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ORIGINAL ARTICLE

Isolating stromal stem cells from periodontal granulation tissues

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Abstract Stem cell therapy is a promising area in regenerative medicine. Periodontal granulation tissues are often discarded during conventional surgery. If stromal stem cells can be isolated from these tissues, they can be used for subsequent surgery on the same patient. Fifteen human periodontal granulation tissue samples were obtained from intrabony defects during surgery. Immunohistochemistry (IHC) was carried out on five of the samples to identify STRO-1, a marker of mesenchymal stem cells. Five samples underwent flow cytometry analysis for the same marker. The remaining five samples were characterized by "colony formation unit-fibroblast" (CFU-f) assay and selected for proliferation assay, flow cytometry of stem cell markers, immunocytochemistry (ICC), multipotent differentiation assays, and repairing critical-size defects in mice. The ratio of STRO-1^+ cells detected by IHC was 5.91±1.50%. The analysis of flow cytometry for STRO-1 was 6.70±0.81%. Approximately two thirds of the CFU-f colonies had a strong reaction to STRO-1 in ICC staining. The cells were multipotent both in vitro and in vivo. Mice given bone grafts and stem cells showed significantly better bone healing than those without stem cells. Multipotent stromal stem cells can be isolated from human periodontal granulation tissues. These cells improve new bone formation when transplanted in mouse calvarial defects. Isolating stem cells from relatively accessible sites without extra procedures is clinically advantageous. This study demonstrated that human periodontal granulation tissues contain isolatable multipotent stem cells. The cells may be a good source for autotransplantation in subsequent treatment.

Keywords Periodontitis · Stem cell · Granulation tissue · STRO-1 · Calvaria

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Introduction

Regenerative medicine, and tissue engineering in particular, has attracted considerable attention from both public and scientific societies due to its enormous economic and therapeutic potential [1]. Successful tissue engineering requires the successful coordination of three elements: the implanted and cultured cells that will create the new tissue, a biomaterial to act as a scaffold or matrix to hold the cells, and biological signaling molecules that direct the cells to form the desired tissue type [2]. Stem cells are cellular sources for tissue engineering because of their two major characteristics: self-renewal and differentiation into multiple cell lineages [3]. The two broad types of mammalian stem cells are embryonic stem cells isolated

from the inner cell mass of blastocysts and postnatal stem cells found in postnatal tissues. These two types of stem cells have their own advantages and disadvantages. The major drawbacks to embryonic stem cells are ethical considerations and the risk of teratoma formation, while the major disadvantages of postnatal stem cells are their limited source, potency, and in vitro life span [4]. Among the different kinds of postnatal stem cells, mesenchymal stem cells (MSCs) are one of the best documented [5]. Research on the plasticity of MSCs shows that they can differentiate into osteoblasts, chondrocytes, adipocytes, cardiomyocytes, myoblasts, and neural cells [6]. Although MSCs from bone marrow have been successfully used for therapeutic purpose, the accessibility and availability of these cells is limited, preventing their wider application in clinical situations. Shi et al. successfully isolated high-quality human postnatal stem cells from tooth-associated tissues, including periodontal ligaments, and dental pulps of permanent and deciduous teeth [7–10]. Dental stem cells are attractive for novel approaches to treat diseases like periodontitis, dental caries, or to improve dental pulp healing and the regeneration of craniofacial bone and teeth. These cells are more accessible and more closely related to dental tissues than bone marrow-derived MSCs [11]. However, these cells are usually collected from the extracted deciduous teeth, impacted third molars, or premolars for orthodontic purposes. Obtaining these cells from patients beyond their late twenties is very unlikely. Periodontitis is a highly prevalent oral disease in adults. Researchers have developed many regenerative procedures to regain periodontal attachment. Although the results of these procedures are predictable and better than open debridement alone, they rarely achieve complete regeneration. Therefore, new alternatives in regenerative medicine involving stem cells are generating great interest. Unfortunately, the postnatal stem cells collected from bone marrow, cord blood, dental pulp, and periodontal ligaments are relatively inaccessible for most adults with chronic periodontitis. Discovering new sources of stem cells in older patients is therefore an important issue. The standard protocol for periodontal surgery involves the removal of the chronically inflamed granulation tissue lateral to the pocket epithelium and in angular bony defects [12]. Granulation tissue can form in connective tissue during wound healing, chronic inflammation, and certain pathological conditions. Interestingly, the granulation tissue fibroblasts from both chronically inflamed periodontal lesions and healing wounds behaved similarly in vitro [13]. Therefore, the chronically inflamed periodontal tissue that is usually removed during surgery might also contain stem cells for wound healing. Because many patients with chronic periodontitis need more than one surgery in their course of their treatment, the isolated stem cells from the granulation tissues in the initial surgery may be a good source for autotransplantation in subsequent operations. This may conveniently improve the clinical outcomes of periodontitis patients. These isolated stem cells can also be stored for other medical applications in the future. Therefore, this study identifies and isolates stromal stem cells in periodontal granulation tissues to prove that they are beneficial for osseous healing.

