Growth/differentiation factor-5 promotes *in vitro*/*vivo* periodontal specific differentiation of induced pluripotent stem cell-derived mesenchymal stem cells

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Abstract. Mesenchymal stem cells (MSCs) derived from induced pluripotent stem cells (iPSCs) represent a promising alternative source of MSCs for effective periodontal regeneration. Scientific evidence has demonstrated that growth/differentiation factor-5 (GDF-5) supports regeneration of periodontal tissues and has a key role in MSC differentiation. The present study investigated the effects of recombinant human GDF-5 (rhGDF-5) on periodontal specific differentiation of iPSC-derived MSCs (iPSC-MSCs) and bone marrow mesenchymal stem cells (BMSCs). rhGDF-5 treatment *in vitro* significantly enhanced the expression levels of marker genes associated with osteogenesis (OCN), fibrogenesis (periostin) and cementogenesis (CAP) in the iPSC-MSCs compared with untreated controls (all $P<0.05$). Interestingly, the rhGDF-5-treated BMSCs failed to exhibit overexpression of periostin and CAP despite highly upregulated expression of OCN. In the presence of rhGDF-5, both the iPSC-MSCs and BMSCs demonstrated marked formation of mineralized nodules. Notably, rhGDF-5 greatly promoted periodontal specific differentiation of the iPSC-MSCs encapsulated in hyaluronic acid (HA) hydrogels *in vivo* as determined by immunohistochemical and immunofluorescence staining. The majority of the PKH67-labeled iPSC-MSCs implanted with rhGDF-5 exhibited strong expression of OCN, periostin and CAP. In conclusion, iPSC-MSCs demonstrate high periodontal specific differentiation potential in response to rhGDF-5 both *in vitro* and *in vivo*. The delivery of iPSC-MSCs and rhGDF-5 with HA hydrogel may have beneficial effects in regenerative periodontal therapy.

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Introduction

Periodontitis is a common infectious and inflammatory disease characterized by irreversible destruction of tooth supporting tissues, including alveolar bone, periodontal ligament and cementum (1). Periodontitis is the predominant cause of tooth loss in adults and has been linked to many systemic diseases, such as diabetes, significantly impairing patients' quality of life and escalating the healthcare burden worldwide (2,3). The ultimate aim of periodontal treatment is to regenerate the defective tissues and restore the function of the periodontium. However, periodontal regeneration has been an elusive endeavor. Current therapeutic approaches, including bone grafting, guided tissue regeneration and use of biological factors, have had limited success in achieving this therapeutic aim (4,5).

With the development of stem cell biology and tissue engineering, recent insights have been focused on cell-based strategies that have a favorable effect on periodontal regeneration (6). The widely studied mesenchymal stem cells (MSCs) hold great promise for tissue regeneration, owing to their multilineage differentiation ability. MSCs from different tissue sources, such as bone marrow mesenchymal stem cells (BMSCs), adipose-derived stem cells and periodontal ligament stem cells, have demonstrated the capacity to promote periodontal regeneration to various degrees in animal studies (7-9). Nevertheless, the utility of MSCs has been partially restricted by limited accessibility, insufficient quantity and aging (5,10). Thus, it is imperative to identify alternative sources of MSCs for effective periodontal regeneration. The generation of induced pluripotent stem cells (iPSCs) by reprogramming somatic cells has provided a practical approach for acquisition of patient-specific stem cells. Currently, iPSCs may be efficiently generated from various

types of easily accessible tissues. Of note, iPSCs have been successfully differentiated into MSC-like cells, which display comparable surface phenotype and differentiation capability to the traditional MSCs (11). Furthermore, iPSC-derived MSCs (iPSC-MSCs) exhibit increased proliferation capacity, avoiding the senescence-related issues in the application of adult MSCs (12,13). Therefore, iPSC-MSCs offer a promising cell source for regenerative therapy, including periodontal regeneration (14).

