

1 Secretomes from Bone Marrow-derived Mesenchymal Stem Cells Enhance Periodontal  
2 Tissue Regeneration

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## Abstract

*Background aims.* Periodontal tissue regeneration using mesenchymal stem cells (MSCs) has been regarded as a future cell-based therapy. However, low survival rates and the potential tumorigenicity of implanted MSCs could undermine the efficacy of cell-based therapy. The use of conditioned media from MSCs (MSC-CM) may be a feasible approach to overcome these limitations. The aim of this study is to confirm the effect of MSC-CM on periodontal regeneration.

*Methods.* MSC-CM were collected during their cultivation. The concentrations of the growth factors in MSC-CM were measured using ELISA. Rat MSCs (rMSCs) and human umbilical vein endothelial cells (HUVEC) cultured in MSC-CM were assessed on wound healing and angiogenesis. The expressions of osteogenetic and angiogenic-related genes of rMSCs cultured in MSC-CM were quantified by real-time RT-PCR analysis. *In vivo*, periodontal defects were prepared in the rat models and the collagen sponges with MSC-CM were implanted.

*Results.* MSC-CM includes IGF-1, VEGF, TGF- $\beta$ 1 and HGF. *In vitro*, wound healing and angiogenesis increased significantly in MSC-CM. The levels of expression of osteogenetic and angiogenic-related genes were significantly upregulated in rMSCs cultured with MSC-CM. *In vivo*, in the MSC-CM group 2 weeks after implantation, immunohistochemical analysis showed several CD31-, CD105-, or FLK-1-positive cells occurring frequently. At 4 weeks after implantation, a regenerated periodontal tissue was observed in MSC-CM groups.

*Conclusions.* The use of MSC-CM may be an alternative therapy for periodontal tissue regeneration because several cytokines included in MSC-CM will contribute to many

processes of complicated periodontal tissue regeneration.

**Key Words:** *periodontal regeneration, mesenchymal stem cell, conditioned medium, paracrine effects, angiogenesis, migration*

## Abbreviations

mesenchymal stem cells (MSCs), human MSCs (hMSCs), platelet-rich plasma (PRP), conditioned media (CM), human bone marrow-derived MSCs (MSC-CM), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), hepatocyte growth factor (HGF), fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF)-BB, bone morphogenetic protein (BMP)-2, stromal-cell-derived factor (SDF)-1, rat periodontal ligament cells (rPDLs), fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), enzyme-linked immunosorbent assay (ELISA), Rat MSCs (rMSCs), human umbilical vein endothelial cells (HUVEC), human diploid fibroblasts (HDF), optimized angiogenesis medium (BM), DMEM-10% FBS (EM; Expansion Medium), *Alkaline phosphatase (ALP)*, *Osteocalcin (OCN)*, *angiopoietin 1 (ANG-1)*, *angiopoietin 2 (ANG-2)*, atelo-collagen sponge (ACS)

## Introduction

The chronic presence of plaque bacteria in the gingival and periodontal tissues results in destruction of structural components of the periodontium (1). This condition leads to the clinical signs of periodontitis, with the breakdown of periodontal tissue, resulting in tooth loosening (2). To regenerate lost periodontal tissue, numerous procedures and products have been developed, for example, autogenous bone grafting (3), guided tissue regeneration (GTR) (4), platelet-rich plasma (5), enamel matrix derivatives (Emdogain<sup>®</sup>) (6), and recombinant human growth factors (7-12). Although these treatments have been reported to be effective in regenerating periodontal tissue, candidates for such treatments are limited, and the amount of tissue that is regenerated cannot be reliably predicted.

Cell therapy with stem cells is a promising approach for treating various refractory diseases. Therefore, periodontal tissue regeneration using mesenchymal stem cells (MSCs) has been regarded as a viable future cell-based therapy for the treatment of periodontal diseases (13, 14).

We previously used a mixture of human MSCs (hMSCs) and platelet-rich plasma (PRP) (hMSCs/PRP) as bone graft materials, with predictable outcomes (15). However, recent studies of MSC transplantation in an acute myocardial infarction revealed that the implanted MSCs did not survive, and only 4.4% of engraftment of MSCs could be found 1 week after transplantation (16). The studies of MSC transplantation in cases of spinal cord injury revealed that the implanted MSCs disappeared from the host tissue 1–2 weeks after transplantation (17). These facts suggest that the implanted cells may contribute to tissue regeneration through them as well as their paracrine effects. Many

secretomes, including growth factors and cytokines, have been reported in the conditioned media (CM) of various MSCs (18-20), which could be responsible for the paracrine effects of stem cells on tissue regeneration. Previous studies have reported the use of CM for experimental regenerative therapies. For example, CM obtained from amniotic fluid-derived MSCs (21) and adipose-derived stem cells (21) significantly enhanced wound healing. Endothelial progenitor cell CM induced neovascularization in a rat hindlimb ischemia model (22).

The use of CM has the added benefit of solving several problems currently encountered in clinical applications of stem cells, such as tumorigenesis (23) and the transmission of infectious diseases.

We previously reported that CM transplantation from human bone marrow-derived MSCs (MSC-CM) promoted bone regeneration in a rat calvarial bone defect model. MSC-CM contributed to accelerated mobilization of endogenous MSCs and endothelial cells for bone regeneration (24, 25). These effects of MSC-CM transplantation were stronger than those of MSC implantation. MSC-CM has plural growth factors and cytokines related to tissue regeneration, such as insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), and hepatocyte growth factor (HGF).

Based on these findings, we hypothesized that the cytokines contained in MSC-CM may enhance mobilization of endogenous MSCs and angiogenesis and promote periodontal tissue regeneration through several steps. At the cellular level, the process of periodontal tissue regeneration requires angiogenesis, cell migration, and proliferation and differentiation into various cell types, particularly osteoblasts and cementoblasts, and the cytokines contained in MSC-CM can contribute these biological steps toward

135 osteogenesis.

136 Angiogenesis is especially crucial to the accelerated regeneration of lost tissues in  
137 periodontal therapy because the process of periodontal tissue regeneration is  
138 complicated, as periodontal wound healing occurs on the nonvascular and nonvital hard  
139 tissues of the root surface (26).

140 The purpose of this study was to confirm the effect of MSC-CM and its role in  
141 periodontal regeneration.

## Materials and Methods

### *Ethics Statement*

All animal protocols were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine (Approval ID number: 25375). Maximum efforts were made to minimize suffering, and all surgery and measurement under inserting catheter were performed under deep anesthesia.

### *Cell preparation*

Human MSCs (hMSCs) were purchased from Lonza Inc. (Walkersville, MD, USA) and cultured in mesenchymal stem cell basal medium (MSCBM; Lonza Inc.) containing MSCGM SingleQuots (Lonza Inc.) at 37 °C in 5% CO<sub>2</sub>/95% air. After primary culture, the cells were subcultured at a density of approximately  $1 \times 10^4$  cells/cm<sup>2</sup>. hMSCs at the 3rd to the 9th passages were used for experiments. Subconfluent hMSCs were trypsinized and used for cell implantation.