Materials and methods

Collecting periodontal granulation tissues

Periodontal granulation tissues were sampled from two- or three-walled intrabony defects in 15 patients during periodontal surgery. All the patients had received periodontal non-surgical treatments and had excellent oral hygiene. Each patient supplied a signed informed consent form approved by the Institutional Review Board of National Cheng Kung University. Five of the samples were fixed in 4% paraformaldehyde (PFA) solution and then embedded in paraffin for subsequent immunohistochemistry (IHC). Five samples were prepared for flow cytometry of STRO-1. The other five samples underwent colony formation unit-fibroblast (CFU-f) assay and were selected for further immunocytochemistry (ICC), flow cytometry of MSC markers, multi-potent differentiation assays, and animal study.

Immunohistochemistry (IHC) analysis of the STRO-1 marker

The STRO-1 antigen is an early marker of different MSC populations that is also expressed by perivascular cells in situ [14, 15]. Therefore, this study used STRO-1 as a marker for putative stem cells in periodontal granulation tissues. Paraffin-embedded granulation tissues were cut into 4-μm sections and placed on silane-coated slides that were then deparaffinized and rehydrated with serial xylene and ethyl alcohol. The slides were then incubated for 10 min in 3% H_2O_2 in methanol to quench endogenous peroxidase activity, and rinsed three times in phosphate-buffered saline (PBS) for 5 min each time. The antigens were retrieved using heat treatment (10 mM citrate buffer, pH 6.0). After the slides were washed with PBS three times for 5 min each and blocked for 20 min, they were incubated with primary antibodies for STRO-1 (R&D Systems, Minneapolis, MN, USA) at a concentration of 2 μg/mL overnight at 4°C. The following day, the slides were incubated with biotin-

conjugated secondary antibodies and streptavidin-horseradish peroxidase following the manufacturer's instructions (Abcam, Cambridge, MA, USA). Finally, peroxidase activity was detected with a chromogen kit (Zymed, San Francisco, CA, USA) and then counterstained with Mayer's hematoxylin.

Flow cytometry of STRO-1

Granulation tissues were soaked in PBS containing 3 mg/mL collagenase type I (GIBCO/Invitrogen, Carlsbad, CA, USA) and 4 mg/mL dispase (GIBCO/Invitrogen) for 30 min at 37°C. The mixture was then transferred to a 70-μm cell strainer (Falcon, BD Labware, Franklin Lakes, NJ, USA) to obtain a single cell suspension. The single cell suspension (1×10^6/mL) was incubated with 2 μg/mL of STRO-1 (IgM) antibody for 1 h on ice. The cells were then incubated with goat anti-mouse IgM conjugated to FITC (1/1,000 dilution, Dako, Carpinteria, CA, USA) for 1 h on ice. After washing with PBS twice, the cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A level of fluorescence exceeding 99% of the corresponding isotype-matched control antibody indicated a positive expression of cells.

Colony formation unit-fibroblast (CFU-f) and bromodeoxyuridine incorporation assay

The granulation tissues were soaked in PBS containing 3 mg/mL collagenase type I and 4 mg/mL dispase for 30 min at 37°C. After passing through a cell strainer and washing two times, single-cell suspensions were seeded into a 10-cm dish at a concentration of 1×10^3 cells/cm^2 with alpha Modification of Eagle's Medium (α-MEM, GIBCO/Invitrogen) supplemented with 15% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Biosource/Invitrogen). The suspensions were then incubated at 37°C in 5% CO_2 without being perturbed for 14 days. After 2 weeks, the media was removed, and the cultures were washed in PBS twice. Half of the dishes were then fixed and stained with Giemsa stain (Sigma Aldrich, St. Louis, MO, USA). Adherent colonies containing more than 50 cells were counted at×10 magnification. The other colonies with low density (<50 cells) were used for control cells in future studies. Half of the dishes were not stained. The high-density (>50 cells) and low-density (<50 cells) colonies were selected using cloning cylinders and subcultured for further immunocytochemistry (STRO-1), flow cytometry, in vitro and in vivo differentiation assays, and animal study. The cells from high-density colonies or low-density colonies were seeded on coverslips at 4×10^4 cells per 13-mm round coverslips (Nunc, Inc., Naperville, IL, USA) and grown for 24 h in growth medium. The cells were pulsed with 10 μM BrdU for 4 h, fixed with ice-cold methanol for 20 min, and processed for immunostaining of BrdU. The number of BrdU-positive cells was expressed as a percentage of total number of cells counted from six replicate cultures.

Immunocytochemistry (ICC) of STRO-1 and flow cytometric analysis of STRO-1, CD146, CD90, and CD44

During subculturing from the procedures of CFU-f, the partitions of the cell suspensions were transferred to a four-welled chamber slide (LabTek Chamber Slide, Thermo Fisher Scientific, Rochester, NY, USA) and incubated in the same condition for 2 days. The cells were then fixed in methanol at −20°C for 5 min and air-dried. After the cells were washed three times in PBS, 3% H_2O_2 in PBS was added to quench endogenous peroxidase. The subsequent procedures without antigen retrieval were the same as the IHC of STRO-1. To further confirm the high-density colonies have specific MSC markers, we performed flow cytometric analysis of STRO-1, CD146/MUC18, CD90, and CD44 on the cells from high-density colonies. Briefly, cells from high-density colonies were trypsinized, and approximately 1×10^5 cells were pelleted in 5-mL polypropylene tubes (BD Bioscience). The protocol for STRO-1 staining had been described above. For the other stainings, the cells were incubated with 2 μg/mL of primary antibodies for 1 h on ice. The cells were then incubated with goat anti-mouse IgG conjugated to FITC (1/1,000 dilution, Dako, Carpinteria, CA, USA) for 1 h on ice. After washing with 5% heat-inactivated FBS in PBS twice, the cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences). An isotype-matched antibody was used as negative control. The primary antibodies for CD146 and CD90 were purchased from Abcam (Cambridge, MA, USA), while the antibody for CD44 was from Thermo Fisher Scientific Inc. (Fremont, CA, USA). All of them were mouse monoclonal antibodies.

