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Original Article

Systemic BMSC homing in the regeneration of pulp-like tissue and the enhancing effect of stromal cell-derived factor-1 on BMSC homing

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Abstract: Pulp regeneration caused by endogenous cells homing has become the new research spot in endodontics. However, the source of functional cells that are involved in and contributed to the reconstituting process has not been identified. In this study, the possible role of systemical BMSC in pulp regeneration and the effect of stromal cell-derived factor-1 (SDF-1) on stem cell recruitment and angiogenesis were evaluated. 54 mice were divided into three groups: SDF-1 group (subcutaneous pockets containing roots with SDF-1 absorbed neutralized collagen gel and the green fluorescent protein (GFP) positive BMSCs transplantation via the tail vein), SDF-1-free group (pockets containing roots with gel alone and GFP + BMSCs transplantation) and Control group (pockets containing roots with gel alone). The animals were sacrificed after the roots were implanted into subcutaneous pockets for 3 weeks. Histomorphometric analysis was performed to evaluate the regenerated tissue in the canal by hematoxylin and eosin (HE) staining. The homing of the transplanted BMSCs was monitored with a fluorescence microscope and immunohistochemical analysis. The expression of ALP in new formed tissue was detected immunohistochemically. Dental-pulp-like tissue and new vessels were regenerated and GFP-positive BMSCs and expression of ALP could be observed in both SDF-1 group and SDF-1-free group. Furthermore, more GFP+ cells, stronger expression of ALP and stronger angiogenesis were found in the SDF-1 group than in the SDF-1-free group. To conclude, systemic BMSC can home to the root canal and participate in dental-pulp-like tissue regeneration. Intracanal application of SDF-1 may enhance BMSC homing efficiency and angiogenesis.

Keywords: Pulp regeneration, cell homing, bone marrow stromal cells, SDF-1, angiogenesis

Introduction

Root canal treatment (RCT), which involves the removal of the infected dental pulp and obturation with bioinert material, is the most popular endodontic treatment for irreversible pulpitis, pulp necrosis and apical periodontitis [1]. In most clinical cases, RCT can effectively control infections and eliminate the suffering of patients. However, endodontically treated teeth will lack of pulp, which has important functions in supplying nutrients and maintaining the vitality of the teeth. Thus the teeth became brittle which are easy to postoperative fracture and re-infections [2]. Therefore, exploring more ideal form of endodontic therapy has been undertaking recently [3].

Pulp regeneration procedures in which inflamed or necrotic pulp tissues are removed and refreshed with healthy pulp tissue to revitalize teeth are expected to provide the better outcome for the teeth with inflamed or necrotic pulp tissues [4]. Among many potential approaches, stem cell transplantation with scafflods has been the important approach in the pulp regeneration. Ectopic dental-pulp-like tissue was observed in several studies by trans-

plantation of dental stem cells [5-9]. Huang and his colleagues demonstrated the formation of vascularized pulp-like tissue and a continuous layer of dentin-like tissue through delivery of dental pulp stem cells with collagen scaffolds in the emptied root canal in SCID mice for 3 months [7]. Cordeiro and his colleagues found that a structure similar to the real dental pulp could be regenerated by seeding the stem cells of deciduous dental pulp and the gel in the cross-sectional tooth slices which were placed in the immunodeficient mice [9].

Although the results are very optimistic, cell transplantation has encountered major difficulties from clinical and commercialization hurdles. Firstly, it's difficult to obtain the dental stem cells. There are difficulties in cell isolation, culture, storage, transport and so on. Secondly, we can't ignore the immune rejection and ethical issues arising from allograft. The last but not the least is the high cost of cell transplantation [10, 11]. From a dentist's perspective, the introduction of a method of high risk and high input to the treatment of non lethal diseases makes little sense.

As an alternative to cell transplantation, cell homing utilizing patient own regenerative mechanisms has entered into the scope of regenerative endodontics. Kim and colleagues showed the regeneration of the pulp-dentin complex and dental pulp-like tissues by cell homing in endodontically treated human teeth which were surgically implanted in a mouse dorsum for 3 weeks [2]. However, the source of functional cells that were involved in and contribute to the reconstituting process had not been identified. We hypothesized that parts of the functional cells might come from remote body stem cell niches (such as bone marrow), migrate with the blood flow and finally reach the tooth canal. To testify this hypothesis, roots containing neutralized collagen gel with or without stromal cell-derived factor-1 (SDF-1) were implanted into mouse subcutaneous pockets and bone marrow stromal cells (BMSCs) labeled with green fluorescent protein (GFP) were transplanted into mouse via the tail vein to evaluate the possible role of systemic BMSC in pulp regeneration. The enhancing effect of SDF-1 on stem cell recruitment and angiogenesis were also be evaluated.