Rat MSCs (rMSCs) was isolated from 7-week-old Wistar/ST rats weighing 180–210 g (Japan SLC, Shizuoka, Japan) as previously reported (27). Briefly, donor rats were sacrificed and the femora were dissected out. Using sterile conditions, the edge of each bone was cut, Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD) was injected into the bone marrow using a 25-gauge syringe, the bone marrow cells were flushed out to the opposite side, and this maneuver was repeated several times. The marrow was then seeded into each tissue culture flask in DMEM containing antibiotic-antimycotic (100 units/mL penicillin G, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B; Gibco) and the medium was supplemented with 10% fetal



bovine serum (FBS). Three days after seeding, floating cells were removed and the medium was replaced with fresh medium. The adherent, spindle-shaped cells were passaged when the cells were approaching confluence. Adherent cells were collected using trypsin/EDTA, resuspended in fresh medium, and transferred to new flasks at a density of  $1 \times 10^4$  cells/cm<sup>2</sup>. rMSCs obtained from cultures at the 2nd to the 4th passages were used for the experiments.

Rat periodontal ligament cells (rPDLCs) were isolated from the periodontal ligament tissue of 7-week-old Wistar/ST rats. Periodontal ligament was gently removed from the middle third of the mandibular molar root surface and digested in an equal volumes of Type I collagenase (3 mg/mL) and Type II dispase (4 mg/mL) for 1 h at 37°C. The resulting cells ( $1 \times 10^4$  cells/cm<sup>2</sup>) were then seeded into each tissue culture flask in  $\alpha$ -MEM culture medium (Sigma-Aldrich Inc., St Louis, MO, USA) supplemented with 10%FBS, 100  $\mu$  M L-ascorbate-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 2mM L-glutamine and antibiotic-antimycotic (100 units/mL penicillin G, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B; Gibco). Single cell colonies were observed and passage (P0) cells were cultured. rPDLCs obtained from cultures at the 3rd to the 5th passages were used for the experiments.

Pluripotency of obtained cells for differentiation into classical mesenchymal lineage cells, including osteoblasts, adipocytes, or chondrocytes, was verified by using previously reported methods. These cells were used as rMSCs in this study (data not shown). The results indicated that these cells had stem cell characteristics.

*Preparation of conditioned media (CM)*

hMSCs that were 70%–80% confluent were re-fed with serum-free medium. The cell-cultured CM were collected after a 48-h incubation. Collected cultured CM were defined as hMSC-cultured CM (MSC-CM) and were stored at 4 or –80 °C before using for the following experiments.

#### *Enzyme-linked immunosorbent assay (ELISA)*

The levels of IGF-1, VEGF, TGF- $\beta$ 1, HGF, fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF)-BB, bone morphogenetic protein (BMP)-2, and stromal-cell-derived factor (SDF)-1 in MSC-CM were investigated using enzyme-linked immunosorbent assay (ELISA). The concentration of these factors was measured using a Human Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, 200  $\mu$  L of MSC-CM, DMEM-0%FBS, or DMEM-30%FBS was added to 96-well microplates that were coated with a monoclonal antibody to the factor of interest and incubated for 2 h. After washing with PBS, a horseradish peroxidase-conjugated cytokine or growth-factor-specific antibody was added to each well, incubated for 2 h, and washed. Substrate solution was added and incubated for 30 min, and the reaction was terminated by addition of the stop solution. Cytokine/growth factor levels were determined by measurement of the optical density at 450nm using a microplate spectrophotometer (Benchmark Plus; Bio-Rad, Hercules, CA).

#### *Wound-healing assay*

The migratory properties of rMSCs and rPDLCs were examined using the CytoSelect

Wound Healing Assay kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, cell suspension was added to the well with a plastic insert in place. The insert was removed from the well after a monolayer of cells had formed, creating a wound gap of 0.9 mm. After washing, cells were incubated at 37 °C for 48 h in MSC-CM with 30% FBS or serum-free DMEM. The extent of wound closure was determined with a light microscope (CK40; Olympus, Tokyo, Japan).

#### *Tube formation assay*

An angiogenesis assay kit (KZ-1000; Kurabo, Osaka, Japan) was used according to the manufacturer's instructions (28). This kit comprised a 24-well culture dish in which human umbilical vein endothelial cells (HUVECs) and human diploid fibroblasts (HDF) were seeded in the optimal condition for capillary tube formation. The optimized angiogenesis medium (BM) in each well was changed on days 1, 4, 7, and 9 with fresh medium containing VEGF (10 ng/mL), MSC-CM, MSC-CM plus anti-VEGF (MAB293) (10 µg/mL) (R&D Systems), or none. The antibody concentration was 10 µg/mL and therefore was 100-fold greater than that for half-maximal inhibition of 10 ng/mL of the recombinant proteins.

After 11 days, cells were fixed in 70% ethanol and incubated with diluted primary antibody (mouse anti-human CD31, 1:4000) for 1 h at 37 °C, and with the secondary antibody (goat anti-mouse IgG alkaline-phosphatase-conjugated antibody, 1:500) for 1 h at 37 °C, with visualization achieved using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Images were obtained from five different fields (5.5 mm<sup>2</sup>/field) for each well, and tube length (the total lengths of the tubes) and joints (the number of capillary connections) were quantified using Angiogenesis Image Analyzer

Ver.2 (Kurabo).

### *Real-time RT-PCR*

rMSCs were cultured with MSC-CM or DMEM-10% FBS (EM; Expansion Medium) for 48 h, and total RNA was extracted using an RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. Real-time RT-PCR analysis was performed as previously described (29). Samples for total RNA determination (50 ng each) were placed into a 50- $\mu$ L-volume RT-PCR tube. The sequences of the specific primers and probes used for the real-time RT-PCR analysis for *Alkaline phosphatase (ALP)*, *Osteocalcin (OCN)*, *Runx2*, *VEGF-A*, *angiopoietin 1 (ANG-1)*, *angiopoietin 2 (ANG-2)*, and *GAPDH* are given in Table 1. RT-PCR reactions and the resulting relative increase in reporter fluorescent dye emission were monitored in real time using the 7000 sequence detector (Perkin-Elmer, Foster City, CA, USA). Signals were analyzed using a sequence detector 1.0 program (Perkin-Elmer). The PCR conditions were as follows: 1 cycle at 50 °C for 2 min, 1 cycle at 60 °C for 30 min, 1 cycle at 95 °C for 5 min, 50 cycles at 95 °C for 20 s, and then 60 °C for 1 min. The relative amount of each mRNA in one sample was obtained by calculation of the respective standard curves. The standard curves for each mRNA were drawn using different concentrations (2000, 400, 80, 16, and 3.2 ng) of the total RNA of rMSCs. The relative expression levels were normalized to GAPDH expression.