To enhance the *in vivo* efficacy of stem cells, the concomitant use of suitable cell carriers and biological factors is essential for creating a favorable environment to support cell attachment, proliferation and differentiation (4). Hyaluronic acid (HA) is a linear, non-sulfated glycosaminoglycan and a primary component of the extracellular matrix. HA hydrogels have been widely engineered for biomedical use due to their biocompatibility and their ability to incorporate and release drugs (15). In addition, HA hydrogels provide a three-dimensional scaffold that allows spatial distribution of stem cells, mimics the native microenvironment and maintains space for mechanical stability (16). Growth/differentiation factor-5 (GDF-5) is a member of the bone morphogenetic protein family and the transforming growth factor-β superfamily. GDF-5 has been recognized as a key regulator for MSC differentiation and development of bone, cartilage and tendon/ligament (17). Notably, GDF-5 expression is associated with periodontal tissue formation and insertion of periodontal ligament fibers in alveolar bone and cementum during tooth root development, suggesting a regulatory role in the establishment of the periodontium (18). Emerging preclinical and clinical evidence demonstrates that GDF-5 may serve as a promising therapeutic agent for periodontal wound healing/regeneration (17,19). A recent study has identified that GDF-5 significantly enhances periodontal specific differentiation of iPSCs (20). However, it remains unclear how and to what extent GDF-5 mediates the cellular differentiation and function of iPSC-MSCs in the scenario of periodontal regeneration.

The present study investigated the effects of recombinant human GDF-5 (rhGDF-5) on periodontal specific differentiation of iPSC-MSCs and BMSCs *in vitro*, and characterized a HA-based delivery system of iPSC-MSCs/rhGDF-5 *in vivo* to offer a potential approach for periodontal regeneration.

Materials and methods

Ethics statement. Approval for the animal experiments in the present study was granted by the Biomedical Ethics Committee of Peking University (Beijing, China).

Human iPSC culture and derivation of iPSC-MSCs. Human iPSCs derived from peripheral blood mononuclear cells were obtained from Frankel Cardiovascular Center, The University of Michigan (Ann Arbor, MI, USA) (21). The iPSCs were cultured on Matrigel-coated 60-mm dishes in iPSCs culture medium containing basal DMEM/F-12, 20% knockout serum replacement, 1 mM GlutaMAX-I supplement, 4 ng/ml basic fibroblast growth factor (bFGF), 1% nonessential amino acids (all from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 0.1 mM β-mercaptoethanol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The differentiation of human iPSCs toward MSC-like cells was performed as described previously (22). In brief, the cell colonies were manually dissected into small clumps and incubated at 37˚C in iPSCs culture medium without bFGF supplement to generate floating embryoid bodies (EBs). The medium was changed every other day. After 10 days of culture, ~10 EBs were inoculated into 6-well plates coated with 0.1% gelatin and cultured at 37˚C in MSC medium comprising basal α-minimum essential medium (Gibco; Thermo Fisher Scientific, Inc.), 20% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 1 mM glutaMAX-I supplement, 1 ng/ml bFGF, 1% nonessential amino acids and 1% penicillin-streptomycin. Cells migrated out from the EBs gradually and MSC-like cells emerged. After 2 weeks of culture, the cells were digested by TrypLE Express (Thermo Fisher Scientific, Inc.) and cultured on 0.1% gelatin-coated 25-cm^2 flasks to establish passage 1 culture. The cells were then serially passaged with trypsin up to passage 4.

Induced differentiation of iPSC-MSCs in vitro. Human BMSCs were obtained from the School of Dentistry, The University of Michigan (Ann Arbor, MI, USA). The iPSC-MSCs and human BMSCs ($5x10^6$ cells) at passage 4 were cultured at 37˚C in MSC medium with or without 200 ng/ml rhGDF-5 (Pepro Tech, Inc., Rocky Hill, NJ, USA) and the media were replenished every other day. The concentration of rhGDF-5 was selected based on a previous study (20). After 2 weeks of incubation, immunofluorescence staining was performed to analyze the protein expression of osteocalcin (OCN), periostin and cementum attachment protein (CAP). Cells were rinsed with phosphate-buffered saline (PBS) and fixed by incubation with 4% paraformaldehyde for 30 min at room temperature, followed by three 5-min washes with PBS. Subsequently, the samples were blocked with blocking buffer (3% bovine serum albumin) (Sigma-Aldrich; Merck KGaA) for 1 h at 37˚C. The cells were then incubated with primary antibodies against OCN (ab13418), periostin (ab14041) (1:100; both from Abcam, Cambridge, UK) and CAP (sc-53947; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at room temperature. Following this, the samples were incubated with Alexa Fluor 488 (A32723) or Alexa Fluor 594 (R37117)-conjugated secondary antibodies (1:100; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at 37˚C. Nuclei were counterstained using DAPI Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Images were captured using a confocal laser scanning microscope (CLSM; Nikon Eclipse C1 Plus Confocal Workstation; Nikon Corporation., Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Gene expression levels of OCN, periostin and CAP in the iPSC-MSCs and BMSCs treated with rhGDF-5 were evaluated by RT-qPCR. Total RNA was isolated from iPSC-MSCs and BMSCs using an RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA) and treated with DNase (Qiagen, Inc.) according to the manufacturer's protocols. Then, cDNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems; Thermo Fisher Scientific, Inc.). Following this, RT-qPCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher

