

reconstructed CT images of rat periodontal tissues before and after defect creation. We confirmed the reproducibility of defect creation by using CT images on both right and left sides (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). PDLSC-amnion or amnion alone was placed to cover the defect completely. Figure 3C shows *in vivo* imaging of PDLSC-amnion transplanted in the rat maxilla and demonstrates fluorescence only in the side transplanted with

PDLSC-GFP-amnion (right side); no signal was observed on the amnion side (left).

CT images of periodontal defects after transplantation

Figure 4 shows 3D reconstructed CT images of periodontal defects 4 weeks after transplantation of amnion alone or PDLSC-amnion. Images are shown from all six

FIG. 2. Fabrication of PDLSC-amnion. **(A)** Decellularized amnion after trimming. **(B)** Schematic view of PDLSCs transferring onto amnion. Cultured PDLSCs were trypsinized and suspended in the culture medium. The PDLSC suspension was added onto the transfer substrate (yellow) and cultured for 3 h. The substrate with PDLSCs (green) was then placed onto amnion (gray) to permit the cell surface to contact the amnion. After addition of the culture medium, amnion with a transfer substrate was incubated for another 20 h and the transfer substrate was removed using forceps. Transferred PDLSCs remained as a monolayer of cells on the amnion. **(C)** A cross-sectional image of PDLSC-GFP-amnion. The frozen section of PDLSC-GFP-amnion was nuclear stained with TO-PRO-3. Transferred PDLSC-GFP showed a monolayer of the cells on amnion (left). No cellular component was observed other than transferred GFP-positive cells in the amnion. GFP, TO-PRO-3, brightfield, and merged images of PDLSC-amnion (right). *Stromal layer of amnion. **(D)** Overhead view of PDLSC-GFP-transferred amnion after trimming and during surgical manipulation of the membrane with tweezers. Trimmed amnion in square **(D-1)**. Amnion with wrinkles **(D-2)**. Folded PDLSC-amnion **(D-3)**. PDLSC-amnion pulled from opposite directions with forceps **(D-4)**. PDLSC-amnion dragged with surgical probe **(D-5)**. Despite the dynamic change of its shape and surgical manipulation, PDLSC-GFP stably adhered to the amnion. Scale bar = 50 µm **(C)** and 300 µm **(D)**. Magnification = $\times 40$ **(C, left)**, $\times 60$ **(C, right)**, and $\times 2$ **(D)**. GFP, green fluorescence protein. Color images available online at www.liebertpub.com/tea

tested animals to demonstrate the overall tendency of periodontal defect healing, including wide inter-animal variation. Overall, the depth of the furcation defect appeared to be shallower on the PDLSC-amnion side than on the amnion side after 4 weeks of transplantation.

We analyzed bony healing after transplantation at the furcation in horizontally sliced images of 3D reconstructed CT data as shown in Figure 5. To confirm that slicing was uniform, which is critical for comparison and quantification, we obtained images that included the posterior tooth in

FIG. 3. Surgical creation of periodontal defects in the rat maxillary first molar. **(A)** Stereoscopic microscopic view of rat periodontal defect creation. The alveolar bone, periodontal ligament, cementum, and surface of dentin were removed using a dental burr. **(B)** Micro-CT image of maxillary first molar palatal and buccal-mesial root. Approximately 2 mm of bucco-palatal depth and 1.5 mm of horizontal depth were created. **(C)** *In vivo* image of PDLSC-GFP-amnion and transplanted rat. PDLSC-GFP-amnion (right side) and amnion alone (left side) were transplanted into a surgically created periodontal defect in rats. Immediately after transplantation, the fluorescence signal was examined using an *in vivo* imager. Only the side transplanted with PDLSC-GFP-amnion showed positive signals. Micro-CT, microcomputed tomography. Color images available online at www.liebertpub.com/tea

horizontal micro-CT sections at the same plane on the right and left sides. More bone filled the furcation on the PDLSC-amnion side than on the amnion-alone side (arrows), although inter-animal variation was also observed. To quantify bone healing, we measured the bone length at furcation in sliced CT images and calculated the amount of filled bone as demonstrated in Figure 5B and Supplementary Table S1. The amount of bone filling was higher in the PDLSC-amnion group than in the amnion-alone group.