### *Rat periodontal defect model*

Adult male Wistar/ST rats were purchased from Japan SLC (Shizuoka, Japan). The rats were housed on a 12 hours light/dark cycle in a temperature-and-humidity-controlled

room with food and water ad libitum. The animals were allowed to acclimatize for at least seven days before the start of the experiments. Surgery was performed in a similar fashion as previously described (14) using magnification loupes. Ten-week-old male Wistar/ST rats weighing 260–290 g were anesthetized by intraperitoneal injection of Somnopentyl<sup>®</sup> (20 mg/kg body weight). With the rat in the supine position, a mucosal incision was made from the gingival sulcus of the second molar mesial palatal side to the first molar mesial palatal side, and an approximately 5-mm incision was made continuously in the mesial direction from the first molar mesial side. After mucosal flap elevation, the periodontal tissue, including the cementum, alveolar bone, and periodontal ligament, was bilaterally excised at the palatal side of the first molar using a dental round bur (ISO standard 010) under irrigation, so that the dimensions of the defect were approximately 1 mm in diameter. After irrigation with physiological saline solution, the experimental materials were then implanted into the defects. An absorbable atelo-collagen sponge (ACS) (TERUDERMIS<sup>®</sup>; Olympus Terumo Biomaterials, Tokyo, Japan) was used as a scaffold and contained 30  $\mu$ L MSC-CM or PBS. The rats with defects were implanted with graft materials: MSC-CM with ACS (MSC-CM group), PBS with ASC (PBS group), or defect only (Defect group). Finally, the mucosal flaps were replaced using 6-0 polydioxanone sutures (PDS II; Ethicon Inc., Somerville, N.J., USA). During the surgery, the body temperature was maintained at 37°C using a homeothermic heating pad. Following the surgery, the rats were administered buprenorphine (0.05 mg/kg, i.m. per 12 hours) for 24 hours to relieve pain. The rats were euthanized by an overdose of ether (Wako, Osaka, Japan) on 2 or 4 weeks after transplantation (n = 8 at each time point in each group).

*Histological analyses*

Explants were decalcified with K-CX solution (Falma Co., Tokyo, Japan), and were then dehydrated using graded ethanol, cleared with xylene, and embedded in paraffin. The specimens were cut in a sagittal direction to make 5- $\mu$ m-thick histological sections in the buccal–palatal plane and were stained with hematoxylin and eosin. Histological analysis was performed using a light microscope (CK40; Olympus, Tokyo, Japan).

*Immunohistochemical staining*

MSC-CM or PBS with ACS was implanted into rat periodontal defects, and samples were collected after 2 weeks. Fresh-frozen sections of these samples were made according to the Kawamoto method using a Multi-Purpose Cryosection Preparation Kit (30). Cryofilm type 2C was applied to the cutting surface of the completely frozen block, which was cut with a tungsten carbide knife at  $-25^{\circ}\text{C}$  in a cryostat chamber (Leica CM3050S; Leica Microsystems, Wetzlar, Germany). The section was fixed with 100% ethanol for 10 min and then washed with PBS for 3 min. CD31, a monoclonal mouse antibody (BD Pharmingen), was used as a marker for rat endothelial cells. CD105, a polyclonal rabbit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), was used as a marker for rat stem cells. Flk-1, a monoclonal mouse antibody (Santa Cruz Biotechnology), was used as a marker for VEGF-R2. An Alexa Fluor 633-conjugated goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR, USA) and an Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Molecular Probes, Inc.) were used as secondary antibodies. After DAPI staining, the section was washed with PBS and mounted between a glass slide and the adhesive film. The section was enclosed by the mounting resin SCMM R2 on the glass slide, and the resin was hardened with UV irradiation for 1

min by means of the UV Quick Cryosection Mounter (ATTO Bio-Instrument, Tokyo, Japan). After fixation, the specimen was observed by a fluorescence microscope (BZ9000; Keyence Co., Osaka, Japan).

### *Statistical analysis*

All values are expressed as the mean  $\pm$  SD. Comparisons of results between experimental groups and control groups were analyzed with Tukey's HSD (Honestly Significant Difference) test. Statistical analyses were performed using the SPSS version 22.0.0 software package. If the *P* -value was  $<0.05$ , the result obtained was considered to be significant.

## **Results**

### *Growth factors included in MSC-CM*

In MSC-CM, the concentrations of IGF-1, VEGF, TGF- $\beta$ 1, and HGF were  $1515.6 \pm 211.8$  pg/mL,  $465.8 \pm 108.8$  pg/mL,  $339.8 \pm 14.4$  pg/mL, and  $20.3 \pm 7.9$  pg/mL, respectively. No other factors were detected in MSC-CM, DMEM (–), or 30% FBS (Table 2).

### *Effects of MSC-CM on rMSC and rPDLC migration and proliferation*

The percentage of rMSCs in the wound area of DMEM (–) was  $9.28 \pm 4.41\%$ . There were  $70.9 \pm 6.8\%$  rMSCs in the wound area of positive control (30% FBS). MSC-CM exerted significant effects ( $p < 0.01$ ) and closed the wound to  $43.4 \pm 10.6\%$  rMSCs

(Figure 1).

The percentage of rPDLCs in the wound area of DMEM (–) was  $2.36 \pm 2.32\%$ , with  $48.01 \pm 6.28\%$  in 30% FBS and  $17.98 \pm 4.14\%$  in MSC-CM. Thus, MSC-CM increased rMSC migration more than four-fold and rPDLC migration more than seven-fold compared with that in DMEM (–). These differences were statistically significant ( $p < 0.01$ ), indicating that MSC-CM enhanced rMSC and rPDLC migration and proliferation (Figure 1).

#### *Effects of MSC-CM on tube formation of HUVECs*

In the presence of BM and BM with anti-VEGF, HUVECs did not demonstrate tube formation. In contrast, BM with MSC-CM or VEGF stimulated tube formation (Figure 2A). The tube lengths were  $18341.59 \pm 3453.14$  pixels,  $20987.50 \pm 2053.97$  pixels,  $11244.35 \pm 1662.13$  pixels, and  $11542.33 \pm 1869.95$  pixels in BM with MSC-CM, in BM with VEGF, in BM with MSC-CM and anti-VEGF, and in BM only, respectively (Figure 2B). The number of joints were  $72.36 \pm 20.72$ ,  $81.81 \pm 15.86$ ,  $42.66 \pm 15.27$ , and  $38.06 \pm 12.42$ , respectively (Figure 2C). The tube lengths and the number of joints were significantly greater in BM with MSC-CM than in BM with MSC-CM plus anti-VEGF and in BM only.

#### *MSC-CM enhanced osteogenic and angiogenic marker gene expression*

The levels of expression of the *ALP*, *OCN*, *Runx2*, *VEGF-A*, *ANG-1*, and *ANG-2* genes were significantly upregulated in rMSCs cultured with MSC-CM compared with rMSCs



cultured in EM (Figure 3).