Differentiation assays for osteogenesis, chondrogenesis, and adipogenesis

To further test if the STRO-1-positive cells had multipotency, the cells of selected clones were transferred to four-well chamber slides (LabTek Chamber Slide) to reach confluence. Osteogenesis, chondrogenesis, and adipogenesis assays were then performed. Two commercial kits from the same company (In Vitro Osteogenesis Assay Kit & Adipogenesis Assay kit, Chemicon/Millipore, Billerica, MA, USA) were used for osteogenesis and adipogenesis assays. After 5 weeks of culture in the conditions

suggested by the manufacture, the cells were fixed in 4% PFA, washed with PBS, and then subjected to ICC and specific stainings. For osteogenesis assay, ICC for osteopontin was performed to determine if the cells expressed the osteoblast marker. Alizarin Red S staining was performed to detect the calcium deposition. For adipogenesis assay, ICC for PPAR-γ was performed to test if the cells expressed the adipocyte marker. Oil Red O staining was performed to detect the oil-droplet accumulation in the cells. The chondrogenesis assay in this study followed the protocol in previous studies [16]. After the cells reached confluence, TGF-β1 was added to the culture medium (α-MEM with 15% FCS) every other day, at a final concentration of 10 ng/mL. The cell culture was maintained in this condition for 4 weeks. The cells were then fixed with ice-cold methanol for 10 min at −20°C, air-dried, washed with distilled water, and covered with Alcian blue (Sigma Aldrich) at pH 2.0. After overnight staining, the cultures were thoroughly washed with distilled water. Alcian blue staining revealed deposition of glycosaminoglycan. The cells were also processed for ICC of type II collagen, which is a chondrocyte cell marker. The primary antibodies for type II collagen (Thermo Fisher Scientific) and PPAR-γ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were mouse monoclonal antibodies, while the antibody for osteopontin (Thermo Fisher Scientific) was epitope specific rabbit antibody. The concentrations of the primary antibodies used in the ICC were all 2 μg/mL.

Animal study

The animal study was performed following the specifications of an approved small animal protocol from the National Cheng Kung University. The animal study consisted of two parts. The first part of the study was to test the in vivo differentiation ability of the putative stem cells from human periodontal granulation tissues. Approximately 2.0×10^6 of putative stem cells from high-density colonies were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic particles (SinBone, Purzer, Taipei, Taiwan) and then transplanted subcutaneously into the dorsal surfaces of five immunocompromised mice (NOD/LtSZ Prkdc). The other sides of dorsal surfaces received the same ceramic particles plus cells from low-density colonies as control. The transplants were recovered at 5 weeks post-transplantation, fixed with 4% formalin, decalcified with buffered 10% EDTA (pH 8.0), and then embedded in paraffin. Sections (4 μm) were deparaffinized and stained with hematoxylin–eosin or underwent IHC for human-specific mitochondria. The primary antibody for human-specific mitochondria was a mouse monoclonal antibody from Abcam (Cambridge). The IHC protocol was the same as described above except that a mouse-on-mouse kit (Vector Laboratories, Burlingame, CA, USA) was used to reduce the background staining from mouse immunoglobulins.

The second part of animal study was to prove that the putative stem cells had potential for future clinical applications in a critical-sized defect model. Approximately 2.0×10^6 ex vivo expanded granulation tissue-derived cells were mixed with 40 mg of HA/TCP ceramic particles (SinBone). The cells were not driven to an osteogenic pathway prior to application. The mixture was then transplanted into a 3.0-mm diameter defect created by a trephine bur on the calvaria of immunocompromised mice (NOD/LtSZ Prkdc) [10]. Ten male mice (8 weeks old, 25.3±1.5 g) were divided into two groups, including test group HA/TCP+granulation tissue-derived stem cells (n=5) and control group (HA/TCP without stem cells, n=5). The five mice in each group were euthanized at 8 weeks post-transplantation. The transplants were recovered, fixed with 4% PFA, decalcified with buffered 10% EDTA (pH 8.0), and then embedded in paraffin. Sections measuring 4 μm were subsequently deparaffinized and stained with hematoxylin and eosin. An image analysis system (ImageJ, NIH, Bethesda, MA, USA) was used to semi-quantitatively compare the new bone deposition area between test and control groups. The percentage of new bone formation was calculated as the new bone deposition area (numerator) divided by the total area in the defined critical size defect (denominator). The residual bone graft was not included in the reported bone values. The average of five individual sections from the same specimen represented the result of that mouse. This study presents the data of two groups as mean percentage±SD. The analysis of new bone formation area was performed by a blinded examiner.

Statistical analysis

The mouse study data was presented as a mean percentage ±SD. The Wilcoxon signed rank test was used to identify any significant differences (at the level of $P<0.05$) between the test and control groups. Statistical analysis was performed using the StaView program (Version 4.5, Abacus Concepts, Berkeley, CA, USA).