Histological observation of rat periodontal tissues after transplantation

In histological sections, newly formed bone at the furcation area, which was observed coronal to the notches, was prominent in the PDLSC-amnion-transplanted side relative to the side treated with amnion alone (Fig. 6A). In the amnion-treated side, the defect was filled mainly with fibroblastic cells, collagen fibers, and blood vessels. Residual amnion, which was pachychromatic on eosin staining, was

found on both the sides. At higher magnifications, a thin, newly formed cementum-like layer was observed on the denuded dentin in PDLSC-amnion-transplanted defects, whereas new cementum and collagen bundles that were connected to the root surface were unclear in amnion-transplanted defects (Fig. 6B, C). The thickness of newly formed cementum in the PDLSC-amnion site was thinner ($3.441 \pm 0.485 \mu\text{m}$) than healthy cementum ($8.046 \pm 0.693 \mu\text{m}$), while it was not measurable in amnion-transplanted sites (Fig. 6C). Single-layered cementoblast-like cells were seen on the new cementum on the PDLSC-amnion-transplanted side, as shown in Figure 6D. Most of the cementoblast-like cells were flat morphology, suggesting their inactive state. Newly formed collagen bundles were evident in the periodontal ligament space, which ran from the denuded dentin surface to the newly formed bone as determined by azan staining (Fig. 6E) on the PDLSC-amnion-transplanted side. The ends of the collagen bundles were inserted into the cementum-like layer formed perpendicularly on denuded dentin, which resembled functional Sharpey's fibers (Fig. 6E). In some

FIG. 4. Periodontal tissue healing after PDLSC-amnion transplantation. Three-dimensionally reconstructed micro-CT image of periodontal defects 4 weeks after transplantation. Data from all six tested rats are shown. CT images of the amnion side (left panel) and PDLSC-amnion side (right panel) are shown. The furcation defect is shallower on PDLSC-amnion-treated side than on the amnion-treated side. The concavity of bone between the mesial and palatal root is small on the PDLSC-amnion side (arrows). Color images available online at www.liebertpub.com/tea

areas, the rest of the amnion interfered with the orientation of collagen bundles running from the cementum to bone.

Discussion

Amnion has been shown to have prominent characteristics as a wound dressing given its antifibrotic, anti-inflammatory, and antimicrobial effects.²¹ Thus, amnion has been clinically applied in ophthalmology and dermatology to treat burns, ulceration, and wounds in the eyes and on the skin.^{22,23} Moreover, amnion has mechanical strength and flexibility as well as low immunogenicity. Therefore, we previously examined the therapeutic potential of cell-transferred amnion in disease models, including hindlimb ischemia and calvaria bone defects.^{24–26} The enhanced wound healing in animals transplanted with endothelial cell- or osteoblast-transferred amnion suggested efficacy as a cell-based regenerative treatment. In the present study, as a new therapeutic target, we applied amnion with PDLSCs, which are promising cell types for periodontal regeneration, in a rat periodontal defect model and examined the therapeutic potential of PDLSC-

amnion in periodontal disease. We showed that the new periodontal tissue formation was enhanced in the defects after transplantation with PDLSC-amnion relative to defects treated with amnion alone in micro-CT and histological analysis.

Advantages of our transplantation material include its mechanical strength against damage incurred by the surgical procedure and its flexibility, which allowed PDLSCs to adapt to the denuded root surface. As shown in Figure 2, cells remained firmly on the membrane surface after the PDLSC-amnion was moved by surgical instruments. Further, we were able to cut the membrane to the desired size to adjust to the recipient site and even fold it into small pieces, while maintaining the transferred cells on the membrane. These unique characteristics of the membrane make it possible to transplant cells into narrow and small recipient sites, including periodontal bony defects. Furthermore, upon transplantation, the amnion conformed to the surface contours because of its pliability, which allowed the transferred stem cells to closely adhere to the transplanted site. Thus, by using the PDLSC-amnion, we transplanted a monolayer of PDLSCs