Materials and methods

Tooth collection and endodontic treatment

This study was approved by the Medical Ethics Committee of School of Stomatology Shandong University. A written consent form was obtained from each patient included in the study. Intact mandibular first premolars with single root canal freshly extracted for orthodontic were collected. Roots were cut off along the crown root junction. Endodontic treatment was performed and the root canal space was expanded. The apical foramen was expanded to 1 mm in diameter. Residual periodontal ligament and peri-apical tissues were scraped away with scalpel and 75% alcohol. The roots were soaked at the room temperature in 10% NaOCl for at least one week. Before the application of the roots, there were a series of treatments with NaOCl, ethylenediaminetetraacetic acid (EDTA) and phosphate-buffered saline (PBS). The roots were first exposed to 0.5 M EDTA for one minute, then washed in sterile PBS for five minutes, and then exposed to 6.15% NaOCl for ten minutes. The roots were then rinsed in sterile PBS for three times and then exposed to 0.5 M EDTA for ten minutes. The roots were washed again in PBS for three times. Finally, in order to remove residual sterilization agents and to avoid microbial contamination, the roots were soaked in PBS which contains penicillin (100 u/ml) and streptomycin (100 μg/ml) at 4°C for 3 days [12, 13].

GFP transfection of BMSCs

The mouse stromal ST2 cells were provided by Medical Genetics Institute of Shandong University. The multiplicity of infection was determined as 60 and the BMSCs (6×10^3 cells/cm^2) were incubated with culture medium in 6-well plates for 24 h. Then the culture medium was replaced with serum-free α-MEM which was mixed with concentrated viral suspension. Eight hours later, the viral suspension was removed and complete culture medium was added. G418 (100 μg/ml) was used to purify the GFP-positive cells. The BMSCs with high level of GFP expression passaged in vitro to generate sufficient cells for in vivo experiments.