Results

Immunohistochemistry and flow cytometry of STRO-1 for periodontal granulation tissues

The histological sections of every specimen exhibited obvious inflammatory cell infiltration and small blood vessels. These findings indicated chronically inflamed tissues. Some of the cells in the connective tissues were positive for IHC staining

of STRO-1 (Fig. 1a, b). Endothelial and epithelial cells did not express STRO-1. The average ratio of positive cells in the five specimens was 5.91±1.50%. The flow cytometry analysis for STRO-1 expression indicated an average positive ratio of 6.70±0.81% from five tested specimens.

CFU-f, BrdU incorporation assay, and flow cytometric analysis of stem cell markers

Due to the stringent oral care of the subjects, no bacterial contamination occurred in each culturing process. After 2 weeks of culture in low-density inoculation (1×10^3 cells/cm^2), each 10-cm culture dish contained an average of 12 colony formation units (>50 cells/colony). The cloning cylinder technique was used to randomly select three colonies from each culture dish and expand the cells. Ten of the 15 selected colonies showed a strong reaction to ICC staining of STRO-1 (data not shown). The two largest colonies from different patients were selected for further differentiation assays and animal study. The low-density colonies were pooled together if needed and used as control cells in the following studies. The BrdU incorporation assay showed that cells from high-density colonies had a significantly higher uptake rate of BrdU than did those from low-density colonies, P=0.004 (Fig. 2). The ex vivo expanded cells from high-density colonies expressed four MSC markers, i.e., STRO-1, CD146/MUC18, CD90, and CD44 (Fig. 3).

Immunocytochemistry (ICC) and specific stainings for osteogenesis, chondrogenesis, and adipogenesis

After culturing in an osteogenic medium for 5 weeks, most of the cells tested positive for osteopontin expression, as determined by ICC staining. The staining was predominately localized in the cytoplasm (Fig. 4a). Alizarin red staining showed calcium deposition in certain focal areas (Fig. 4b). After culturing in a chondrogenic medium, many cells were immunoreactive for type II collagen (Fig. 4c). Positive staining results appeared localized in the cytoplasm in a parallel and fragmented linear form at×400 magnification. This staining pattern differed from that of osteopontin. Alcian blue staining showed a positive reaction for glycosaminoglycan deposition in some focal areas (Fig. 4d). Five weeks after adipogenesis culture, most of the cells tested positive for PPAR-γ staining. The staining appeared predominately in the cell nucleus, and the pattern was unlike those of osteopontin and type II collagen (Fig. 4e). Adipogenic differentiation was visually apparent by the accumulation of lipid containing vacuoles based on Oil Red staining (Fig. 4f). These results demonstrate that the cells selected from periodontal granulation tissue were multipotent in differentiation.

Animal studies

To validate the capacity of putative stem cells to differentiate into functional periodontium-associated cells, ex vivo-expanded cells from high-density colonies were transplanted into immunocompromised mice. A cementum/PDL-like structure was generated. Cells from human granulation tissues were responsible for cementum/PDL-like tissues formation surrounding the transplants, as shown by the reactivity of these cells with human-specific mitochondria antibody. Interestingly, adjacent small blood vessels were also immunoreactive for human-specific mitochondria. Human cells were detect-

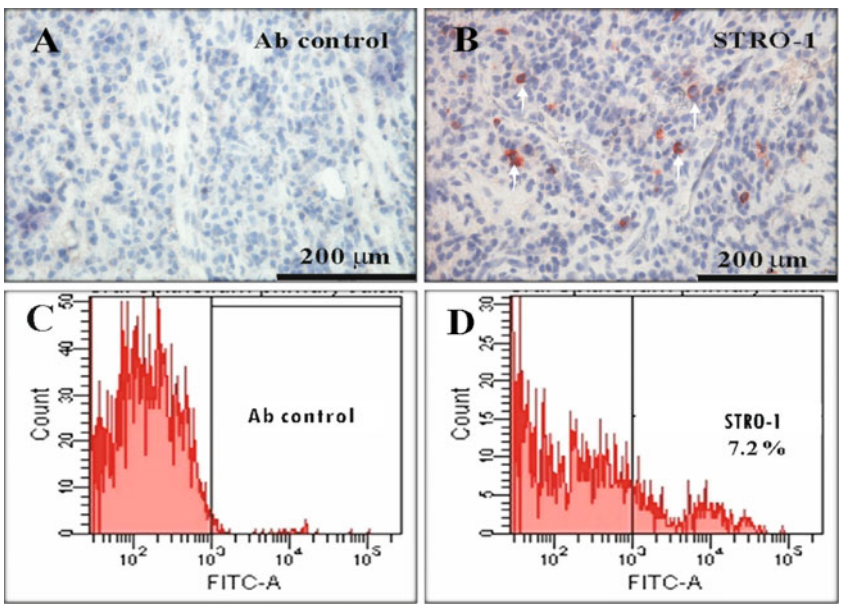

Fig. 1 Immunohistochemistry and flow cytometry of STRO-1 in periodontal granulation tissues. Abundant leukocytes and small blood vessels appeared in the periodontal granulation tissues. Some of the stromal cells were immunoreactive for STRO-1, while no staining was detected in the antibody control slides (**a**, **b**). *Scale bar*=200 μm. Flow cytometry analysis of STRO-1 for one representative tissue indicated that approximately 7.2% of the dissociated cells tested positive for STRO-1 (**c**, **d**)