FIG. 5. Periodontal tissue healing after PDLSC-amnion transplantation in horizontally sectioned CT images. **(A)** Horizontal micro-CT section from periodontal defects 4 weeks after transplantation. Data from six all tested rats are shown. CT images of the amnion-treated side and PDLSC-amnion-treated side are shown. Increased bone filling was observed at the furcation in the PDLSC-amnion-treated side relative to the side treated with amnion alone (arrows), although inter-animal variation was also observed. **(B)** Quantification of horizontal bone healing at the furcation. Schematic representation of parameters for measurement. Two tangent lines were drawn at the mesial and distal sides of the palatal and mesiobuccal roots (red). A perpendicular line was drawn on the distal tangent line at the center of the furcation (blue). On the blue line, the length from each red line to the bone margin (a and b) was measured. The amount of bone filling was calculated as $(a/(a+b)) \times 100$. The amount of bone filling was higher in the PDLSC-amnion-treated group than in the group treated with amnion alone. $*p < 0.05$. Color images available online at www.liebertpub.com/tea

FIG. 6. Histological images of rat periodontal tissues 4 weeks after PDLSC-amnion or amnion transplantation. **(A)** Hematoxylin-eosin staining of cross sections of sites transplanted with amnion (left) or PDLSC-amnion (right) in the rat maxillary first molar. Periodontal defects filled with PDLSC-amnion demonstrated higher bone regeneration than defects filled with amnion alone. Black arrows show the surgically created notch that represents the bottom of the created defects. Red arrows show the coronal margin of the alveolar bone 4 weeks after transplantation. **(B)** High magnification image of periodontal tissues at the bottom of the defect amnion (left)- and PDLSC-amnion-transplanted rats. Newly formed thin cementum-like layer and bone were evident coronal to notches in the PDLSC-amnion-transplanted site (right). In the amnion-transplanted site (left), new cementum formation was not observed on denuded dentin, and the defect was filled mainly with collagen fibers and blood vessels. Arrow, notch; D, dentin; B, bone; PDL, periodontal ligament. **(C)** Higher magnification images of newly formed cementum in the amnion- and PDLSC-amnion-transplanted site and healthy rat. Newly formed cementum layer (between two arrows) was observed on PDLSC-amnion-transplanted root surface (center), while the layer was not observed in amnion-transplanted site (left, arrow). The thickness of the new cementum layer in PDLSC-amnion was thinner than that observed in healthy cementum (right, arrows) D, dentin; PDL, periodontal ligament. **(D)** Close-up image of newly formed cementum in the PDLSC-amnion-transplanted site. New cementum (yellow arrow) and cementoblast-like cells (red arrows) were found on the denuded dentin surface. D, dentin. **(E)** Azan staining of the bottom of the defect in a PDLSC-amnion-transplanted site. Azan-positive collagen bundles were evident in the periodontal ligament space, which runs from the denuded dentin surface to the newly formed bone. The end of the collagen bundles was inserted into the new cementum (yellow arrows). Black arrow, notch; D, dentin; B, bone; PDL, periodontal ligament. Magnification= $\times 3$ (A), $\times 8$ (B), $\times 30$ (D), and $\times 12$ (E). Color images available online at www.liebertpub.com/tea

directly over the denuded root surface. It is difficult to achieve close contact between cells and the transplantation site when using other scaffold materials such as hydroxyapatite, tricalcium phosphate, and collagen gels. The observed enhancement of periodontal regeneration by PDLSC-amnion may have resulted from close contact between dentin and transplanted PDLSCs in the periodontal defect. We did not determine the fate of transplanted cells or mechanisms underlying the observed enhancement of periodontal regeneration. There may be two possible mechanisms regulating the regeneration we observed in the present study. First is the direct differentiation of transplanted PDLSCs into periodontal tissue forming cells, including osteoblasts, cementoblasts, and periodontal ligament fibroblasts. The second is the indirect mechanism such as the stimulation of host cells with regenerative potential by the transplanted PDLSCs through paracrine factors. Recent studies have revealed that the transplanted MSC enhances the wound healing processes through the production of growth factors, cytokines, and extracellular matrices. Although PDLSC-amnion has advantage in PDLSC transplantation in the form of direct contact of PDLSCs to the root surface, which expects the first mechanism mentioned above, the second paracrine mechanism may also be involved in the regeneration by the PDLSC-amnion. Inukai *et al.* have shown that the conditioned medium from cultured bone marrow MSC induced regeneration of periodontal tissues in dog and suggested the potential of paracrine factors from MSC to regenerate periodontal tissues.²⁹ Further studies are underway to address these points.