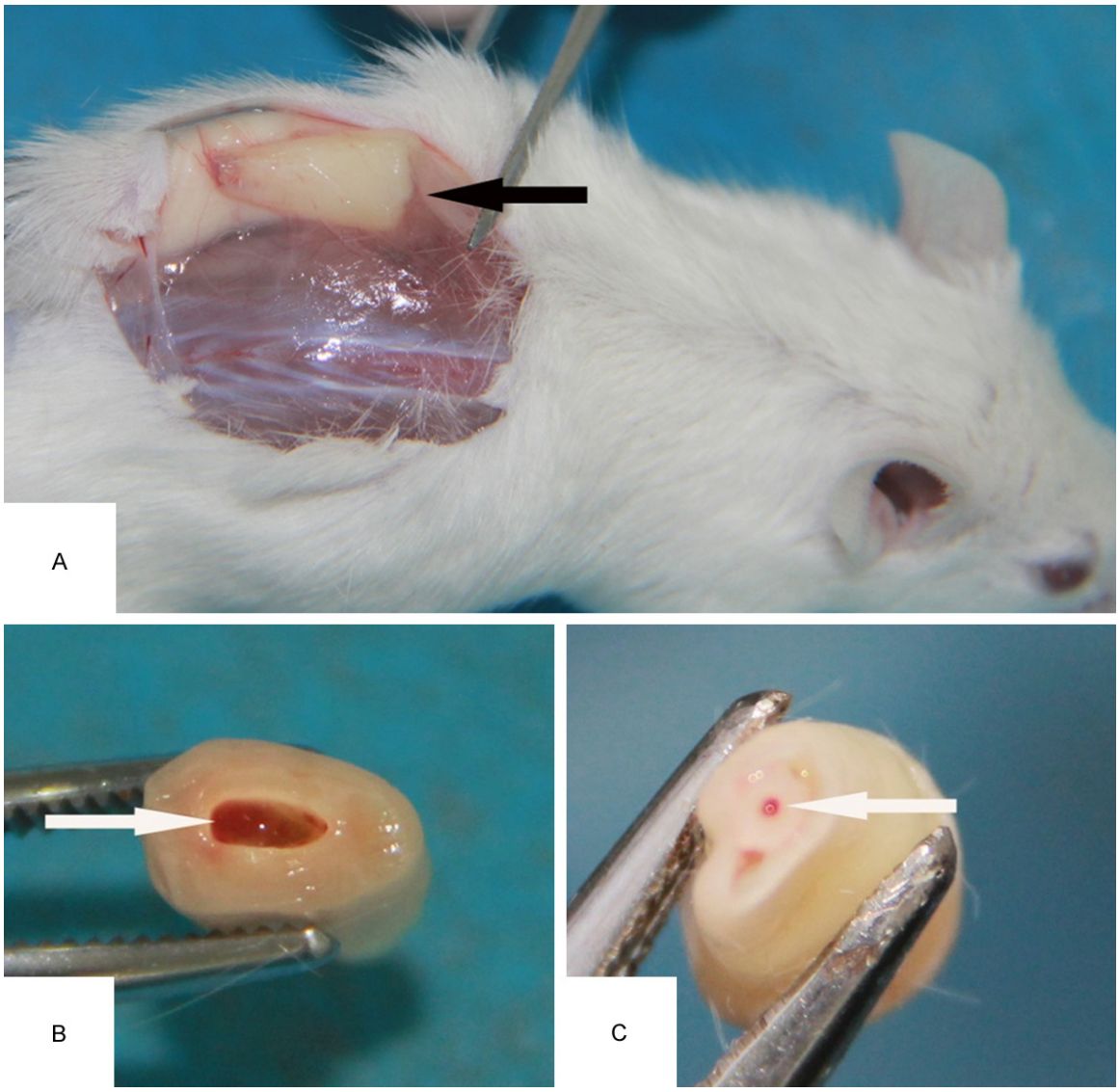

Figure 1. A: Tooth root at the time of retrieval from the subcutaneous space of mice. B: The highly vascular appearance of the tissues engineered in the root canal. C: Red pigmentation appearance from apical foramen.

Cytokine delivery

SDF-1 (Santa Cruz, America) at a dose of 100 ng/ml was selected for its strong chemotaxis [14]. SDF-1 was absorbed in 2 mg/ml collagen gel solution (Gibco, America) which was neutralized and injected into the canal space of endodontically treated human roots. Then the roots full of collagen gels with or without SDF-1 were incubated at 37°C for 1 h to induce gelation.

Animal surgery and BMSC transplantation

Before transplantation, the GFP-positive BMSCs were collected and re-suspended in α-MEM at a density of 1×10^4 cells/μl. Each mouse received approximately 1×10^6 cells in 100 μl α-MEM via the tail vein in SDF-1 group and SDF-1-free group [15]. 5-7 week-old male mice were anesthetized and linear incisions approximately 1 cm in length in the dorsum was made to create a subcutaneous pocket by blunt dissection. Endodontically treated human roots with or without SDF-1 absorbed were implanted into subcutaneous pockets. Three weeks after the transplantation, specimens were obtained for histological and immunohistochemical analysis (**Figure 1**).

Tissue preparation and histomorphometric assessment

Retrieved specimens were fixed in 4% phosphate-buffered paraformaldehyde for 24 h.

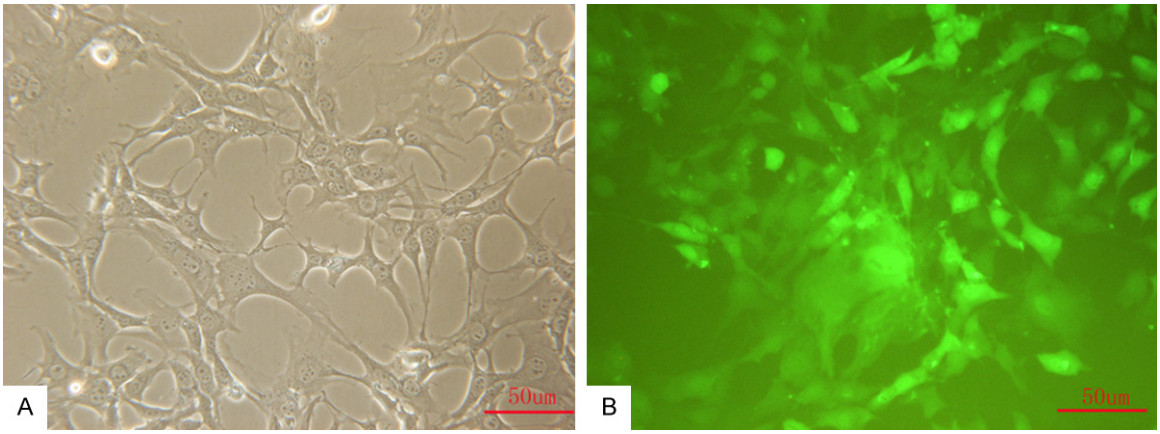

Figure 2. A: In vitro culture of BMSCs. B: Expression of GFP in BMSCs cultured in vitro.

Then these specimens were decalcified in 15% ethylene iaminetetraacetic acid until the dentin offered no resistance to cutting with a blade (3-4 months) and embedded in paraffin. 5 μm-thick histological sections adhered to glass slides were used for hematoxylin & eosin (H&E)-staining. All sections were observed and captured under a light microscope (Olympus, Japan). For each specimen, 3 HE-stained sections were selected for quantitative analysis of neovessels using a software package (Image-Pro plus 6.0, USA). The percentage of the newly formed vascular area was defined as the ratio of new vascular area to the total new tissue area.