#### *MSC-CM induced periodontal tissue regeneration*

Two weeks after MSC-CM implantation into the periodontal tissue defect, a small amount of bone regeneration from the residual bone was evident, and a cell mass had accumulated adjacent to the regenerated bone (Figure 4A, 4B). In contrast, the PBS and Defect groups did not show bone or periodontal tissue regeneration (Figure 4C–4F). In addition, particularly in the Defect group, inflammatory cell infiltration was seen in the periodontal defect site.

Four weeks after MSC-CM implantation into the periodontal tissue defect, the regenerated bone and other periodontal tissues were observed in the defect (Figure 5A, 5B). A cementum-like structure was noted on the superficial surface of the dentin, and the regenerated bone exhibited alveolar crista. Moreover, a periodontal-ligament-like structure was seen between the cementum and the regenerated bone. In contrast, the PBS and Defect groups did not have any periodontal tissue, except for regeneration of a small amount of the alveolar bone (Figure 5C–5F).

#### *Immunohistochemical analysis for CD31, CD105, and FLK-1 expression in periodontal tissue defects*

In the MSC-CM group, numerous CD31-, CD105-, or FLK-1-positive cells occurred particularly frequently on the periphery of PDL, surface of the alveolar bone, and dental root. In contrast, there were fewer CD31-, CD105-, or FLK-1-positive cells in both the

381    PBS and Defect groups (Figure 6).

## Discussion

The results from this study suggested that several cytokines and chemokines present in MSC-CM promote periodontal tissue regeneration through various processes, such as mobilization of endogenous MSCs, angiogenesis, and differentiation.

Recently, attempts have begun to establish treatments to accelerate periodontal tissue regeneration by local application of human recombinant cytokines to stimulate proliferation and differentiation into hard tissue-forming cells, such as cementoblasts and osteoblasts (31) from endogenous MSCs, as well as promote periodontal tissue regeneration. Direct local application of combination of factors, such as PDGF and IGF-I (7), BMP-2 (8), TGF- $\beta$  (9), osteogenic protein (OP)-1 (10), and brain-derived neurotrophic factor (BDNF) (11), stimulates and promotes regional periodontal tissue regeneration *in vivo*. In addition, the clinical trials of FGF-2 and PDGF-BB for periodontal tissue regeneration in human have been reported (12). However, application of a single growth factor has limited tissue regeneration ability, and the amount of tissue that is regenerated cannot be predicted (32). In addition, application of a single growth factor such as BMP-2 requires superphysiological doses (33) and may induce a severe inflammatory response (34). Therefore, a combination of several different factors will likely be better for optimizing bone regeneration (35).

The present results of the wound-healing assay show that MSC-CM enhanced migration and proliferation of rMSCs and rPDLCs. In addition, to confirm that endogenous MSCs migrated to the site where MSC-CM was implanted, we performed immunohistochemical staining of the periodontal defects of rats with anti-CD105 antibodies after a 2-week implantation. A large number of CD105-positive cells,

reported to be specific markers of MSCs (36), existed in periodontal defects compared with the PBS and Defect groups. These results indicate that MSC-CM has the potential to mobilize endogenous MSCs and to promote periodontal tissue regeneration. In our previous study (24), the injected MSCs from the rat caudal vein migrated into the calvarial bone defect where MSC-CM was implanted. Similarly, in the results of immunohistochemical analysis for CD44 expression as markers of MSCs (36) in the bone defects of rats, numerous CD44-positive cells and tubular formations were detected in the calvarial bone defects of the MSC-CM group compared with the PBS and Defect groups, indicating that the migration of endogenous MSCs and angiogenesis were induced by MSC-CM (24). IGF-1 induces osteoblast proliferation and migration (37, 38) and enhances periodontal regeneration by stimulating PDL cells through the PI3K pathway (39).

The results of real-time RT-PCR in this study and the levels of expression of osteogenic marker genes, *ALP*, *OCN*, and *Runx2*, were significantly upregulated in rMSCs cultured with MSC-CM compared with rMSCs cultured in EM. This indicates that MSC-CM promotes osteoblastic differentiation of rMSCs. From histological findings of this study, MSC-CM had dramatic effects on periodontal tissue regeneration. In the MSC-CM groups after a 2-week implantation, bone regeneration from the residual bone was evident, but the other groups showed no bone regeneration. Four weeks after MSC-CM implantation, the regenerated bone exhibited alveolar crista. From these finding, it was obvious that MSC-CM promoted stem cell differentiation into the osteoblastic lineage after endogenous cell mobilization and bone regeneration in the periodontal tissue defect had occurred.

VEGF is the main regulator of angiogenesis as well as contributes to osteogenesis (40).

TGF- $\beta$ 1 increases bone formation by recruiting osteoprogenitor cells and stimulating their proliferation and differentiation into osteocytes (41).

Compared with the results from a previous study using the same rat model with MSC implantation, the histological results from this study at 4 weeks after implantation were equivalent to those from the other study at 8 weeks after implantation (14). In the MSC-CM groups, a regenerated bone and other periodontal tissues such as a cementum-like structure and a periodontal-ligament-like structure were observed after a 4-week implantation. Conversely, these were observed after a 4-week MSC implantation.

To regenerate periodontal tissue destroyed by periodontitis, several events including angiogenesis, cell migration, and proliferation and differentiation of various cell types, particularly osteoblasts and cementoblasts will be required. This process is complicated by the fact that periodontal wound healing occurs on the nonvascular and nonvital hard tissues of the root surface. Thus, periodontal tissue regeneration critically relies on the re-establishment and proper function of the damaged vascular system.

In the present study, to confirm the effects of MSC-CM on angiogenesis *in vitro*, we performed the tube formation assay, which showed that MSC-CM strongly promotes angiogenesis. From the results of the real-time RT-PCR in this study, the levels of expression of angiogenic marker genes, *VEGF-A*, *ANG-1*, and *ANG-2*, were significantly upregulated in rMSCs cultured with MSC-CM compared with rMSCs cultured in EM. *ANG1* causes chemotaxis of endothelial cells and stimulates angiogenesis (42-43). *ANG2* also stimulates angiogenesis in the presence of VEGF (44). *In vivo*, in the MSC-CM groups after a 2-week implantation, immunohistochemical staining showed that many CD31- and FLK-1-positive cells existed in periodontal

defects compared with the PBS and Defect groups. The results that MSC-CM with anti-VEGF antibody did not promote tube formation of HUVECs and that more FLK-1-positive cells existed in the MSC-CM groups than in other groups in the immunohistochemical analysis indicate that VEGF exerts the effects of MSC-CM primarily on angiogenesis. VEGF is well known to enhance angiogenesis in tissue regeneration by promoting proliferation and migration of vascular endothelial cells (45). Other factors known to relate to angiogenesis, such as HGF and TGF- $\beta$ 1, were also detected in MSC-CM in the present study. HGF reportedly potentiates the angiogenic effect of VEGF by inducing its upregulation (46, 47). TGF- $\beta$ 1 plays an important role in the process whereby pericytes exert a stabilizing effect on newly formed vessels (43). Several recent studies have suggested that perhaps almost all MSCs are normally resident as pericytes before isolation and that MSCs express aspects of the pericyte phenotype (48, 49). In other reports, MSCs have also been shown to differentiate into SMA<sup>+</sup> pericytes after implantation into a hind-leg ischemia model (50). It is presumed, because of the results of the wound-healing assay in this study, that MSC-CM enhances the migration and proliferation of pericytes and contributes to angiogenesis. Thus, we predict that MSC-CM directly promotes angiogenesis by some cytokines as well as indirectly mediates endogenous MSCs, including pericytes, to participate in angiogenesis.