Scientific, Inc.) using a TaqMan Universal PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) containing AmpliTaq Gold DNA Polymerase. The TaqMan primers and probes specific for OCN (Hs01587813_g1), periostin (Hs01566734_m1), CAP (Hs00171965_m1) and GAPDH (Hs99999905_m1) (all from Applied Biosystems; Thermo Fisher Scientific, Inc.) were adopted. The reactions were performed in triplicate with a final volume of 30 μl containing 15 μl of TaqMan 2X Universal PCR Master mix, 2 μl of cDNA and 1.5 μl of TaqMan primers and probes. The thermal cycling conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Relative gene expression levels were normalized against GAPDH and analyzed with the $2^{-\Delta\Delta Cq}$ method (23).

Alizarin Red S staining. After 4 weeks of culture in the presence of rhGDF-5, the iPSC-MSCs and BMSCs were rinsed with PBS and fixed in 10% formalin for 30 min at room temperature. Cells were then washed with PBS three times, each for 5 min, and stained with 40 mM Alizarin Red S (pH 4.2) for 20 min at room temperature. Excess dye was removed by washing the cells three times with PBS. The deposited mineralized matrix was stained in red by Alizarin Red S.

Hydrogel preparation and cell encapsulation. The commercially available HyStem-C hydrogels (ESI BIO, Alameda, CA, USA) were prepared according to the manufacturer's instructions. Briefly, degassed, deionized water was used to dissolve Glycosil (thiol-modified hyaluronan), Gelin-S (thiol-modified collagen) and Extralink [thiol-reactive Polyethylene (glycol) Diacrylate crosslinker] in individual vials. The iPSC-MSCs were labeled with PKH67 Green Fluorescent Cell Linker (Sigma-Aldrich; Merck KGaA). Subsequently, equal volumes of Glycosil and Gelin-S were mixed prior to addition of the labeled cells ($5x10^5$ ells/ml) and 200 ng/ml rhGDF-5. To form the hydrogel, Extralink was added to the mixture in a 1:4 volume ratio.

Surgical procedure. Three intervention groups were created: i) i PSC-MSCs + rhGDF-5 + hydrogel; ii) iPSC-MSCs + hydrogel; iii) rhGDF-5 + hydrogel. The hydrogel constructs (n=4 for each group) were subcutaneously implanted into the dorsal surface of 6-8-week old male athymic nude mice (weight, 18-22 g; Charles River Laboratories, Wilmington, MA, USA) under general inhalation anesthesia using 2% isoflurane. Each mouse received 2 implants at random. The mice were maintained under standard conditions (12-h light/dark cycle, 22˚C, 60% humidity) with free access to chow and water. After 6 weeks, the mice were sacrificed and the implants were harvested for further analysis.

Immunohistochemical and immunofluorescence staining. For immunohistochemical staining, the Cell and Tissue Staining kit [horseradish peroxidase-amino ethylcarbazole (HRP-AEC) System; R&D Systems, Inc., Minneapolis, MN, USA] was used, according to the manufacturer's instructions. The harvested specimens were incubated in 10% formalin overnight at room temperature and embedded in paraffin. Sections (5 μm thickness) were deparaffinized, rehydrated and then immersed in 3% H_2O_2 for 10 min to quench the endogenous peroxidase activity. After blocking at room temperature, sections were incubated with the aforementioned primary antibodies against OCN, periostin and CAP overnight at room temperature followed by incubation with biotinylated secondary antibodies for 1 h at room temperature. Staining was visualized by HRP-AEC reaction under an Olympus IX71 microscope (Olympus Corp., Tokyo, Japan).