Detection of GFP positive BMSCs

Detection of GFP positive BMSCs was done under an inverted fluorescence microscopy (Olympus, Japan). The sections were stained with 4', 6-diamino-2-phenylindole (DAPI). The number of the migrated GFP-positive cells was counted using Image J2x Software (National Institutes of Health, USA). Three fields were selected randomly of each representative sections and the homing efficiency was estimated by the proportion (GFP-positive cells counterstained with DAPI/total number of DAPI labeled cells).

Immunohistochemical analysis

Purified rabbit polyclonal antibody against GFP (1:600; Abcam, Britain) and rabbit monoclonal antibody alkaline phosphatase (ALP) (1:800; Abcam, Britain) were used for the immunodetection. After a series of processing of de-waxing, hydration and termination of endogenous peroxidase, the sections were successively incubated with the primary antibodies overnight at 4°C. The next day the slides were incubated with anti-rabbit secondary antibody (ZSGB-BIO, China). Finally,the sections were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (ZSGB-BIO, China) method and counter stained with Mayer's haematoxylin.

Statistical analysis

Statistical analysis was performed using a statistical package (SPSS 19.0, SPSS Inc, USA). Since there was no detectable dental-pulp-like tissue regenerated in Control group, data of homing GFP+ cells, new blood vessel area, integrated optical density (IOD) of GFP and ALP in SDF-1 group and SDF-1 free group were analyzed using Students't tests. Values of *P* lower than 0.05 were considered statistically significant.

Results

Obtainment of GFP-positive BMSCs

Green fluorescence of GFP in BMSCs was observed 48 hours after Lentiviral transfection and got the highest fluorescent light at 72-96 hours. G418 (100 μg/ml) was used to purify the GFP-positive cells. The morphology of BMSCs with high level of GFP expression did not change significantly after infection, the growth state were good and the cells stably expressed GFP with passage (**Figure 2**).