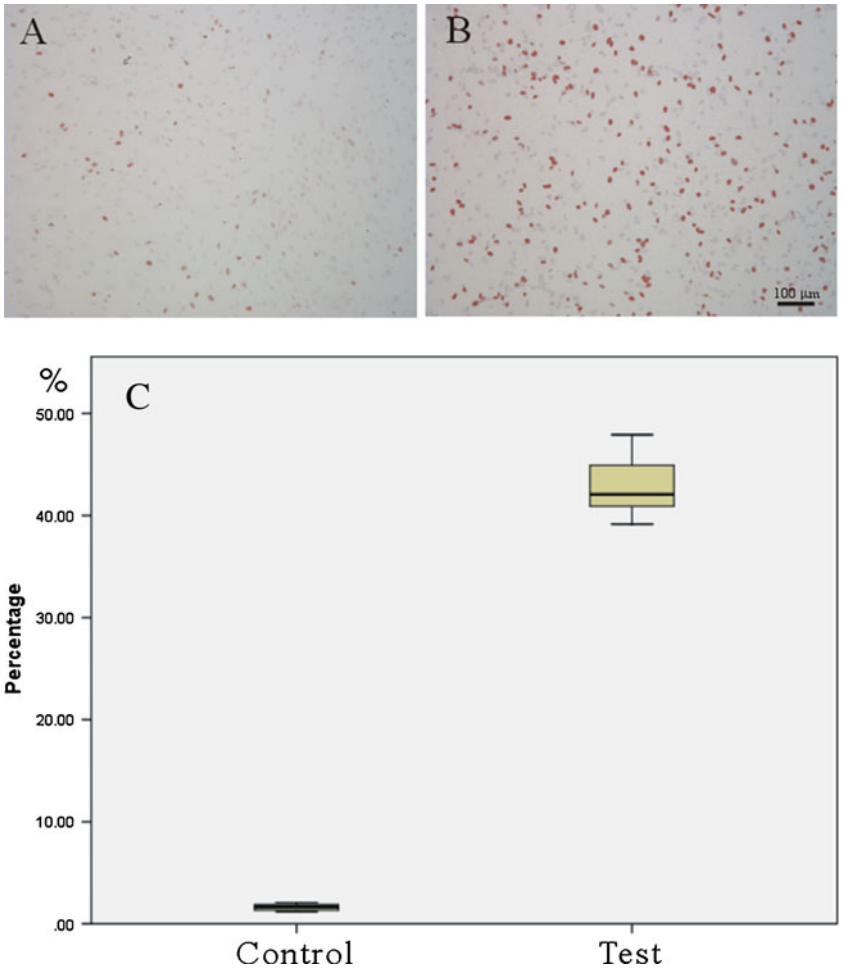

Fig. 2 Bromodeoxyuridine (BrdU) incorporation by periodontal granulation tissue-derived cells. The photomicrographs are the representative photographs from a low-density colony (**a**) and a high-density colony (**b**). Hematoxylin was used as counterstaining. *Scale bar*= 100 µm. After quantification, the percentage of BrdU-positive cells in high-density colonies is significantly higher than that in low-density colonies (**c**)

able in the tissue sections from the control specimens, but no cementum-like or PDL-like tissues could be found (Fig. 5).

For calvaria repairing experiment, after 8 weeks of wound healing, most of the osseous repair in the control group appeared patchy and irregular. Small osseous

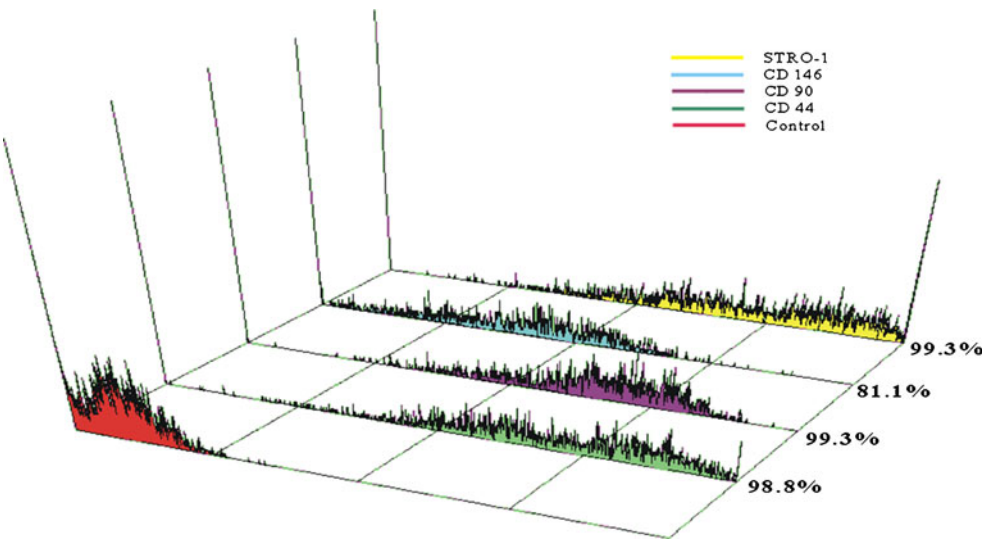

Fig. 3 Flow cytometric analysis of mesenchymal stem cell markers for putative stem cell clones from the periodontal granulation tissues. The cells from the high-density colonies were processed for flow cytometric assays of STRO-1, CD146, CD90, and CD44. The presented data was the result for one representative clone