For immunofluorescence staining, the harvested specimens were fixed with 4% paraformaldehyde for 15 min at room temperature and cryosectioned at a thickness of 5 mm. After blocking with 5% bovine serum albumin for 30 min at 37˚C, sections were incubated with the aforementioned primary antibodies against OCN, periostin and CAP at 4˚C overnight followed by incubation with Alexa Fluor 594-conjugated secondary antibody (1:100) for 1 h at 37˚C. Nuclei were counterstained using DAPI Fluoromount-G. Images were captured using a CLSM. The percentage of OCN-, periostin- and CAP-positive cells in fluorescence-labeled donor cell populations were calculated for four implants.

Statistical analysis. At least three samples were used for each quantitative experiment. All quantitative data were expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Student's t-test was applied to analyze differences between the groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of rhGDF-5 on periodontal specific differentiation of iPSC-MSCs in vitro. To investigate the differentiation capacity of the iPSC-MSCs and BMSCs in response to rhGDF-5, the expression of marker genes associated with periodontal tissue formation, including OCN, periostin and CAP, were examined by RT-qPCR and immunofluorescence staining. The iPSC-MSCs presented a fibroblastic-like morphology. The rhGDF-5 treatment (200 ng/ml) significantly enhanced the mRNA expression levels of OCN, periostin and CAP in the iPSC-MSCs at weeks 1, 2 and 3 ($P<0.05$; Fig. 1A) compared with the corresponding untreated control groups. As the incubation continued from week 1 to 3, the inductive effect of rhGDF-5 on OCN and CAP expression gradually increased. With reference to the marked overexpression of OCN and CAP, the enhancement of periostin expression was relatively weak (Fig. 1A). For BMSCs, the cells were cultured with rhGDF-5 for 2 weeks. BMSCs treated with rhGDF-5 exhibited significantly higher mRNA expression levels of OCN ($P<0.05$), comparable to that in the iPSC-MSCs, compared with the untreated control; however, no significant difference was observed between the periostin and CAP expression levels of the control and treatment groups (Fig. 1B).

The formation of mineralized matrix deposits was assessed by Alizarin Red S staining. After 4 weeks of culture in the presence of rhGDF-5, both the iPSC-MSCs and BMSCs demonstrated marked formation of mineralized nodules compared with the control groups. No deposits were observed in the untreated controls (Fig. 1C).

Immunofluorescence analysis, after 2 weeks, revealed intense expression of OCN, periostin and CAP in the