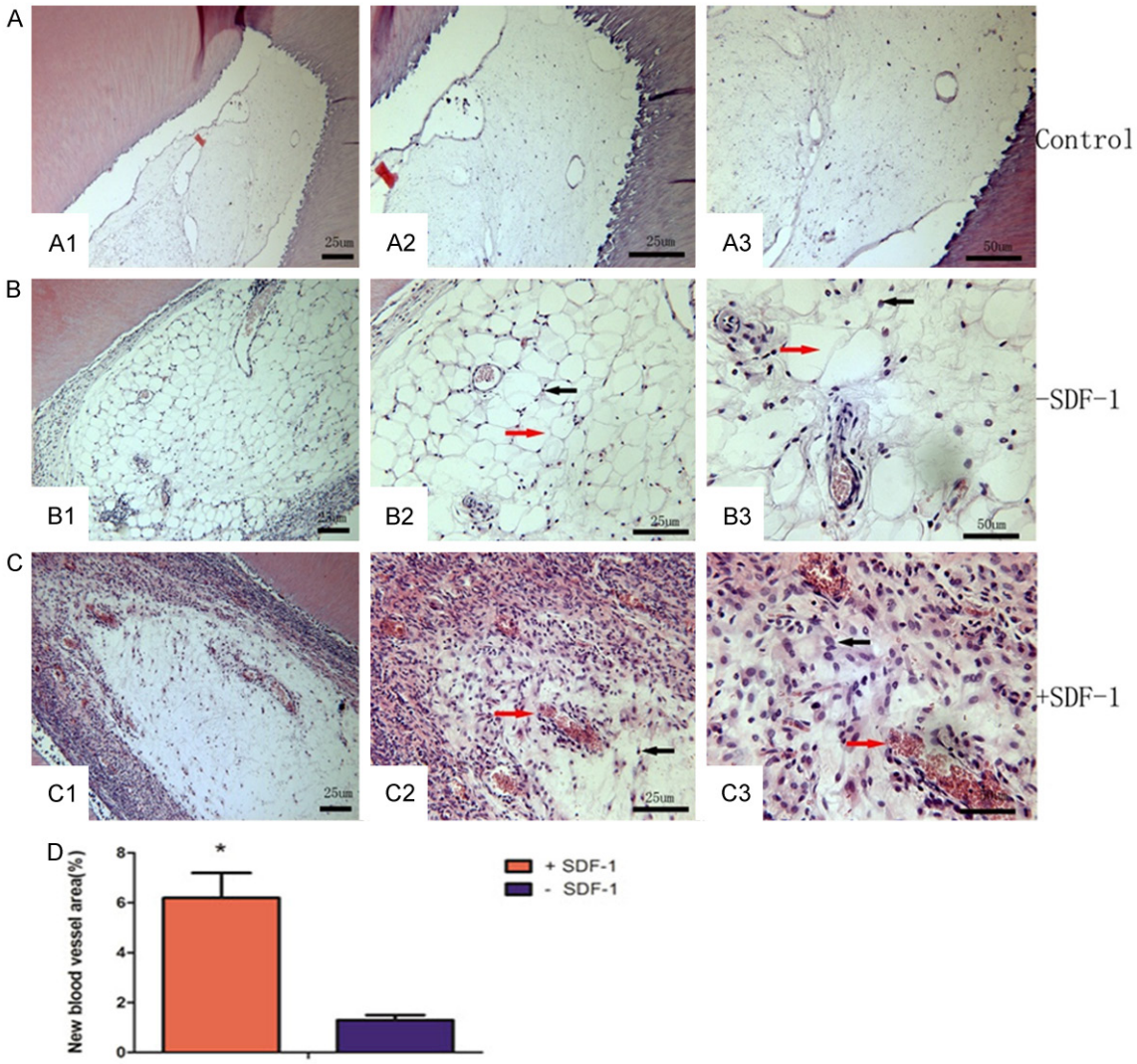

Figure 3. Histological observation and histomorphometric analysis of regenerated tissue in root canal. A: Close-up image showing no organized cells and blood vessels in endodontically treated root canal. B1: Close-up image showing a small amount of cells and erythrocyte-filled blood vessels. B2, B3: Collagen scaffold exhibits grid-like morphology (as red arrowheads indicated) and some cells arranged between the grids (as black arrowheads indicated). C1: Close-up image showing abundant cells and erythrocyte-filled blood vessels in endodontically treated root canal. C2, C3: Multiple cells (as black arrowheads indicated) and blood-vessel-like structures (as re arrowheads indicated) in regenerated connective tissue. D: Comparison of new blood vessel area between SDF-1 group and SDF-1-free group. $^{*}P < 0.05$.

Regeneration of ectopic dental-pulp-like tissue in vivo

Human roots filled with scaffolds implanted into the dorsum regenerated ectopic dental-pulp-like tissue in roots canal both in SDF-1 group and in SDF-1-free group. However, there was no detectable dental-pulp-like tissue regenerated in Control group. H&E staining microscopically showed that root canals filled with collagen scaffold alone and without cell transplantation showed no organized cell and blood vessel (**Figure 3A**). In SDF-1-free group, root canals filled with collagen scaffold alone and cell transplantation showed a small amount of cells. Residual amount of collagen scaffold exhibits grid-like morphology and some cells were arranged between the grids. A small amount of erythrocyte-filled blood vessels could be observed under high power micro-