From the results of real-time RT-PCR in this study, the levels of expression of osteogenic marker genes, *ALP*, *OCN*, and *Runx2*, were significantly upregulated in rMSCs cultured with MSC-CM compared with rMSCs cultured in EM, indicating that MSC-CM promoted the osteoblastic differentiation of rMSCs.

The human PDL contains a subpopulation of stem cells that are responsible for

maintaining and regenerating periodontal tissue structure and function (31). These cells exhibit multipotency, as demonstrated by their ability to differentiate into osteoblasts, fibroblasts, and cementoblasts and form cementum- and PDL-like tissues. In addition, progenitor cells responsible for alveolar bone formation lie in PDL or around the blood vessels. From the present results of immunohistochemical analysis in periodontal tissue defects after a 2-week implantation, in the MSC-CM group, numerous CD31-, CD105-, or FLK-1-positive cells occurred particularly frequently on the periphery of PDL, surface of the alveolar bone, and dental root. These results indicated that MSC-CM effectively promoted periodontal tissue regeneration because it contributed to the angiogenesis, mobilization, and differentiation into cementoblasts and osteoblasts of endogenous MSCs, leading to the regenerated alveolar bone, cementum tissue, and PDL.

From the results of this study, it was suggested that MSC-CM contributes to upregulation of several processes of periodontal tissue regeneration through the angiogenesis and mobilization of endogenous MSCs, and thus enhanced periodontal regeneration. Using MSC-CM for periodontal tissue regeneration may be effective because several cytokines, including MSC-CM, contribute several processes to the complex system of periodontal tissue regeneration.

In this study, we also used hMSCs and their cultured conditioned media for periodontal tissue regeneration because our aim was to apply MSC-CM to human patients and we investigated MSC-CM for drug discovery as a preclinical trial. We previously reported that MSC-CM promoted bone regeneration in a rat calvarial bone defect model (24, 25). In these studies, it was suggested that MSC-CM suppress T-lymphocyte proliferation and MSC-CM is useful for xenogeneic transplantation (24). Additionally, there was less

infiltration of inflammatory cells in the MSC-CM group compared with the other group without immunosuppressive drug in histological analysis (25). As a result, transplantation of MSC-CM to rats didn't enhance the immune response but contributed periodontal tissue regeneration in this study. If MSC-CM treatment protocol is to be established for periodontal regeneration, it is essential that effective and therapeutic doses of MSC-CM as well as the safety of the therapy should be carefully established. Further investigation regarding these matters including transplantation to large animals is now in progress.

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## ***Declaration of interest:***

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.



## References

1. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet*. 2005;366:1809-20.
2. Kikuchi T, Mitani A, Noguchi T. The mechanism of bone resorption in chronic inflammation of periodontal disease. *Clin Calcium*. 2006;16:241-47.
3. Gantes B, Martin M, Garrett S, Egelberg. Treatment of periodontal furcation defects. (II). Bone regeneration in mandibular class II defects. *J Clin Periodontol*. 1988;15:232-9.
4. Villar CC, Cochran DL. Regeneration of periodontal tissues: guided tissue regeneration. *Dent Clin North Am*. 2010;54:73-92.
5. Anitua E, Troya M, Orive G. An autologous platelet-rich plasma stimulates periodontal ligament regeneration. *J Periodontol*. 2013;84:1556-66.
6. Esposito M, Grusovin MG, Papanikolaou N, Coulthard P, Worthington HV. Enamel matrix derivative (Emdogain (R)) for periodontal tissue regeneration in intrabony defects. *Cochrane Database Syst Rev*. 2009:69.
7. Lynch SE, Williams RC, Polson AM, Howell TH, Reddy MS, Zappa UE, et al. A combination of platelet-derived and insulin-like growth factors enhances periodontal regeneration. *J Clin Periodontol*. 1989;16:545-8.
8. Kinoshita A, Oda S, Takahashi K, Yokota S, Ishikawa I. Periodontal regeneration by application of recombinant human bone morphogenetic protein-2 to horizontal circumferential defects created by experimental periodontitis in beagle dogs. *J Periodontol*. 1997;68:103-9.
9. Mohammed S, Pack ARC, Kardos TB. The effect of transforming growth factor

- 545        beta one (TGF-beta(1)) on wound healing, with or without barrier membranes, in a  
546        Class II furcation defect in sheep. J Periodontal Res. 1998;33:335-44.
- 547    10. Giannobile WV, Ryan S, Shih MS, Su DL, Kaplan PL, Chan TCK. Recombinant  
548        human osteogenic protein-1 (OP-1) stimulates periodontal wound healing in class  
549        III furcation defects. J Periodontol. 1998;69:129-37.
- 550    11. Takeda K, Shiba H, Mizuno N, Hasegawa N, Mouri Y, Hirachi A, et al.  
551        Brain-derived neurotrophic factor enhances periodontal tissue regeneration. Tissue  
552        Eng. 2005;11:1618-29.
- 553    12. Nevins M, Giannobile WV, McGuire MK, Kao RT, Mellonig JT, Hinrichs JE, et al.  
554        Platelet-derived growth factor stimulates bone fill and rate of attachment level gain:  
555        Results of a large multicenter randomized controlled trial. J Periodontol.  
556        2005;76:2205-15.
- 557    13. Tsumanuma Y, Iwata T, Washio K, Yoshida T, Yamada A, Takagi R, et al.  
558        Comparison of different tissue-derived stem cell sheets for periodontal regeneration  
559        in a canine 1-wall defect model. Biomaterials. 2011;32:5819-25.
- 560    14. Tobita M, Uysal AC, Ogawa R, Hyakusoku H, Mizuno H. Periodontal tissue  
561        regeneration with adipose-derived stem cells. Tissue Eng Part A. 2008;14:945-53.
- 562    15. Yamada Y, Ueda M, Naiki T, Takahashi M, Hata KI, Nagasaka T. Autogenous  
563        injectable bone for regeneration with mesenchymal stem cells and platelet-rich  
564        plasma: Tissue-engineered bone regeneration. Tissue Eng. 2004;10:955-64.
- 565    16. Nakamura Y, Wang XH, Xu CS, Asakura A, Yoshiyama M, From AHL, et al.  
566        Xenotransplantation of long-term-cultured swine bone marrow-derived  
567        mesenchymal stem cells. Stem Cells. 2007;25:612-20.
- 568    17. Ide C, Nakai Y, Nakano N, Seo TB, Yamada Y, Endo K, et al. Bone marrow

stromal cell transplantation for treatment of sub-acute spinal cord injury in the rat.  
Brain Res. 2010;1332:32-47.