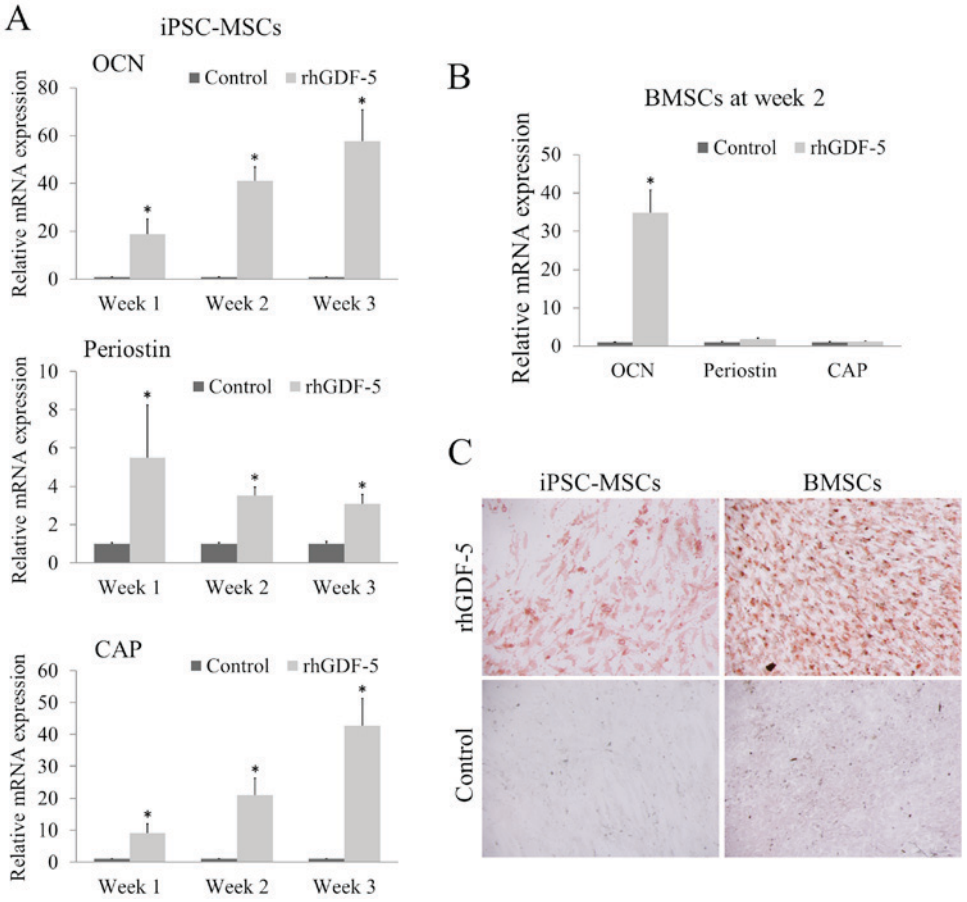

Figure 1. Effects of rhGDF-5 on gene expression and mineralization in iPSC-MSCs and BMSCs *in vitro*. Relative mRNA expression levels of OCN, periostin and CAP in (A) iPSC-MSCs at weeks 1-3 and (B) BMSCs at week 2 in response to rhGDF-5 treatment (200 ng/ml), relative to GAPDH. (C) Mineralized nodule formation evaluated by Alizarin Red S staining at week 4. Data are presented as the mean + standard deviation. *P<0.05 vs. the untreated control group. rhGDF-5, recombinant human growth/differentiation factor-5; iPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; BMSCs, bone marrow mesenchymal stem cells; OCN, osteocalcin; CAP, cementum attachment protein.

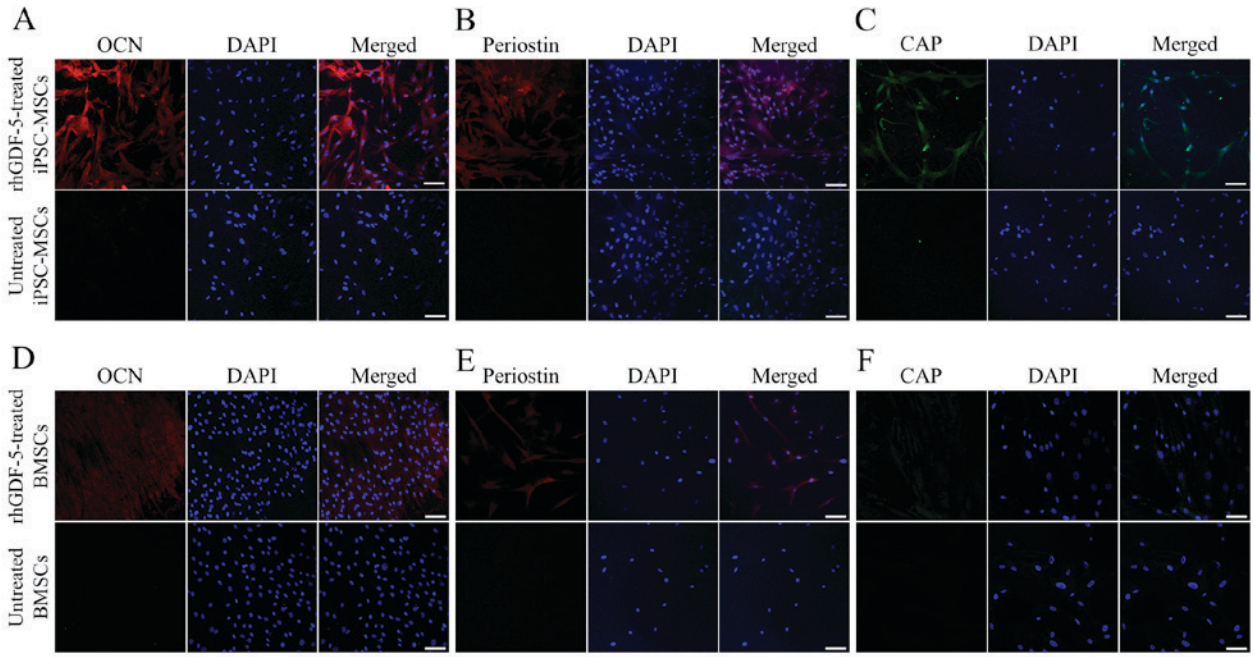

Figure 2. Confocal imaging of iPSC-MSCs and BMSCs treated with rhGDF-5 (200 ng/ml) for 2 weeks *in vitro*. Expression of (A) OCN, (B) periostin and (C) CAP in iPSC-MSCs in response to rhGDF-5. Expression of (D) OCN, (E) periostin and (F) CAP in rhGDF-5-treated BMSCs. Nuclei were stained blue with DAPI. Scale bar, 50 μm. rhGDF-5, recombinant human growth/differentiation factor-5; iPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; BMSCs, bone marrow mesenchymal stem cells; OCN, osteocalcin; CAP, cementum attachment protein.