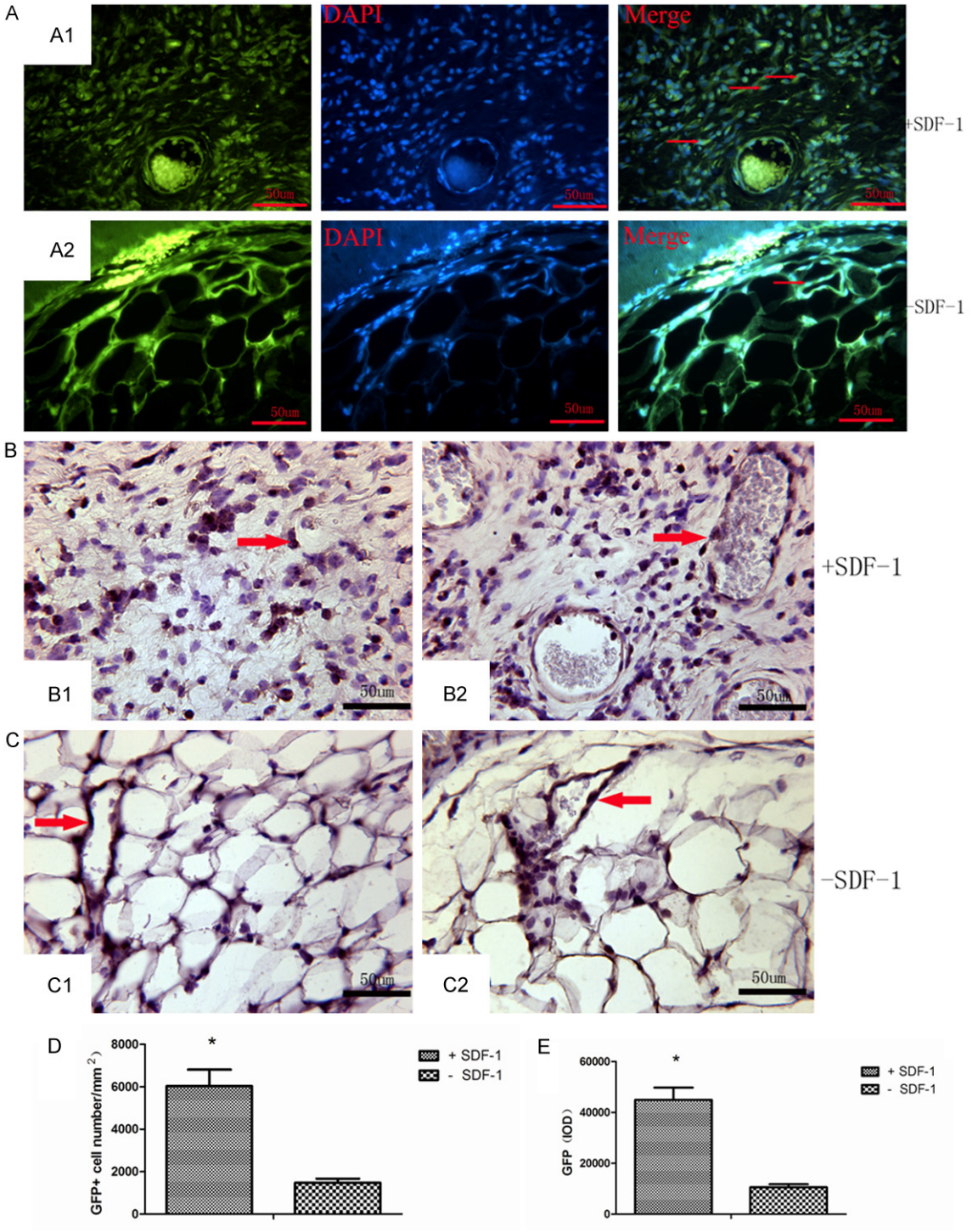

Figure 4. Detection of GFP + BMSCs within the generated tissue. A: Observation under fluorescence microscopy. Expression of GFP is shown in green and DAPI-stained nuclei in blue (as red arrowheads indicated). B1: Detection of GFP expression via immunohistological staining. Abundant GFP positive cells. Arrows indicate positive GFP staining. B2: Large number of GFP-positive cells could be found at the vessel wall and beside the perivascular. C1, C2: A small number of GFP positive cells expressing. D: Comparison of homing GFP+ cells between SDF-1 group and SDF-1-free group. $^{*}P < 0.05$. E: Comparison of GFP IOD between SDF-1 group and SDF-1-free group. Scale bars, 50 μm. $^{*}P < 0.05$.

scope (**Figure 3B**). Contrastingly, the root canals full of collagen gels with SDF-1 and cell transplantation yielded abundant cells and the grid-like morphology of collagen scaffold disappeared. A large number of erythrocyte-filled blood vessels could be observed under high power microscope (**Figure 3C**). The quantitative analysis showed that the newly formed blood vessels area in SDF-1 group and SDF-1-free group was 6.13% and 1.52% respectively and this differences was statistically significant (**Figure 3D**).

The appearance of BMSC homing

Homing cells were observed by DAPI staining and GFP immunohistochemical analysis. First, the slices were observed using fluorescence microscopy. GFP-positive cells showed cytoplasm in green and DAPI-stained nuclei in blue. The results demonstrated GFP+ cells were observed in the slices in both SDF-1 group and SDF-1-free group. The number of GFP+ cells in SDF-1 group was significantly more than the SDF-1-free group (**Figure 4A**). Similar results were observed in immunohistochemical analysis. We detected the positive expression of GFP via immunohistochemical analysis in both groups. Strong expression of GFP was detected in root canals of SDF-1 group. However, the expression of GFP was much weaker in SDF-1-free group (**Figure 4B**, **4C**). As a whole, the GFP+ cells in the new organization distributed more evenly, however, relatively concentrated GFP+ cells appeared at the vessel wall and beside the perivascular tissue in both SDF-1 group and SDF-1-free group (**Figure 4B2**, **4C2**). Cell counting and IOD of GFP showed that SDF-1 delivery homed significantly more GFP+ cells into canal than without SDF-1 delivery, which further indicated systemical BMSC can home to the root canal and participate in dental-pulp-like tissue regeneration (**Figure 4D**, **4E**)

Immunohistochemicai analysis of ALP

ALP is regarded as an early marker of osteogenic differentiation and hard tissue formation [16]. We can found the expression of ALP in new regenerated tissue. ALP expression was stronger in the SDF-1 group than in the SDF-1-free group (**Figure 5**).