As shown in Figure 6, we observed newly formed periodontal ligament fibers that ran perpendicularly to dentin in animals transplanted with PDLSC-amnion. Similar fibers have been reported previously in PDLSC-transplanted animal studies and suggested to be the functional periodontal attachment apparatus.^{9,13} Thus, our histological results indicate proper regeneration by PDLSC-amnion. However, we also found remaining amnion in the periodontal ligament space at 4 weeks after surgery, and the remaining amnion interfered with periodontal ligament orientation as shown in Figure 6. In general, a scaffold material for cell transplantation has to maintain the cells in good condition during grafting and disappear after a certain period, so as not to inhibit regenerative processes. It is conceivable that the rest of the amnion will disappear shortly after 4 weeks; however, further studies with long-term observations are necessary to confirm this.

Chronic periodontitis patients are mainly in middle to old age and it has been demonstrated that stem cell functions of PDLSCs from aged donors were reduced compared with PDLSCs from young donors, including differentiation capacity and cementum/periodontal ligament-like tissue formation.³⁰ Therefore, for the treatment of chronic periodontitis patients using PDLSC-amnion, we may need to consider an alternative method for cell-based treatment, including allogeneic transplantation from young donors. It has been demonstrated that allogeneic PDLSC transplantation into swine periodontal defect models did not induce immunorejection by showing no enhancement of T-cell and B-cell proliferation and immunoglobulin production and suggested the possibility of periodontal regeneration using allogeneic PDLSCs.^{8,31} In this study, we used PDLSCs de-

rived from relatively young donors (age, 14–28 years) and transplanted them into adult rats (7 weeks old). It is needed to take the limitation of the animal model into consideration for the estimation of the result of this study. Although there are a lot of hurdles to overcome, including ethical issues, allogeneic PDLSCs from young donors might have the possibility to treat aged periodontal patients using cell transfer technology.

It is widely known that the amniotic membrane has anti-inflammatory and antiscarring ability when transplanted in wound models.^{21,22,23} In the present study, we found that PDLSC-transferred-amnion demonstrated enhanced regeneration of periodontal tissues. Thus, it is possible to speculate that our findings, at least in part, were derived from the effect of the amniotic membrane. In our preliminary experiment, we could not find a difference in the extent of periodontal regeneration between defect alone (no transplantation) and amnion transplantation by using the same periodontal defect model (data not shown). Therefore, we concluded that the periodontal regeneration we found in this study was mostly derived from transferred PDLSCs. PDLSCs were shown to have the ability to differentiate into cementoblasts that form the cementum, which is a crucial tissue in periodontal regeneration.^{32–37} Further, it was shown that PDLSCs form a cementum-like tissue on the surface of hydroxyl-apatite particles ectopically in nude mice.¹⁶ Moreover, it has been demonstrated that PDLSCs form cementum-like tissue when they are transplanted onto root surfaces coated with dentin noncollagenous proteins *in vivo*.³⁸ These data strongly suggest that PDLSCs have the ability to recognize their microenvironment (i.e., dentin surface) and differentiate into cementoblasts. Our PDLSC-amnion allows close contact between PDLSCs and the dentin surface, which enables the interaction between PDLSCs and their regenerative microenvironment and may favor periodontal regeneration.

Conclusion

In conclusion, we fabricated novel PDLSC transplant materials by using decellularized amnion and cell transfer technology and transplanted them into surgically created periodontal defects in rats. PDLSC-amnion significantly enhanced the formation of periodontal tissues *in vivo*. These data suggest that the transplantation of PDLSC-amnion could be a new regenerative therapy for periodontal disease.

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Disclosure Statement

No competing financial interests exist.

References

1. Page, R.C., Offenbacher, S., Schroeder, H.E., Seymour, G.J., and Kornman, K.S. Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontol* 2000 **14**, 216, 1997.