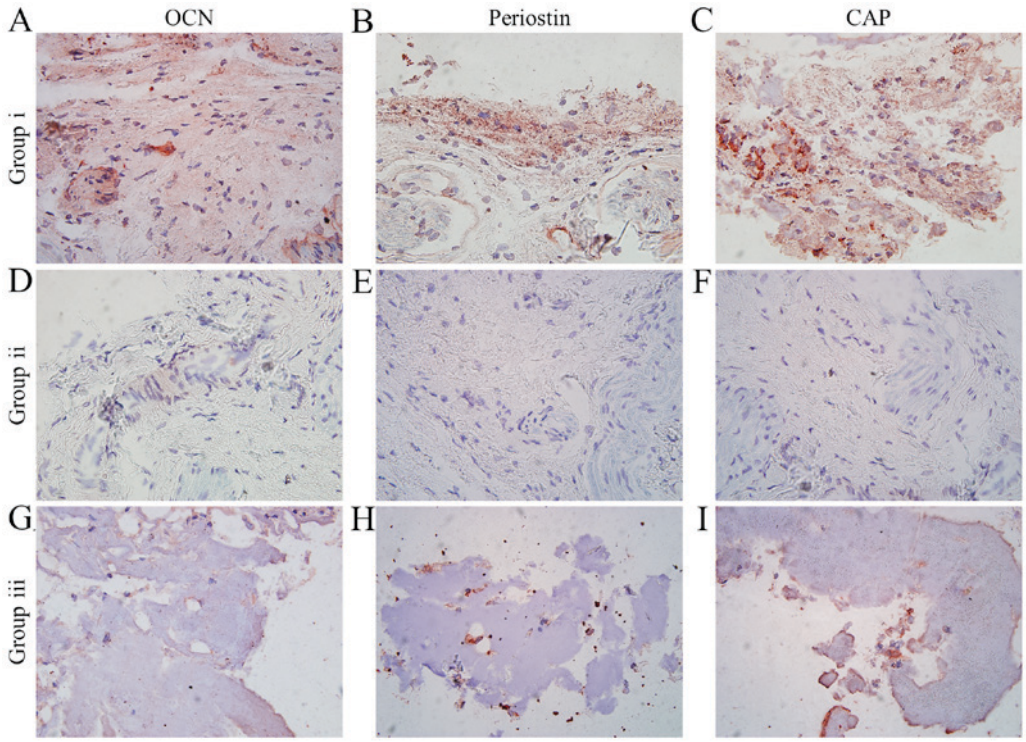

Figure 3. Immunohistochemical analysis (magnification, x40) of engineered hyaluronic acid hydrogels 6 weeks after subcutaneous transplantation in the three intervention groups. Staining of (A) OCN, (B) periostin and (C) CAP in Group i. Staining of (D) OCN, (E) periostin and (F) CAP in Group ii. Staining of (G) OCN, (H) periostin and (I) CAP in Group iii. Group i, iPSC-MSCs + rhGDF-5 + hydrogel; Group ii, iPSC-MSCs + hydrogel; Group iii, rhGDF-5 + hydrogel; rhGDF-5, recombinant human growth/differentiation factor-5; iPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; OCN, osteocalcin; CAP, cementum attachment protein.

rhGDF-5-treated iPSC-MSCs (Fig. 2A-C). The BMSCs treated with rhGDF-5 for 2 weeks displayed strong OCN staining and weak periostin staining, whereas CAP expression was not detected (Fig. 2D-F). The staining of the markers was negative in the untreated controls.