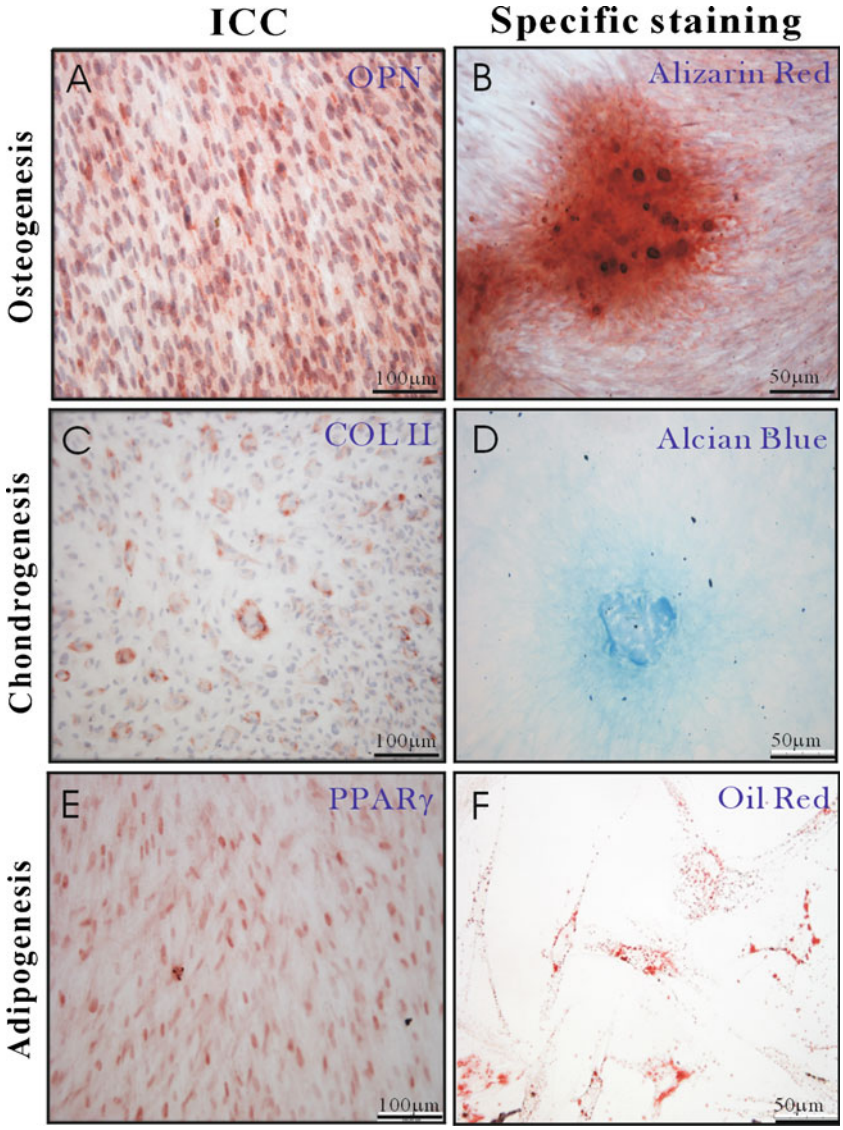

Fig. 4 Immunocytochemistry and specific staining for the potencies of osteogenesis, chondrogenesis, and adipogenesis in selected clones of periodontal granulation tissues. Osteopontin (OPN), collagen type II (COL II), and PPAR-γ are cell markers for osteoblasts, chondrocytes, and adipocytes, respectively (**a**, **c**, **e**). Alizarin Red, Alcian blue, and Oil red are specific stains for detecting calcium deposition, cartilage formation, and lipid droplet deposition, respectively (**b**, **d**, **f**). *Scale bar*=100 μm in the *left column* and 50 μm in the *right column*

deposits were intervened by soft tissues and bone grafts (Fig. 6a). The new bone formation in the test group was significantly better than that in the control group. The percentage of new bone formation in the test group was 47.2±6.5%, while that in the control group was 33.5± 6.9%. The *P* value was 0.04 (Fig. 6b).

Discussion

Chronic periodontitis is a highly prevalent oral disease. Improving periodontal regeneration remains a problem for all dentists. Combining MSCs with biomaterials may improve periodontal regeneration [17, 18]. However, there are limitations to obtaining MSCs from established sites such as bone marrow and umbilical cord [11]. The former requires a second surgical site while the latter requires long-term cell storage. Therefore, this study attempts to locate a more accessible source of stem cells from the oral cavity and verify their properties. Dr. Shi's laboratory first identified dental pulp stem cells (DPSCs) in 2000 [7]. These DPSCs have better properties than bone marrow-derived MSCs in terms of proliferation and clonogenic formation rate. DPSCs and bone marrow MSCs exhibit similar bone formation abilities [7]. In a later study, the same group successfully isolated stem cells from human exfoliated deciduous teeth and gave them the abbreviation SHED [8]. These SHED have a better proliferation rate than the DPSCs and bone marrow MSCs. The flow cytometry assay results in this study indicate that the percentage of SHED was about 10%. Although the cell properties are good, the long-term storage time and limited