18. Barcelos LS, Duplaa C, Krankel N, Graiani G, Invernici G, Katare R, et al. Human  
CD133(+) Progenitor Cells Promote the Healing of Diabetic Ischemic Ulcers by  
Paracrine Stimulation of Angiogenesis and Activation of Wnt Signaling. Circ Res.  
2009;104:1095-U199.

19. Cai LY, Johnstone BH, Cook TG, Tan J, Fishbein MC, Chen PS, et al. IFATS  
Collection: Human Adipose Tissue-Derived Stem Cells Induce Angiogenesis and  
Nerve Sprouting Following Myocardial Infarction, in Conjunction with Potent  
Preservation of Cardiac Function. Stem Cells. 2009;27:230-7.

20. Perin EC, Silva GV. Autologous Cell-Based Therapy for Ischemic Heart Disease:  
Clinical Evidence, Proposed Mechanisms of Action, and Current Limitations.  
Catheter Cardiovasc Interv. 2009;73:281-8.

21. Yoon BS, Moon JH, Jun EK, Kim J, Maeng I, Kim JS, et al. Secretory Profiles and  
Wound Healing Effects of Human Amniotic Fluid-Derived Mesenchymal Stem  
Cells. Stem Cells Dev. 2010;19:887-902.

22. Di Santo S, Yang ZJ, von Ballmoos MW, Voelzmann J, Diehm N, Baumgartner I, et  
al. Novel Cell-Free Strategy for Therapeutic Angiogenesis: In Vitro Generated  
Conditioned Medium Can Replace Progenitor Cell Transplantation. PLoS One.  
2009;4:11.

23. Wislet-Gendebien S, Poulet C, Neirinckx V, Hennuy B, Swingland JT, Laudet E, et  
al. In Vivo Tumorigenesis Was Observed after Injection of In Vitro Expanded  
Neural Crest Stem Cells Isolated from Adult Bone Marrow. PLoS One. 2012;7:13.

24. Osugi M, Katagiri W, Yoshimi R, Inukai T, Hibi H, Ueda M. Conditioned Media

593 from Mesenchymal Stem Cells Enhanced Bone Regeneration in Rat Calvarial Bone  
594 Defects. *Tissue Eng Part A*. 2012;18:1479-89.

595 25. Katagiri W, Osugi M, Kawai T, Ueda M. Novel Cell-Free Regeneration of Bone  
596 Using Stem Cell-Derived Growth Factors. *Int J Oral Maxillofac Implants*.  
597 2013;28:1009-16.

598 26. Carmeliet P. Angiogenesis in health and disease. *Nat Med*. 2003;9:653-60.

599 27. Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ. Engraftment and  
600 migration of human bone marrow stromal cells implanted in the brains of albino rats  
601 - similarities to astrocyte grafts. *Proc Natl Acad Sci U S A*. 1998;95:3908-13.

602 28. Bishop ET, Bell GT, Bloor S, Broom IJ, Hendry NF, Wheatley DN. An in vitro  
603 model of angiogenesis: basic features. *Angiogenesis*. 1999;3:335-44.

604 29. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome*  
605 *Res*. 1996;6:986-94.

606 30. Kawamoto T. Use of a new adhesive film for the preparation of multi-purpose  
607 fresh-frozen sections from hard tissues, whole-animals, insects and plants. *Arch*  
608 *Histol Cytol*. 2003;66:123-43.

609 31. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahimi J, et al. Investigation  
610 of multipotent postnatal stem cells from human periodontal ligament. *Lancet*.  
611 2004;364:149-55.

612 32. Shirakata Y, Takeuchi N, Yoshimoto T, Taniyama K, Noguchi K. Effects of Enamel  
613 Matrix Derivative and Basic Fibroblast Growth Factor with beta-Tricalcium  
614 Phosphate on Periodontal Regeneration in One-Wall Intrabony Defects: An  
615 Experimental Study in Dogs. *Int J Periodontics Restorative Dent*. 2013;33:641-U99.

616 33. Kawasaki K, Aihara M, Honmo J, Sakurai S, Fujimaki Y, Sakamoto K, et al. Effects

of recombinant human bone morphogenetic protein-2 on differentiation of cells isolated from human bone, muscle, and skin. *Bone*. 1998;23:223-31.

34. Perri B, Cooper M, Lauryssen C, Anand N. Adverse swelling associated with use of rh-BMP-2 in anterior cervical discectomy and fusion: a case study. *Spine J*. 2007;7:235-9.

35. Patel ZS, Young S, Tabata Y, Jansen JA, Wong MEK, Mikos AG. Dual delivery of an angiogenic and an osteogenic growth factor for bone regeneration in a critical size defect model. *Bone*. 2008;43:931-40.

36. Javazon EH, Beggs KJ, Flake AW. Mesenchymal stem cells: Paradoxes of passaging. *Experimental Hematology*. 2004;32:414-25.

37. Spencer EM, Liu CC, Si ECC, Howard GA. In vivo actions of insulin-like growth factor-I (IGF-I) on bone formation and resorption in rats. *Bone*. 1991;12:21-6.

38. Li Y, Yu X, Lin S, Li X, Zhang S, Song YH. Insulin-like growth factor 1 enhances the migratory capacity of mesenchymal stem cells. *Biochem Biophys Res Commun*. 2007;356:780-4.

39. Han XZ, Amar S. Role of insulin-like growth factor-1 signaling in dental fibroblast apoptosis. *J Periodontol*. 2003;74:1176-82.

40. Kaigler D, Krebsbach PH, West ER, Horger K, Huang YC, Mooney DJ. Endothelial cell modulation of bone marrow stromal cell osteogenic potential. *FASEB J*. 2005;19:665-+.

41. Janssens K, ten Dijke P, Janssens S, Van Hul W. Transforming growth factor-beta 1 to the bone. *Endocr Rev*. 2005;26:743-74.

42. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, et al. Isolation of Angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning.

641 Cell. 1996;87:1161-9.

642 43. Witzenbichler B, Maisonpierre PC, Jones P, Yancopoulos GD, Isner JM.  
643 Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific  
644 receptor tyrosine kinase Tie2. J Biol Chem. 1998;273:18514-21.

645 44. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand S, Radziejewski C, et al.  
646 Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis.  
647 Science. 1997;277:55-60.

648 45. Karamysheva AF. Mechanisms of angiogenesis. Biochemistry (Mosc).  
649 2008;73:751-62.

650 46. Van Belle E, Witzenbichler B, Chen DH, Silver M, Chang L, Schwall R, et al.  
651 Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via  
652 induction of vascular endothelial growth factor - The case for paracrine  
653 amplification of angiogenesis. Circulation. 1998;97:381-90.