2. Pihlstrom, B.L., and Michalawiz, B.S. Periodontal diseases. *Lancet* **366**, 1809, 2005.
3. Ankrum, J., and Karp, J.M. Mesenchymal stem cell therapy: two steps forward, one step back. *Trends Mol Med* **16**, 203, 2010.
4. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143, 1999.
5. Liu, Z.J., Zhuge, Y., and Velazquez, O.C. Trafficking and differentiation of mesenchymal stem cells. *J Cell Biochem* **106**, 984, 2009.
6. Iwata, T., Yamato, M., Tsuchioka, H., Takagi, R., Mukobata, S., Washio, K., Okano, T., and Ishikawa, I. Periodontal regeneration with multi-layered periodontal ligament-derived cell sheets in a canine model. *Biomaterials* **30**, 2716, 2009.
7. Park, J.Y., Jeon, S.H., and Choung, P.H. Efficacy of periodontal stem cell transplantation in the treatment of advanced periodontitis. *Cell Transplant* **20**, 271, 2011.
8. Ding, G., Liu, Y., Wang, W., Wei, F., Liu, D., Fan, Z., An, Y., Zhang, C., and Wang, S. Allogeneic periodontal ligament stem cell therapy for periodontitis in swine. *Stem Cells* **28**, 1829, 2010.
9. Yang, Y., Rossi, F.M., and Putnins, E.E. Periodontal regeneration using engineered bone marrow mesenchymal stromal cells. *Biomaterials* **31**, 8574, 2010.
10. Li, H., Yan, F., Lei, L., Li, Y., and Xiao, Y. Application of autologous cryopreserved bone marrow mesenchymal stem cells for periodontal regeneration in dogs. *Cells Tissues Organs* **190**, 94, 2009.
11. Kawaguchi, H., Hirachi, A., Hasegawa, N., Iwata, T., Hamaguchi, H., Shiba, H., Takata, T., Kato, Y., and Kurihara, H. Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. *J Periodontol* **75**, 1281, 2004.
12. Tobita, M., Uysal, A.C., Ogawa, R., Hyakusoku, H., and Mizuno, H. Periodontal tissue regeneration with adipose-derived stem cells. *Tissue Eng Part A* **14**, 945, 2008.
13. Tsumanuma, Y., Iwata, T., Washio, K., Yoshida, T., Yamada, A., Takagi, R., Ohno, T., Lin, K., Yamato, M., Ishikawa, I., Okano, T., and Izumi, Y. Comparison of different tissue-derived stem cell sheets for periodontal regeneration in a canine 1-wall defect model. *Biomaterials* **32**, 5819, 2011.
14. Jiang, J., Wu, X., Lin, M., Doan, N., Xiao, Y., and Yan, F. Application of autologous periosteal cells for the regeneration of class III furcation defects in Beagle dogs. *Cyto technology* **62**, 235, 2010.
15. Kawase, T., Okuda, K., Kogami, H., Nakayama, H., Nagata, M., Sato, T., Wolff, L.F., and Yoshie, H. Human periosteum-derived cells combined with superporous hydroxyapatite blocks used as an osteogenic bone substitute for periodontal regenerative therapy: an animal implantation study using nude mice. *J Periodontol* **81**, 420, 2010.
16. Seo, B.M., Miura, M., Gronthos, S., Bartold, P.M., Batouli, S., Brahimi, J., Young, M., Robey, P.G., Wang, C.Y., and Shi, S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364**, 149, 2004.
17. Wada, N., Menicanin, D., Shi, S., Bartold, P.M., and Gronthos, S. Immunomodulatory properties of human periodontal ligament stem cells. *J Cell Physiol* **219**, 667, 2009.
18. Gronthos, S., Mrozik, K., Shi, S., and Bartold, P.M. Ovine periodontal ligament stem cells: isolation, characterization, and differentiation potential. *Calcif Tissue Int* **79**, 310, 2006.
19. Mrozik, K.M., Zilm, P.S., Bagley, C.J., Hack, S., Hoffmann, P., Gronthos, S., and Bartold, P.M. Proteomic characterization of mesenchymal stem cell-like populations derived from ovine periodontal ligament, dental pulp, and bone marrow: analysis of differentially expressed proteins. *Stem Cells Dev* **19**, 1485, 2010.
20. Niknejad, H., Peirovi, H., Jorjani, M., Ahmadiani, A., Ghanavi, J., and Seifalian, A.M. Properties of the amniotic membrane for potential use in tissue engineering. *Eur Cell Mater* **15**, 88, 2008.
