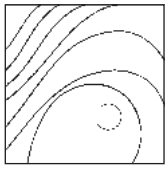
The Ability of Human Periodontium-Derived Stem Cells to Regenerate Periodontal Tissues: A Preliminary In Vivo Investigation



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Periodontium-derived stem cells (pdSCs) can be cultured as dentospheres and differentiated into various cells of the neuronal lineage such as glial cells, thereby demonstrating their stem cell state. This study investigated whether pdSCs could be differentiated into the osteogenic lineage and, if so, whether these cells are able to regenerate periodontal tissue in vivo in an athymic rat model. Human adult pdSCs were isolated during minimally invasive periodontal surgery and expanded in vitro. To induce osteogenic differentiation, expanded pdSCs were cultured for 3 weeks in osteogenic differentiation media. Staining for alkaline phosphatase expression was positive, suggesting osteogenic differentiation. For in vivo studies, pdSCs were delivered onto suitable collagen sponges and implanted into periodontal defects on the right buccal cortex of the mandible in 16 immunodeficient nude rats. Histologic analysis of samples from the test side revealed reformation of periodontal ligament–like tissue, collagen fibers, and elements of bone, but no functional periodontal tissue regeneration. The data show that human adult pdSCs are capable of regenerating elements of bone and collagen fibers in an in vivo animal model. (Int J Periodontics Restorative Dent 2011;31:e94–e101.)

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Stem cells differ from other types of cells in the body. All stem cells, regardless of their source, have general properties: They are capable of dividing and self-renewing themselves for long periods, they are unspecialized, and they can give rise to specialized cell types.[1] In a recent study,[2] it was hypothesized that stem cells may be present in the dental follicle and may be capable of differentiating into cells of the periodontium. At the same time, the human third molar pad has been shown to possess neural crest–derived cells that represent multipotent stem/progenitor cells.[3] It has been speculated that adult dental pulp tissue may also contain a population of multipotential stem cells, which are clonogenic, highly proliferative, and capable of regenerating tissue.[4,5] Stromal-like cells were reestablished in culture from primary multipotential stem cell transplants and retransplanted into immunocompromised mice to generate a dentin pulp–like tissue, demonstrating their self-renewal capability.[6] The periodontal ligament (PDL) is a group of specialized

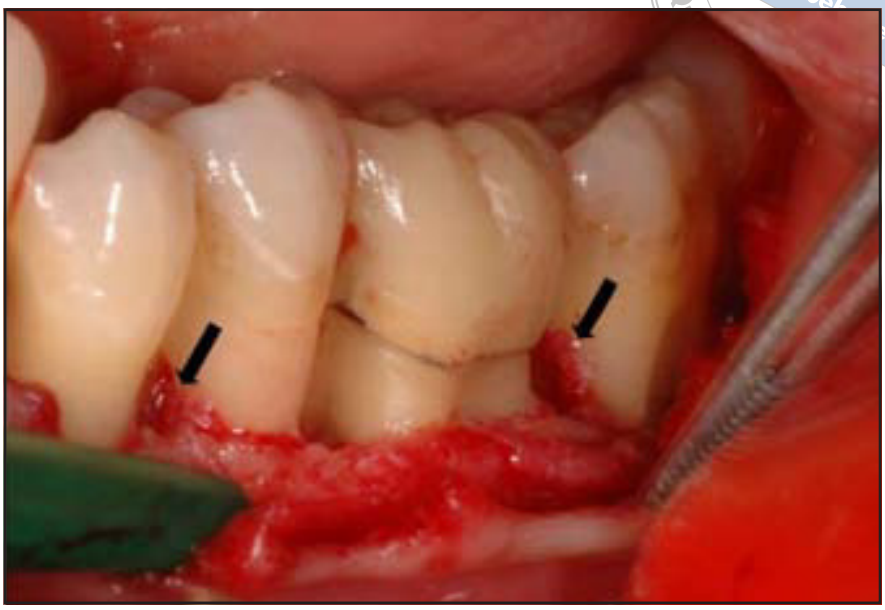


Fig 1 *Minimally invasive isolation of periodontal tissues during periodontal regenerative surgical therapy. Arrows = areas of tissue containing pdSCs.*

connective tissue fibers essentially attaching cementum to the alveolar bone and maintaining tooth support. It has been demonstrated that the human PDL may contain progenitor cells capable of differentiation into cementoblasts or osteoblasts in vitro.[7–10] Taken together, the results of these studies demonstrated the capacity of multipotent postnatal stem cells from human PDL, or periodontium-derived stem cells (pdSCs), to generate a cementum-like tissue in vivo, thus representing a new therapeutic option for periodontal regeneration.[11–20]