Effects of rhGDF-5 on periodontal specific differentiation of iPSC-MSCs encapsulated in HA hydrogels in vivo. The capability of rhGDF-5 to support the periodontal specific differentiation of iPSC-MSCs was further evaluated *in vivo*. The cells and rhGDF-5 were embedded in HA hydrogels for subcutaneous transplantation in nude mice. After 6 weeks, the implants were retrieved and no adverse local responses, such as inflammation, were observed. Immunohistochemistry results demonstrated strong positive staining for OCN, periostin and CAP in the newly formed tissues by the composite of iPSC-MSCs, rhGDF-5 and HA hydrogels (Fig. 3A-C). However, very low expression was detected in the hydrogels incorporating iPSC-MSCs (Fig. 3D-F) or rhGDF-5 alone (Fig. 3G-I).

To track the donor cells in the specimens, the iPSC-MSCs pre-labeled with PKH67 were also examined by immunofluorescence staining. Co-localization of the markers with PKH67 correlated with the immunohistochemical analysis. The majority of the iPSC-MSCs implanted with rhGDF-5 exhibited strong expression levels of OCN, periostin and CAP. The OCN-, periostin- and CAP-positive cells accounted for 90.17±9.98, 77.29±10.65 and 83.73±10.33%, respectively, of the PKH67-labeled donor cells (Fig. 4). By contrast, expression of the three markers was not detected in the iPSC-MSCs or the ingrown indigenous cells of the groups containing hydrogels incorporating either iPSC-MSCs or rhGDF-5 alone (Fig. 4).

Discussion

Periodontal regeneration remains a substantial challenge in management of periodontitis due to the complex architecture and function of periodontium. Tissue engineering approaches employing multipotent progenitor cells, signaling molecules and bioactive scaffolds have been recognized as promising therapeutics for reliable and predictable periodontal regeneration (4,5). The development and repair of periodontal tissues involve an orchestrated process of proliferation and differentiation of periodontal progenitor cells for osteogenesis, fibrogenesis and cementogenesis (4,24). To regenerate the three major components of periodontal tissues, it is therefore crucial to identify an optimum cell source with the capacity to differentiate into functional periodontal cells, and to provide a microenvironment in favor of effective periodontal specific differentiation of the implanted cells.

Previous studies have adopted multiple induction conditions to prompt periodontal specific differentiation of adult stem cells and characterize their differentiation capacity (25,26). iPSC-MSCs have been recognized as a promising cell source for periodontal regeneration (14). In the present study, iPSC-MSCs were generated and their differentiation capacity under rhGDF-5 treatment was examined. OCN is an osteoblast-specific protein implicated in bone mineralization, and has been commonly used as a marker for the osteogenic differentiation of progenitor cells at the late stage (24,27). Periostin