Discussion

Our results showed that transplanted BMSC could home and take part in re-cellularization and re-vascularization in endodontically treated human teeth implanted into the dorsum of rats. Meanwhile, SDF-1 exerted significantly promoting effect on this process. Tissue engineering strategy based on three basic components, mesenchymal stem cells (MSCs), scaffolds, and morphogens has been widely studied in regenerative endodontics. The potential of transplantation of dental pulp stem cells (DPSC), stem cells of human exfoliated teeth (SHED), periodontal ligament stem cells (PDLSC), stem cells from apical papilla (SCAP) and dental follicle progenitor cells (DFPC) in pulp regeneration process has been well evaluated. However, this exogenous cell-based therapy faces an altitude of challenge in clinical transformation in regenerative endodontics [17]. As a substitution for cell transplantation, the in situ tissue engineering which utilizes endogenous regenerative potential provides the better prospects for widely “minimal invasive” applications [11]. In situ tissue engineering strategy depends on endogenous cell homing, functional stimulation and local tissue responses. For in situ pulp regeneration of pulpless root canal, endogenous stem cells, an injectable, bioactive scaffold, and chemoattractants and growth factors are required. Regarding the source of endogenous stem cells, the recruitment of stem cells from resident periapical region plays an important role [18]. However, the effect of MSCs delivered via the blood stream on pulp regeneration has not been identified. To our limited information, for the first time, the present study provided direct evidence that systemic BMSC can home to the root canal and participate in dental-pulp-like tissue regeneration. Although similar studies have not been reported, the study of stem cell homing principle in other medicine areas supports our conclusion. Jiang and his colleagues demonstrated that intravenously injected of MSCs can improve heart function and increase the regeneration of cardiac cells and blood vessels [19]. Dreger and colleagues showed that intravenous application of MSCs can be used as a practical path to provide stem cells for fracture nonunion and delayed union treatment [20]. Moreover, several experiments demonstrated that intravenous administration of BMSCs could be detected in the bone wound site created in the mandibles and calvaria, implying that transplanted BMSCs could migrate to bone defect and participate in bone