A method to isolate and expand a stem cell population from periodontal granulation tissue has been described recently.[21] These pdSCs were positive for the neural stemness markers Nestin and Sox2 and can differentiate into various cell types of the neuronal lineage, including glial cells.[21] However, whether pdSCs are also capable of differentiating into the osteogenic lineage and regenerating periodontal tissue in vivo is unknown and therefore the focus of this study.

Method and materials

Isolation and ex vivo expansion of pdSCs

Human adult pdSCs were isolated from patients with periodontal disease by minimally invasive surgery at the Department of Periodontology, Faculty of Dental Medicine, University of Witten/Herdecke, as described previously (Fig 1).[22] All patients (age range, 23 to 54 years) gave informed consent in accordance with human ethical board approval for human PDL cell procurement (University of Witten/Herdecke, board protocol 54/2009). Cells were isolated from extracted periodontal tissues and expanded ex vivo for 10 days, as described previously.[21] Primary pdSC spheres appeared at days 8 to 10. For subcultivation, primary spheres were dissociated using Accutase (PAA). The subculturing protocol consisted of cell culture passage every 3 to 4 days with changing of the entire culture medium. The spheres were investigated immediately before subculturing (T0), and the subcultured cells were investigated 1 week (T1) and 2 weeks (T2) after subculturing.

Osteogenic differentiation

The medium was switched to osteogenic differentiation medium, first dissociating the pdSCs from T2 using Accutase to receive a single

Fig 2 *Surgical procedure at a test site.*

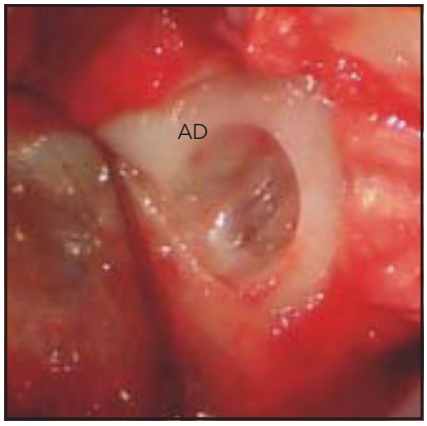


Fig 2a *Preparation of the artificial defect* (AD) *reaching the distal root of the first molar.*

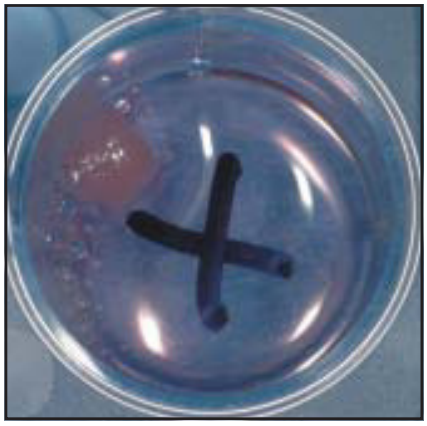


Fig 2b *Collagen sponge with predifferentiated pdSCs.*

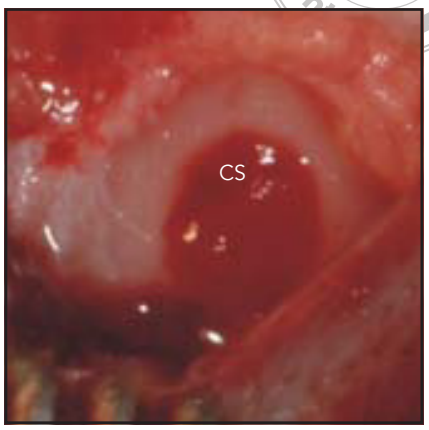


Fig 2c *Application of the congruent collagen sponge* (CS) *in the artificial defect.*

cell suspension. Subsequently, cells (1×10^5 to 2×10^5) were seeded onto cover slips and cultured for 3 weeks in Dulbecco Modified Eagle medium (Invitrogen) supplemented with 10% fetal calf serum (PAA) and 1% penicillin/streptomycin (PAA). To induce osteogenic differentiation, β-glycerophosphate (10 mM), L-ascorbic acid 2-phosphate (50 μM), and dexamethasone 21-phosphate (100 nM) (all Sigma-Aldrich) were added to the media. Subsequently, differentiated cells were fixed with 4% paraformaldehyde. Expression of alkaline phosphatase was determined by FastRed (Sigma) staining according to the manufacturer's instructions. FastRed staining was visualized by using a Leica TCS SP5 confocal laser scanning microscope (Leica Bensheim).