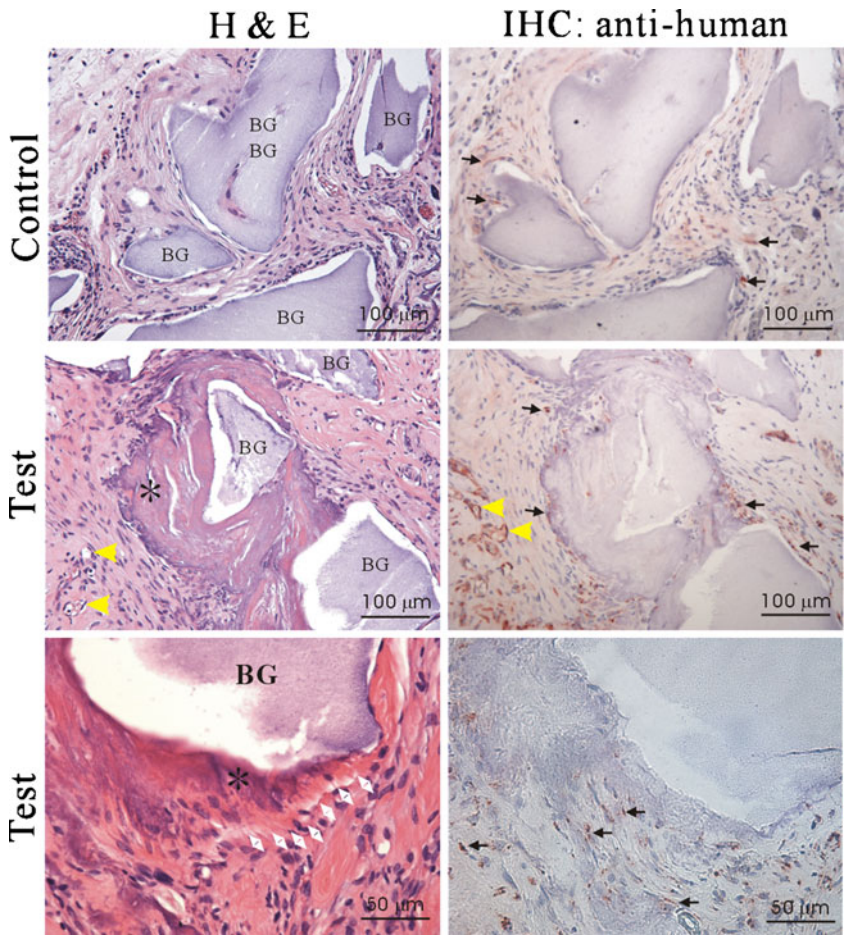

Fig. 5 Transplantation of putative stem cells subcutaneously into the dorsal surface of SCID (NOD/LtSZ Prkdc) mice. The cells from low-density colonies were mixed with the same bone graft and used as control. H&E staining reveals cementum-like (indicated by *asterisk*) and periodontal ligament-like (indicated by *white diamond*) substances surrounding the bone grafts in test group. BG denotes residual bone graft. The IHC of human-specific mitochondria was to track the human cell source. The *black arrows* indicated cells immunoreactive for human-specific mitochondria. The *yellow arrowheads* indicated small blood vessels

source for adults limit the application of SHED. In this study, the STRO-1^+ cells were detected by IHC and flow cytometry in periodontal granulation tissues from human adults. The STRO-1^+ cell ratio was estimated about 5% to 7% in the periodontal granulation tissues. Previous research shows that the self-renewal ability of MSCs decreases with age [19, 20]. This may explain why periodontal granulation tissues had a lower ratio of stem cells than SHED from children.

During the experiments in this study, we found that the isolated stem cells underwent senescence after passage 5 of culture (data not shown). This self-renewal ability failed to meet original expectations. Recent studies show that culture in hypoxia (about 5% O_2) can improve the proliferation rate and differentiation potential in MSCs [21, 22]. In the hypoxia culture condition, most hypoxia-regulated genes were transcriptionally upregulated by hypoxia-inducible factor-1α (HIF-1α) [23]. This condition improved the MSC proliferation rate, doubled the MSC clonogenic formation ability [22], increased the osteoblast and chondrocyte differentiation capability [24], and inhibited adipogenesis [25]. Future research on this topic should change the culture condition to hypoxia and observe whether the aging effect can be diminished for the granulation tissue-derived stem cells.

The differentiation assays in this study used functional staining and ICC to test the potency of isolated granulation cells. Osteogenesis, chondrogenesis, and adipogenesis can all be induced under appropriate culture conditions. Although the in vitro study proves the multipotency of granulation tissue-derived stem cells, chondrocytes and adipocytes are not the main contributor cells of periodontal regeneration. In the in vivo differentiation assay, the granulation tissue-derived stem cell generated cementum-like/PDL-like structures as well as small blood vessels. In the future study, we will target PDL fibroblast and cementoblast specific markers, such as periostin and cementum attachment protein in the generated tissues to confirm the cell identity.