654 47. Xin XH, Yang SY, Ingle G, Zlot C, Rangell L, Kowalski J, et al. Hepatocyte growth  
655 factor enhances vascular endothelial growth factor-induced angiogenesis in vitro  
656 and in vivo. Am J Pathol. 2001;158:1111-20.

657 48. Corselli M, Chen CW, Crisan M, Lazzari L, Peault B. Perivascular Ancestors of  
658 Adult Multipotent Stem Cells. Arterioscler Thromb Vasc Biol. 2010;30:1104-9.

659 49. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, et al. A perivascular  
660 origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell.  
661 2008;3:301-13.

662 50. Al-Khaldi A, Al-Sabti H, Galipeau J, Lachapelle K. Therapeutic angiogenesis using  
663 autologous bone marrow stromal cells: Improved blood flow in a chronic limb  
664 ischemia model. Ann Thorac Surg. 2003;75:204-9.

## Figure Legends

Figure 1. MSC-CM promoted migration and proliferation of rMSCs and rPDLs in the wound-healing assay.

(A) Migration of rMSCs. Wounds were generated as described in “Materials and Methods” (scale bar: 500  $\mu$ m).

(B) The level of cellular fill within the wound area in response to MSC-CM was compared with the wound-fill response in the presence of 30% FBS or serum-free DMEM as control after 48 h. The migration of rMSCs and rPDLs cultured in MSC-CM was enhanced compared with rMSCs and rPDLs cultured in DMEM (-). ( $n = 5$  for each group) Data are presented as means  $\pm$ SD. \*\* $p < 0.01$ .

Figure 2. MSC-CM promoted tube formation of HUVECs.

(A) Tube formation of HUVECs was compared in BM, and in BM with MSC-CM, VEGF (10 ng/mL), and MSC-CM plus anti-VEGF (10  $\mu$ g/mL). After 11 days, developing new blood vessels were observed under a microscope and photographed. BM with MSC-CM or VEGF (10 ng/mL) stimulated tube formation (scale bar: 500  $\mu$ m).

(B) The total length of blood vessels was analyzed using angiogenesis-measuring software. There were statistically significant differences between the length of blood vessels in the MSC-CM group and that in the other groups. ( $n = 10$  for each group) Data are presented as means  $\pm$ SD. \* $p < 0.05$ ; \*\* $p < 0.01$ .

Figure 3. MSC-CM enhanced osteogenic and angiogenic marker gene expression.

The mRNA levels of (A) *Alkaline phosphatase (ALP)*, (B) *Osteocalcin (OCN)*, (C) *Runx2*, (D) *VEGF-A*, (E) *angiopoietin 1 (ANG-1)*, and (F) *angiopoietin 2 (ANG-2)* genes in rMSCs cultured in MSC-CM or DMEM-10% FBS (EM; Expansion medium) were assayed by real-time RT-PCR. Cells underwent lysis for extraction of total RNA on day 7 of culture in MSC-CM or EM, and equal amounts of total RNA (50 ng) were analyzed. The mRNA expression levels of *ALP*, *OCN*, *Runx2*, *VEGF-A*, *ANG-1*, and *ANG-2* were determined relative to the level of *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* mRNA in each sample and were quantified by calculation based on their standard curves as described in “Materials and Methods”. For quantitative comparison of the levels of gene expression of the different samples, we calculated the expression coefficient for each mRNA on the ordinate by dividing the absolute level of expression of each mRNA (*ALP*, *OCN*, *Runx2*, *VEGF-A*, *ANG-1*, and *ANG-2*) with the absolute level of expression of *GAPDH* mRNA in each sample. Each point represents the mean value calculated from five independent replicates in which the difference was <10%. An asterisk indicates a significant difference between the EM and MSC-CM groups for the indicated gene. Data are presented as means  $\pm$ SD;  $n = 5$ . \* $p < 0.05$ ; \*\* $p < 0.01$ .

Figure 4. Hematoxylin and eosin-stained histological cross-section of the periodontal tissue defects 2 weeks after implantation.



(A) The defects in the MSC-CM group show a small amount of alveolar bone regeneration (scale bar: 200  $\mu$ m). (B) High magnification of the defects in the MSC-CM group reveals that the columnar cells were found on the surface of the regenerated bone (scale bar: 50  $\mu$ m). (C, E) No alveolar bone regeneration was found in the PBS and Defect groups (scale bar: 200  $\mu$ m). (D) High magnification of the defects in the PBS group. (F) High magnification of the defects in the Defect group show the presence of inflammatory cellular infiltration (scale bar: 100  $\mu$ m). \*alveolar bone, \*\*dental root, \*\*\*regenerated bone

Figure 5. Hematoxylin and eosin-stained histological cross-section of the periodontal tissue defects 4 weeks after implantation.

(A) In the MSC-CM group, a cementum-like structure and the alveolar bone with alveolar crista had regenerated (scale bar: 200  $\mu$ m). (B) High magnification of the defects in the MSC-CM group showed a periodontal-ligament-like structure located perpendicularly between the cementum-like structure (arrow) and the alveolar bone (scale bar: 100  $\mu$ m). (C) Alveolar bone regeneration was observed at a lower level in the PBS group (scale bar: 200  $\mu$ m). (D) A periodontal ligament-like structure was not seen between the alveolar bone and the dentin surface in the PBS group (scale bar: 100  $\mu$ m). (E) Little bone regeneration in the Defect group. In addition, the volume of the gingival was relatively decreased (scale bar: 200  $\mu$ m). (F) In the Defect group, dense collagen fibers and granulation tissue occupied the space between the dentin surface and the alveolar bone (scale bar: 100 mm). \*alveolar bone, \*\*dental root, \*\*\*regenerated bone

737 Figure 6. Immunohistochemical analysis of periodontal tissue defects 2 weeks after  
738 implantation.

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740 Two weeks after implantation into the periodontal defects, tissue specimens were  
741 analyzed using immunohistostaining for: CD31 (RED), a marker for rat endothelial  
742 cells; CD105 (GREEN), a marker for rat stem cells; and FLK-1 (RED), a marker for  
743 VEGF-R2. Cell nuclei were labeled with DAPI (blue) (scale bar: 200  $\mu$ m). \*alveolar  
744 bone, \*\*dental root

Table1. Primer and Probe Sequences Used in the Real-Time Polymerase Chain Reaction