In vivo assay of periodontal tissue formation

The in vivo regenerative capacity of human adult pdSCs was investigated by using 10-week-old athymic nude rats. All animal experiments were held according to the approval of the German Animal Rights Organization. An artificial bone defect (approximately $2.5 \times 2.5 \times 2$ mm^3) was prepared on the test and control side at the level of the distal root of the first molars (Fig 2). Four days before transplantation, human adult pdSCs were dissociated and resuspended in osteogenic differentiation medium. Subsequently, osteogenic predifferentiated pdSCs (1×10^5 cells/mL) were plated on Opti-Maix collagen sponges, which were cultured for an additional 4 days in osteogenic differentiation media at 37°C and 5% carbon dioxide. Collagen sponges without pdSCs served as controls in the split-mouth model.

The collagen sponges with pdSCs were then transferred to the bone defects created on the test sides, whereas the collagen lattices without cells were transferred to defects created on the control sides. To obtain full coverage of the surgical areas, two layers of surgical sutures were used. Doxycycline (2 mg/mL) was administered orally for 14 days. After 10 days, the sutures were removed. In total, 17 rats were used, whereby 16 rats were considered as a test group and 1 rat served as a control (no implantation of pdSCs). The regenerative capacity of pdSCs was investigated for 2, 6, and 8 weeks. The animals were grouped according to observation period.

Histology

The mandible segments obtained from the animals were first fixed in formalin (4%) followed by 2.5%

glutaraldehyde and then washed with ethanol (70% for 45 minutes). Thereafter, they were decalcified with Rapid Decalcifier (Cell Path) solution (hydrochloric acid) for a fast decalcification. The segments were then embedded in paraffin using a Jung Histokinette 2000 tissue processor (Leica). Serial histologic sections with a thickness of 4 to 5 µm were cut. Afterward, the sections were placed on microscope slides and dried for 24 hours at a temperature of 37°C. Staining was carried out using azan blue staining.

Results

Prior to osteogenic differentiation and animal experimentation, the isolated and propagated pdSCs were analyzed for the expression of the (stem cell) marker panel as described previously.[21] Expanded pdSCs were postive for Nestin, Sox2, and CNPase, but negative for Notch1, GFAP, CD117, CD45, and Oct4 (data not shown), which is identical to previously published data.[21]

Osteogenic differentiation

The pdSC capacity to differentiate into the osteogenic lineage, which is a prerequisite for pdSC-based regenerative purposes, was investigated by subculturing T2 pdSCs. After 3 weeks of induction, cultured T2 pdSCs had formed extensive amounts of FastRed-stained mineral deposits throughout the cell layers. Interestingly, pdSCs grew as single cells or small colonies, whereas the majority formed large cell clusters (Fig 3). In any case, induction of osteogenic differentiation, as indicated by alkaline phosphatase staining, was detectable in pdSCs.

Histology

The developmental potential of the T2 pdSCs was assessed in vivo following transplantation in the athymic rat model. Transplants were recovered after 2, 4, and 6 weeks and then processed for histology. Five rats were excluded from the study because of postoperative death and contamination. All other rats (n = 11) were successfully included without any surgical complications before, during, or after the operation. The rats were first divided randomly into three groups (n = 4 each); the control rat belonged to group 1. Light microscopic sections taken from the control rat clearly demonstrated all histologic features related to a normal periodontium, namely the PDL, the supporting bone, and the dental hard tissue (Fig 4). Within the PDL, collagen fibers and blood vessels were seen. An overview of a representative section from a test site depicting the drilling hole as well as the experimental notch is given in Fig 5.