21. Díaz-Prado, S., Muiños-López, E., Hermida-Gómez, T., Cicioni, C., Rendal-Vázquez, M.E., Fuentes-Boquete, I., de Toro, F.J., and Blanco, F.J. Human amniotic membrane as an alternative source of stem cells for regenerative medicine. *Differentiation* **81**, 162, 2011.
22. Lo, V., and Pope, E. Amniotic membrane use in dermatology. *Int J Dermatol* **48**, 935, 2009.
23. Altan-Yaycioglu, R., Akova, Y.A., and Oto, S. Amniotic membrane transplantation for treatment of symblepharon in a patient with recessive dystrophic epidermolysis bullosa. *Cornea* **25**, 971, 2006.
24. Akahori, T., Kobayashi, A., Komaki, M., Hattori, H., Nakahama, K., Ichinose, S., Abe, M., Takeda, S., and Morita, I. Implantation of capillary structure engineered by optical lithography improves hind limb ischemia in mice. *Tissue Eng Part A* **16**, 953, 2010.
25. Yoshida, T., Komaki, M., Hattori, H., Negishi, J., Kishida, A., Morita, I., and Abe, M. Therapeutic angiogenesis by implantation of a capillary structure constituted of human adipose tissue microvascular endothelial cells. *Arterioscler Thromb Vasc Biol* **30**, 1300, 2010.
26. Tsugawa, J., Komaki, M., Yoshida, T., Nakahama, K., Amagasa, T., and Morita, I. Cell-printing and transfer technology applications for bone defects in mice. *J Tissue Eng Regen Med* **5**, 695, 2011.
27. Fujisato, T., Minatoya, K., Yamazaki, S., Meng, Y., Niwaya, K., Kishida, A., Nakatani, T., and Kitamura, S. Preparation and recellularization of tissue engineered bioscaffold for feat valve replacement. In: Mori, H., and Matsuda, H., eds. *Cardiovascular Regeneration Therapies Using Tissue Engineering Approaches*. Tokyo: Springer, 2005, p. 83.
28. Wilshaw, S.P., Kearney, J.N., Fisher, J., and Ingham, E. Production of an acellular amniotic membrane matrix for use in tissue engineering. *Tissue Eng* **12**, 2117, 2006.
29. Inukai, T., Katagiri, W., Yoshimi, R., Osugi, M., Kawai, T., Hibi, H., and Ueda, M. Novel application of stem cell-derived factors for periodontal regeneration. *Biochem Biophys Res Commun* **430**, 763, 2013.
30. Zhang, J., An, Y., Gao, L.N., Zhang, Y.J., Jin, Y., and Chen, F.M. The effect of aging on the pluripotential capacity and regenerative potential of human periodontal ligament stem cells. *Biomaterials* **33**, 6974, 2012.
31. Liu, O., Xu, J., Ding, G., Liu, D., Fan, Z., Zhang, C., Chen, W., Ding, Y., Tang, Z., and Wang, S. Periodontal ligament stem cells regulate B lymphocyte function via programmed cell death protein 1. *Stem Cells* **31**, 1371, 2013.
32. Bosshardt, D.D. Are cementoblasts a subpopulation of osteoblasts or a unique phenotype? *J Dent Res* **84**, 390, 2005.
33. Lekic, P., and McCulloch, C.A. Periodontal ligament cell population: the central role of fibroblasts in creating a unique tissue. *Anat Rec* **245**, 327, 1996.
34. Komaki, M., Iwasaki, K., Arzate, H., Narayanan, A.S., Izumi, Y., and Morita, I. Cementum protein 1 (CEMP1) induces a

- cementoblastic phenotype and reduces osteoblastic differentiation in periodontal ligament cells. *J Cell Physiol* **227**, 649, 2012.
35. Paula-Silva, FW., Ghosh, A., Arzate, H., Kapila, S., da Silva, L.A., and Kapila, Y.L. Calcium hydroxide promotes cementogenesis and induces cementoblastic differentiation of mesenchymal periodontal ligament cells in a CEMP1- and ERK-dependent manner. *Calcif Tissue Int* **87**, 144, 2010.
36. Zeichner-David, M. Regeneration of periodontal tissues: cementogenesis revisited. *Periodontol 2000* **41**, 196, 2006.
37. Grzesik, W.J., and Narayanan, A.S. Cementum and periodontal wound healing and regeneration. *Crit Rev Oral Biol Med* **13**, 474, 2002.
38. Ma, Z., Li, S., Song, Y., Tang, L., Ma, D., Liu, B., and Jin, Y. The biological effect of dentin noncollagenous proteins (DNCPs) on the human periodontal ligament stem cells (HPDLSCs) *in vitro* and *in vivo*. *Tissue Eng Part A*. **14**, 2059, 2008.

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