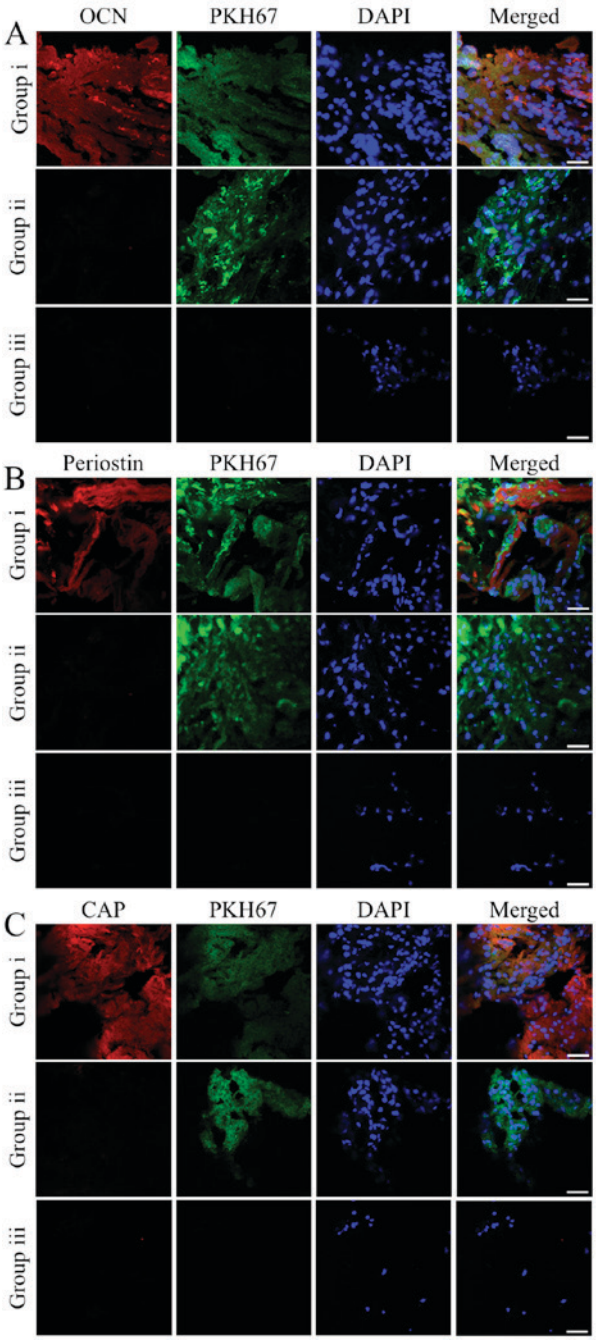

Figure 4. Immunofluorescence analysis of the co-localization of OCN/periostin/CAP with PKH67-labelled subcutaneously-transplanted iPSC-MSCs after 6 weeks. Expression of (A) OCN, (B) periostin and (C) CAP in PKH67-labelled iPSC-MSCs in the three intervention groups. Nuclei were stained blue with DAPI. Scale bar, 50 μm. Group i, iPSC-MSCs + rhGDF-5 + hydrogel; Group ii, iPSC-MSCs + hydrogel; Group iii, rhGDF-5 + hydrogel; rhGDF-5, recombinant human growth/differentiation factor-5; iPSC-MSCs, induced pluripotent stem cell-derived mesenchymal stem cells; OCN, osteocalcin; CAP, cementum attachment protein.

is highly expressed in periodontal ligament fibroblasts and has an important role in periodontal tissue integrity (28). CAP is a cementoblast-related marker and its production is restricted to cementum (29). In the present study, iPSC-MSCs incubated with rhGDF-5 for 1-3 weeks displayed significantly higher gene expression levels of OCN, periostin and CAP compared with the untreated control groups. The immunostaining results corresponded with the RT-qPCR data. This is consistent with a previous observation on the overexpression of OCN, periostin and CAP in rhGDF-5-treated iPSCs (20). Notably, the rhGDF-5 treatment failed to enhance the expression of periostin and CAP in the BMSCs, despite significantly increased expression of OCN compared with the untreated control. Previous research has demonstrated that GDF-5 was able to stimulate osteogenic differentiation of BMSCs in a subcutaneous rat model (30). Although BMSCs may aid periodontal regeneration, a recent meta-analysis indicated that BMSCs have no favorable effect on periodontal ligament formation as determined by subgroup analysis (6). In addition, in the present study, the 4-week rhGDF-5 incubation greatly induced mineralization in both the iPSC-MSCs and BMSCs, whereas there were barely discernible mineralized deposits in the untreated controls. Collectively, these data highlight the potential of rhGDF-5 to induce periodontal specific differentiation of iPSC-MSCs, and suggest that iPSC-MSCs may have a greater capacity than BMSCs to differentiate into periodontal cells in response to rhGDF-5.

Furthermore, the present study evaluated the differentiation potential of iPSC-MSCs delivered by HA hydrogels with or without rhGDF-5 in a subcutaneous murine model. HA hydrogels have been demonstrated to enhance periodontal treatment outcomes when applied alone or in conjugation with cells/factors (31,32). In the present study, the engineered hydrogels demonstrated successful biocompatibility 6 weeks after transplantation. The newly formed tissues by the HA hydrogels containing iPSC-MSCs and rhGDF-5 displayed strong production of OCN, periostin and CAP; however, the markers were not detected in the hydrogels incorporating either iPSC-MSCs or rhGDF-5 alone. Furthermore, the introduction of rhGDF-5 induced expression of the markers in the majority of the implanted iPSC-MSCs. This further indicates that rhGDF-5 may promote the differentiation of iPSC-MSCs into periodontal cells *in vivo*.

In conclusion, iPSC-MSCs displayed a high capacity of periodontal specific differentiation in response to rhGDF-5 both *in vitro* and *in vivo*. With reference to BMSCs, iPSC-MSCs may be a more effective cell source for use in periodontal treatment. The incorporation of iPSC-MSCs and rhGDF-5 in HA hydrogel is likely to offer a promising therapeutic approach for periodontal regeneration. Further investigations are warranted to elucidate the mechanisms underlying the effects of GDF-5 and to evaluate the regenerative potential of iPSC-MSCs/rhGDF-5/HA hydrogel composites in more clinically relevant models.

Acknowledgements

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