Throughout the experimental period, healing of the defects in both transplanted and nontransplanted control sites was completed uneventfully. Considering test animals, the control sites showed a reformation of PDL-like tissue, collagen fibers, and elements of bone at different levels in every section after 6 weeks. At 6 weeks postsurgery, this extensive new bone formation, leading to ankylosis with partial root resorption, was observed in all control sites. Blood vessels could also be seen in the new PDL tissue and in the bone tissue. The collagen fibers were located perpendicular to the newly formed bone. The collagen matrix was completely absorbed and replaced with other tissue (Fig 6).

At test sites where collagen sponges with pdSCs were transplanted, a reformation of PDL-like tissue, elements of bone, and osteocyte lacunae in the bone tissue could be seen after 6 weeks (Fig 7). Some putative transplanted cells were observed to attach onto root dentin surfaces. Blood vessels and collagen fibers could also be shown in the PDL tissue. In the regenerated PDL tissues, immature thin fibers were obliquely arranged parallel to the bone surface and not in a perpendicular direction. Such a fibril anchoring was never observed in the control sites. These observations were consistent for all four rats sacrificed 6 weeks postsurgery. However, a "functional" periodontium was not evident. Down-growth of junctional epithelium was observed to a slight degree over the investigation period. In several test sites, new formation could be observed (Fig 8).

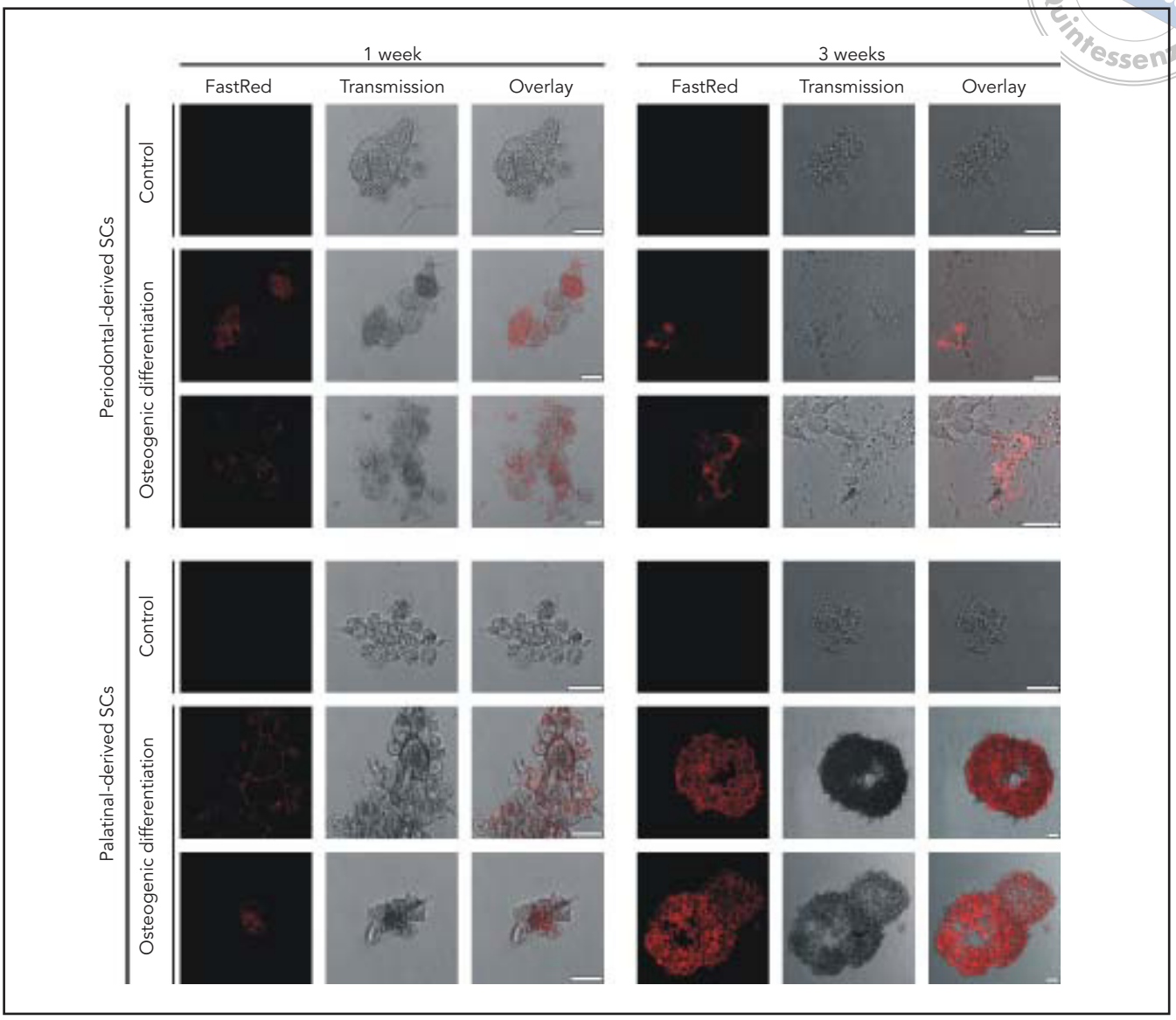
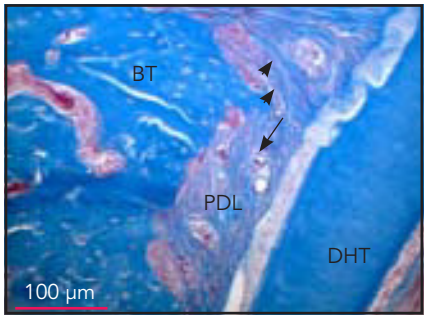