This study uses a mouse model of critical size osseous defect at calvaria to test the in vivo function of the isolated periodontal stem cells instead of periodontal defects in bigger animals, such as monkeys, pigs, and dogs. This is because larger immunocompromised animals were unavailable, and periodontitis does not naturally occur in mice.

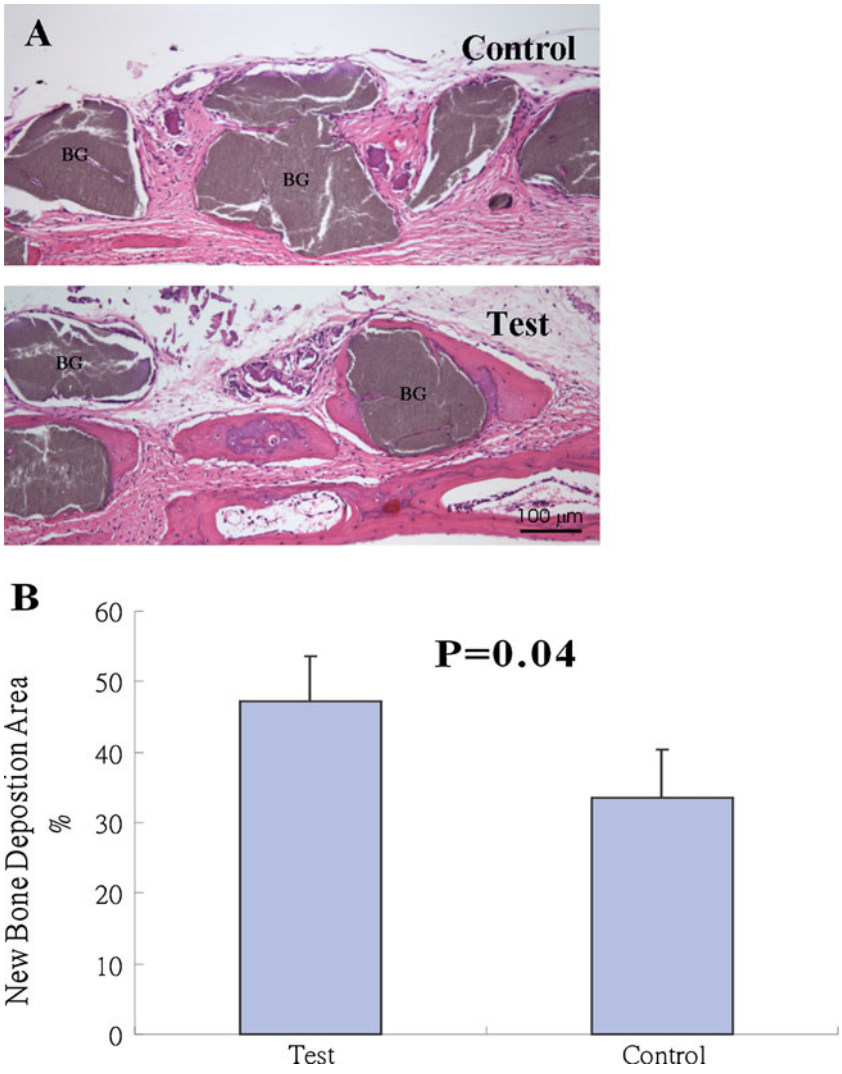

Fig. 6 Comparison of osseous repair in critical-sized defects between control and test mouse calvarias. H&E staining shows that the test group showed greater new bone formation than the control group. BG denotes residual bone graft. *Scale bar*= 100 μm (**a**). Quantification using an image analysis system (ImageJ) revealed a *P* value of 0.04 (**b**)

The critical size defect in the calvaria of adult mice (8 weeks old) was approximately 3 mm in diameter [10, 26]. Compared with Seo's study [10], the defects in our study had thinner new bone formation than their SHED-treated defects. Adipose-like tissues formed in two of the defects transplanted with granulation tissue stem cells. These tissues might be derived from the spontaneous stem cells differentiation. The adipose tissues would be a negative effect if we apply the granulation tissue stem cells for periodontal regeneration in the future. Previous research shows that the alkaline phosphatase activity of bone marrow MSCs increased after 1 week of osteogenic induction and remained until osteoblast maturation [27]. Various methods can inhibit the adipocyte differentiation in vivo. First, granulation tissue stem cells can be cultured in an osteogenic medium for 1 week to upregulate the alkaline phosphatase before grafting into the defects. This may stimulate the granulation tissue stem cells to form osteoprogenitor cells and differentiate into osteoblasts to repair defects. Second, combining stem cells with osteogenic proteins, such as bone morphogenetic protein (BMP)-2 and −7, can further enhance osteogenic results. Third, culturing the granulation tissue stem cells in hypoxia condition can increase the osteogenic potential and reduce the adipogenic potential [24, 25].

For a true periodontal regeneration, periodontal ligament (PDL), alveolar bone, and cementum should all be newly formed. Bone is only one of the components in periodontal regeneration. This study reports the successful isolation and characterization of human granulation tissue stem cells. These cells improved osseous repair in critical size defects in mice. This is the first step in proving their clinical functions. In the future, we will use bigger animal models to test the potential of granulation tissue stem cells to differentiate into PDL fibroblasts and cementoblasts in periodontal

defects. This approach would mimic the periodontal regeneration process more closely.

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Conflicts of interest The authors declare that there are no conflicts of interest in this study.

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