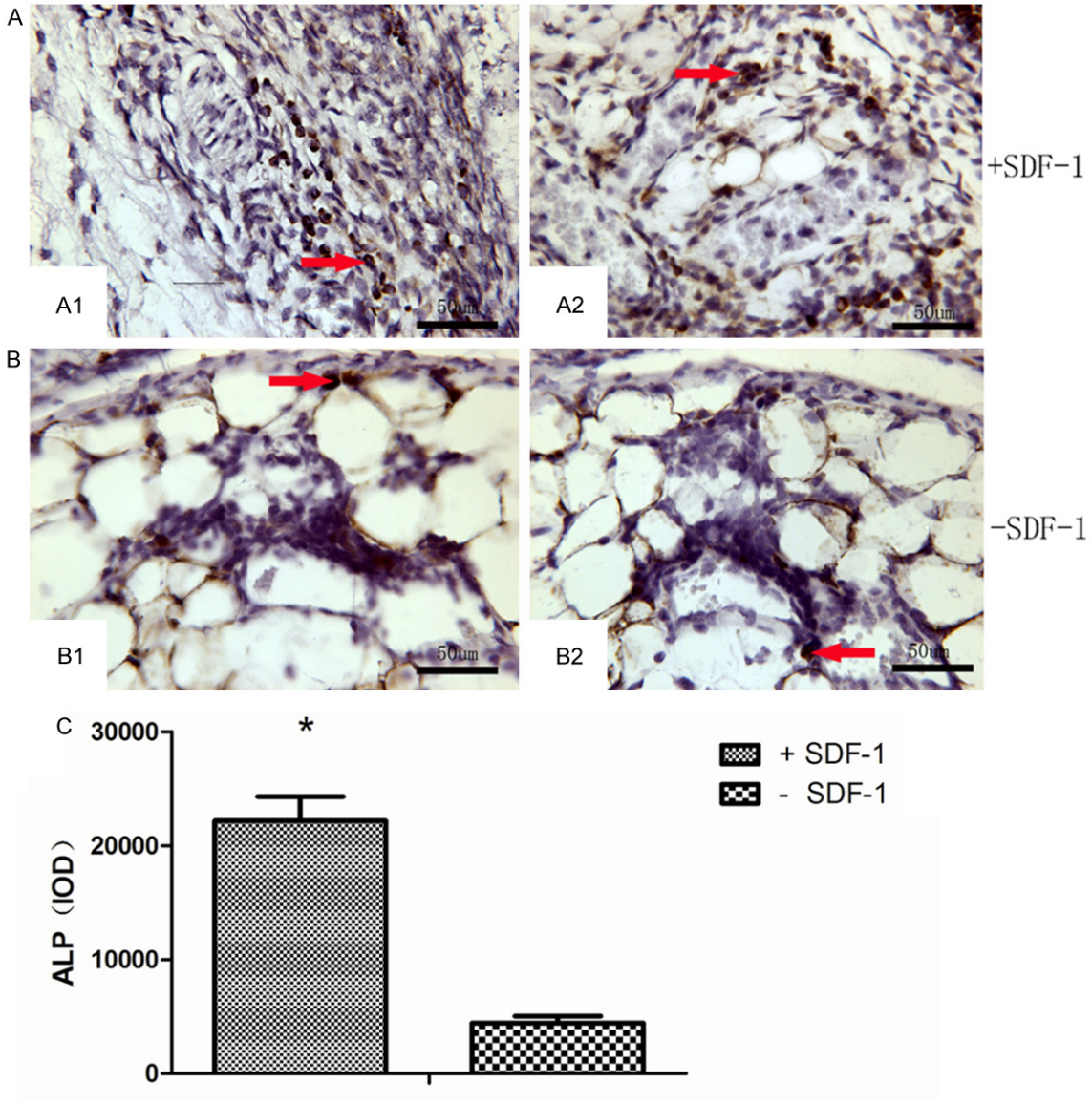

Figure 5. Detection of ALP expression within the generated tissue. A: Detection of ALP expression via immunohistological staining. Abundant ALP positive cells. Arrows indicate positive ALP staining. A2: Relatively concentrated ALP-positive cells beside the perivascular tissue but not at the vessel wall (as red arrowheads indicated). B: A small number of ALP positive cells expressing. C: Comparison of ALP IOD between SDF-1 group and SDF-1-free group. Scale bars, 50 μm. $^{*}P < 0.05$.

regeneration in orocraniofacial region [15, 21, 22].

Although it is not practical just now to utilize intravenous infusion of MSCs in pulp regeneration, the fact that systemic MSCs home and participate in pulp regeneration demonstrated in present study helps to understand the resource of functional cells responsible for pulp regeneration. More practically, this fact emphasizes the importance of evaluation of systemic conditions when homing strategy is used in regenerative endodontics because some systemic diseases such as hyperlipidemia can influence MSC homing [15]. Controlling these general conditions, at least temporally, will benefit in such regenerative procedure.

Given cell homing has rationality and good prospects in pulp regeneration, promoting stem cell homing, whether from local area or from systemic circulation or from both, should be an

important strategy to enhance pulp regeneration. SDF-1 is a chemoattractant protein that can be secreted by bone marrow stromal cells, other related mesothelial cells and epithelial cells. Many studies showed that SDF-1 was a pivotal chemokine that collectively participates in the BMSC mobilization and migration to damaged tissue such as heart, kidney and liver tissue [23-25]. Our experiment showed that the number of GFP-positive cells in SDF-1 group was significantly more than in SDF-1 free group, implying that SDF-1 has a strong chemotaxis on BMSCs towards endodonticaly root canal. Enhanced ALP-positive expression with increased number of GFP-positive cells in SDF-1 group suggests that endogenous BMSC participate in odontoblastic differentiation and the regeneration of dental pulp like tissue. These results were in concordance with the study by Suzuki et al, demonstrating that SDF-1 or basic fibroblast growth factor (bFGF) could induce recellularization and new vessel formation in endodontically treated teeth in rat model [26].

MSCs are believed to not only directly differentiate into different cell types including osteoblasts, adipocytes and chondroblasts, but also secrete trophic factors that exert chemotactic, mitotic, and differentiation-modulating effects [27]. In our experiment, a large number of GFP-positive cells could be found at the vessel wall and in the perivascular tissue. At the same time, relatively concentrated ALP-positive cells could be found beside the perivascular tissue but not at the vessel wall. This result suggests that homing BMSCs and ALP expression cells derived mainly from exogenously infused BMSCs. Meanwhile, it implies that recruited BMSCs can directly differentiate into vascular endothelial cells and ALP expression cells, thereby forming cell basis for further pulp regeneration.

Angiogenesis is the process of new blood vessel development from pre-existing capillaries which impacts significantly on many important disease states such as cancer, ischemic cardiovascular disease, wound healing and inflammation [28]. Successful tissue engineering depends on the establishment of an effective vascular network, capable of providing the tissue with oxygen, nutrients and immune cells, while removing byproducts and waste [17], therefore, a key issue of pulp regeneration is angiogenesis for it can keep the long-term stability of the newly formed tissues [29]. In our study, the newly formed blood vessels area of SDF-1 group was significantly greater than that of SDF-1-free group. The conclusion was consistent with the previous demonstrations that SDF-1 was chemotactic for BMSCs and endothelial cells, both of which were crucial for angiogenesis. It has been proved that SDF-1 can bind to cell surface receptors CXCR4 guiding mature or immature progenitors to induce progenitor cell mobilization and participation of angiogenesis [30].

In summary, this experiment confirmed that systemic BMSC could home to the root canal and participate in dental-pulp-like tissue regeneration and SDF-1 exerted enhancing effect on this process. However, the differentiation of homing cells needs further study and the significance of systemic BMSC homing in pulp regeneration requires to be further explored.

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Disclosure of conflict of interest

None.

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