Fig 3 *Results of osteogenic differentiation. After 1 and 3 weeks of subculturing, T2 pdSCs had formed extensive amounts of FastRed-stained mineral deposits throughout the cell layers.*

Figs 4a and 4b *Histologic sections taken from the untreated control rat demonstrating normal periodontium. Collagen fibers* (black triangles) *and blood vessels* (black arrow) *can be seen within the PDL. BT = bone tissue; DHT = dental hard tissue; PDL = periodontal ligament (azan blue staining).*



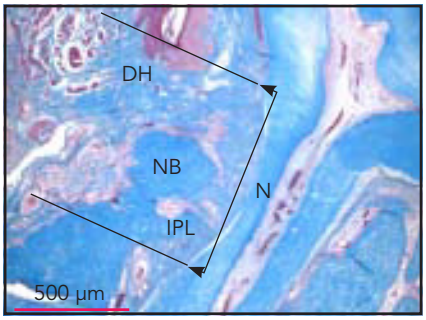


Fig 5 (left) *Overview of a histologic section from a test site. The boundaries of the drilled hole* (DH) *and the experimental notch* (N) *are marked. Note the formation of new bone* (NB) *and the irregular periodontal-like ligament* (IPL) *(azan blue staining).*

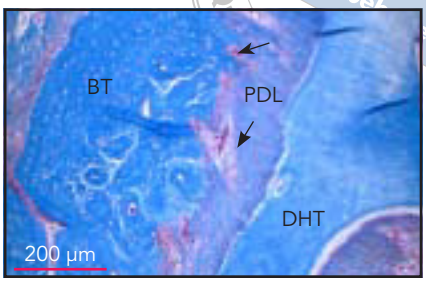
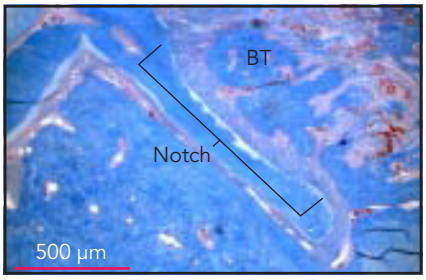
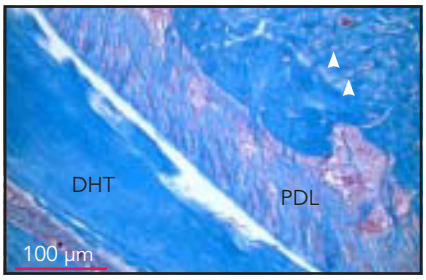


Fig 6 (left) *Histologic section taken from a control site after 6 weeks showing bone tissue elements* (BT), *periodontal ligament–like tissue* (PDL), *dental hard tissue* (DHT; *ie, dentin), and integration of collagen fibers within the PDL with new blood vessels* (arrows) *(azan blue staining).*



Figs 7a and 7b *Histologic section taken from a test site showing bone tissue elements* (BT), *osteocyte lacunae features* (white triangles), *periodontal ligament–like tissue* (PDL) *with collagen fibers and blood vessels, and dental hard tissues* (DHT; *ie, dentin) (azan blue staining).*



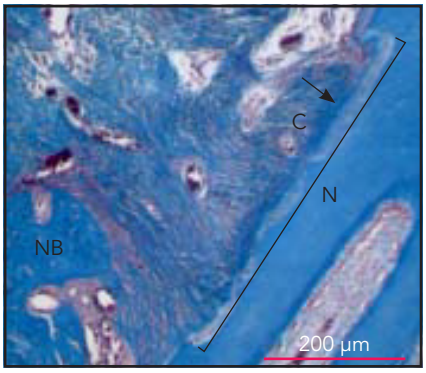
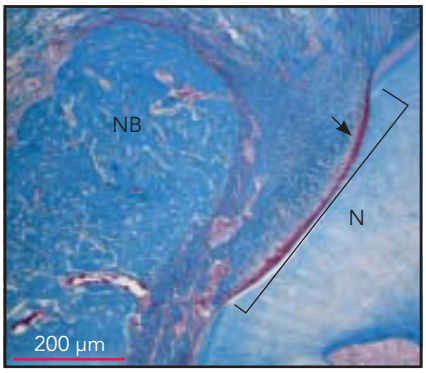


Fig 8a (left) *New cementum* (C, arrow) *formation within the experimental notch* (N) *in a test site. Note the new bone formation* (NB).

Fig 8b (right) *Down-growth of the junctional epithelium at a test site. NB = new bone; N = experimental notch; arrow = new cementum.*



Discussion

The periodontium is an unusual complex tissue composed of two hard (cementum and bone) and two soft (gingiva and PDL) tissues.[23] Periodontium-derived ligament stem cells (PDL stem cells), which have been isolated from the root surface of extracted teeth, were first described by Seo et al[18] and further distinguished by others.[11,14,15,17,19,21,24–28] In contrast, the current authors used human adult pdSCs that had been isolated from patients who suffered from a chronic type of periodontitis with a severe degree of inflammation.[21] The stemness of these cells was verified by the detection of stemness markers, such as Nestin and Sox2, as well as by the cell's capacity to differentiate into the neuronal lineage.[21] Several studies demonstrated that mesenchymal stem cells are capable of differentiating into osteoblast-like cells, cementoblast-like cells, and adipocytes.[4,18,19,29] These data are in agreement with results demonstrating that human adult pdSCs isolated from granulation tissue and subsequently expanded ex vivo are capable of

differentiating into the osteogenic lineage. Since osteogenic differentiation is a prerequisite for stem cells to regenerate bone tissue, the current data (see Fig 3) suggest that pdSCs from granulation tissue might have a potential value for stem cell–based bone tissue engineering in vivo. This is further supported by the recent findings of Arnold et al,[30] who demonstrated that after 1 week in culture, pdSCs produced calcium-rich extracellular matrix, suggesting that pdSCs can differentiate into cementoblasts or osteoblasts to maintain periodontal regeneration.

The investigation of the regenerative capacity of human stem cells in an animal model prerequisites the necessity of immunocompromised animals to avoid the rejection of the stem cell graft. Thus, the authors chose the athymic rat model, in accordance with Seo et al.[18,19] All of the animals showed a primary reformation of PDL-like tissue at test sites. In a pilot study, Zhao et al[31] demonstrated that cementoblasts have a marked ability to induce mineralization in periodontal wounds, while implanted dental follicle cells seem to inhibit periodontal healing. In the experimental sites of the current animal model, a cementum layer was observed on the root surfaces (Fig 8). This may suggest that this layer was comparatively immature and newly deposited onto previously denuded root surfaces. Obviously, even in this case, a "functional periodontium" seemed not to be regenerated.

Conceptually, the delivery of pdSCs to the denuded area in periodontal defects may serve as a viable approach to promote ideal periodontal tissue regeneration. When implanted into immunocompromised rats in association with a conductive carrier material, sphere-expanded human pdSCs possessed the potential to develop periodontal tissues. Of particular importance was the observation that the human pdSCs could produce both mineralized and soft connective tissues with many morphologic features similar to to cementum-like layers containing inserted Sharpey fibers. This strongly implies that this tissue is of a periodontal nature.

Conclusion

This in vivo study clearly showed that human adult pdSCs transplanted into an athymic rat model were able to regenerate periodontal tissue elements at different levels. However, prior to the ultimate use of pdSCs in human trials, further in vivo animal studies should be conducted to optimize the cell's regenerative capacity.

Acknowledgments

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