Gene		Sequence	Accession No.
<i>ALP</i>	(F)	GACAGTCATTGAATACAAAAC	NM_053356
	(R)	ACGGAATTCTTGGTTAGTA	
	(probe)	TAAGCCATCTCGCCTGCCAT	
<i>OCN</i>	(F)	GACTCTGAGTCTGACAAA	NM_013414
	(R)	AGTCCATTGTTGAGGTAG	
	(probe)	CGGAGTCTATTCACCACCTTACTG C	
<i>Runx2</i>	(F)	CCTCTTATCTGAGCCAGA	NM_053470
	(R)	GCAGTGTCATCATCTGAA	
	(probe)	CATCCATCCATTCCACCACGC	
<i>VEGF-A</i>	(F)	ATCCCGGTTTAAATCCTG	NM_031836
	(R)	GGAACATTTACACGTCTG	
	(probe)	CACTGTGAGCCTTGTTCAGAGC	
<i>ANG1</i>	(F)	GAAGGAGGAGAAAGAAAAC	NM_053546
	(R)	TCTGCTAAGTTGCTTCTC	
	(probe)	TGGTTACTCGTCAGACATTCATCA TCC	
<i>ANG2</i>	(F)	CTCTGTATGAGCACTTCTA	NM_134454
	(R)	GATGCTACTGATTTTGCC	
	(probe)	CGGCGAGGAGTCCAACTACA	
<i>GAPDH</i>	(F)	GTTCCAGTATGACTCTACC	NM_017008
	(R)	TCACCCCATTTGATGTTA	
	(probe)	TTCAACGGCACAGTCAAGGC	

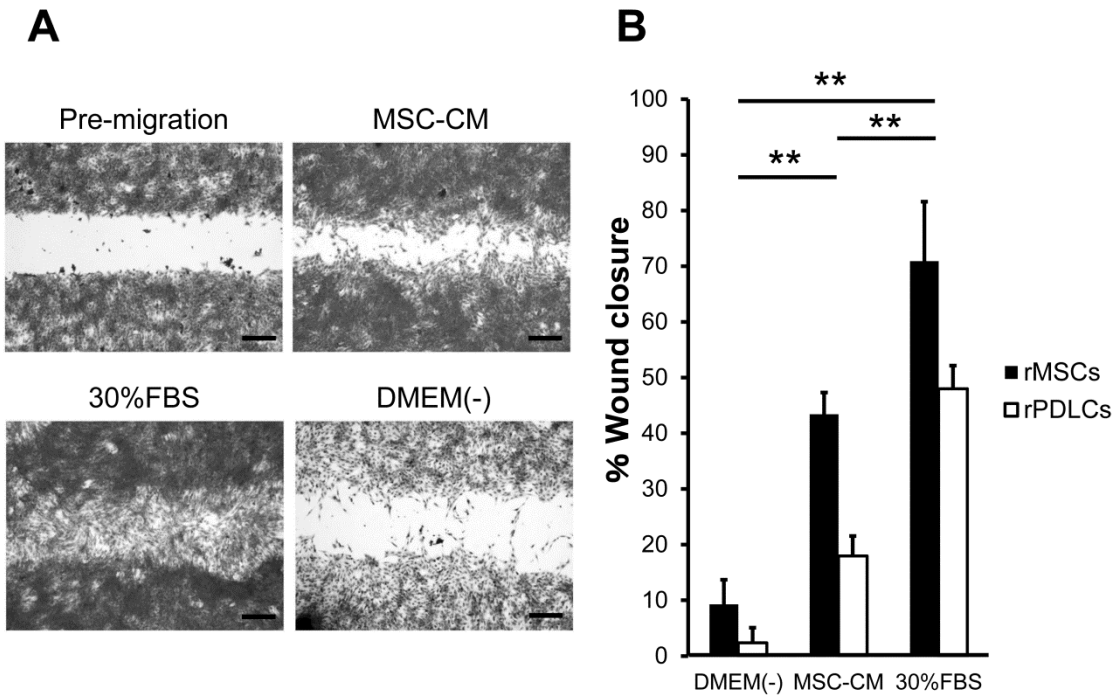
*ALP*, Alkaline phosphatase; *OCN* Osteocalcin; *ANG-1*, angiopoietin 1; *ANG-2*, angiopoietin 2.

Table2. The Levels of Cytokines Present in MSC-CM

Factors	Concentration (pg/mL)
IGF-1	1515.6±211.83
VEGF	465.84±108.81
TGF-β1	339.82±14.41
HGF	20.32±7.89
PDGF-BB	N.D
BMP-2	N.D
FGF-2	N.D
SDF-1	N.D

BMP-2, bone morphogenetic protein-2; IGF-1, insulin-like growth factor-1; VEGF, vascular endothelial growth factor; TGF-β1, transforming growth factor-β1; HGF, hepatocyte growth factor ; PDGF, plateletderived growth factor; FGF, fibroblast growth factor; SDF-1, stromalcell-derived factor-1; ND, not detected.

781 Fig1



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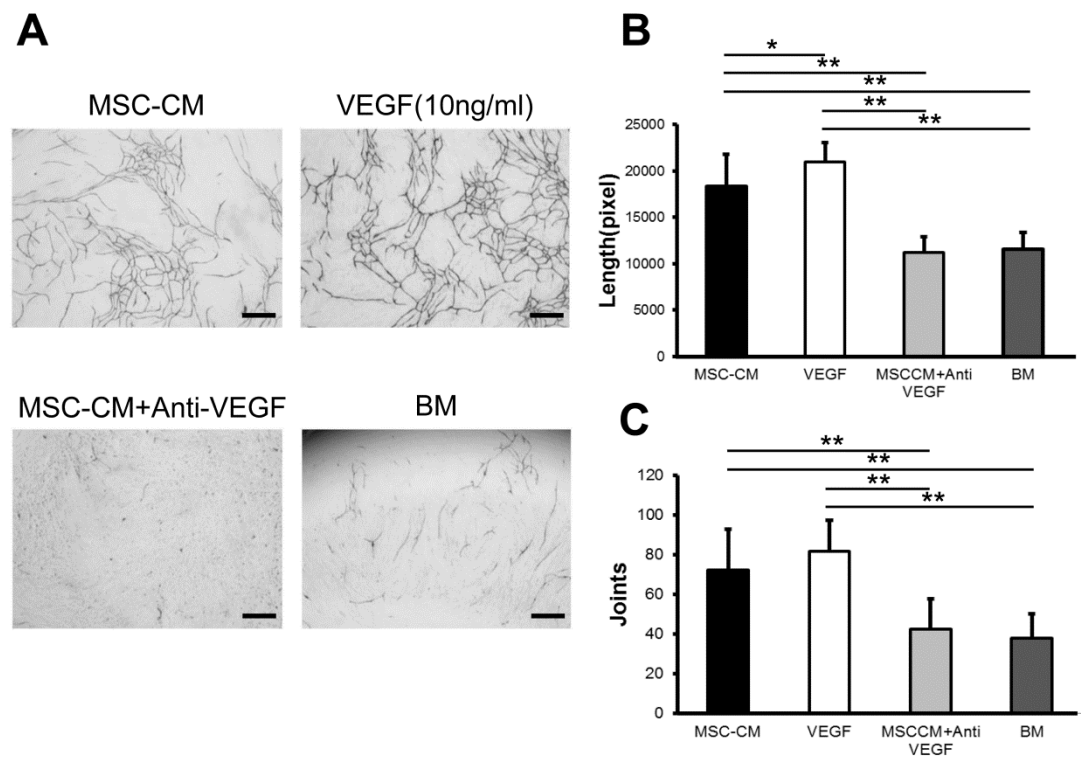
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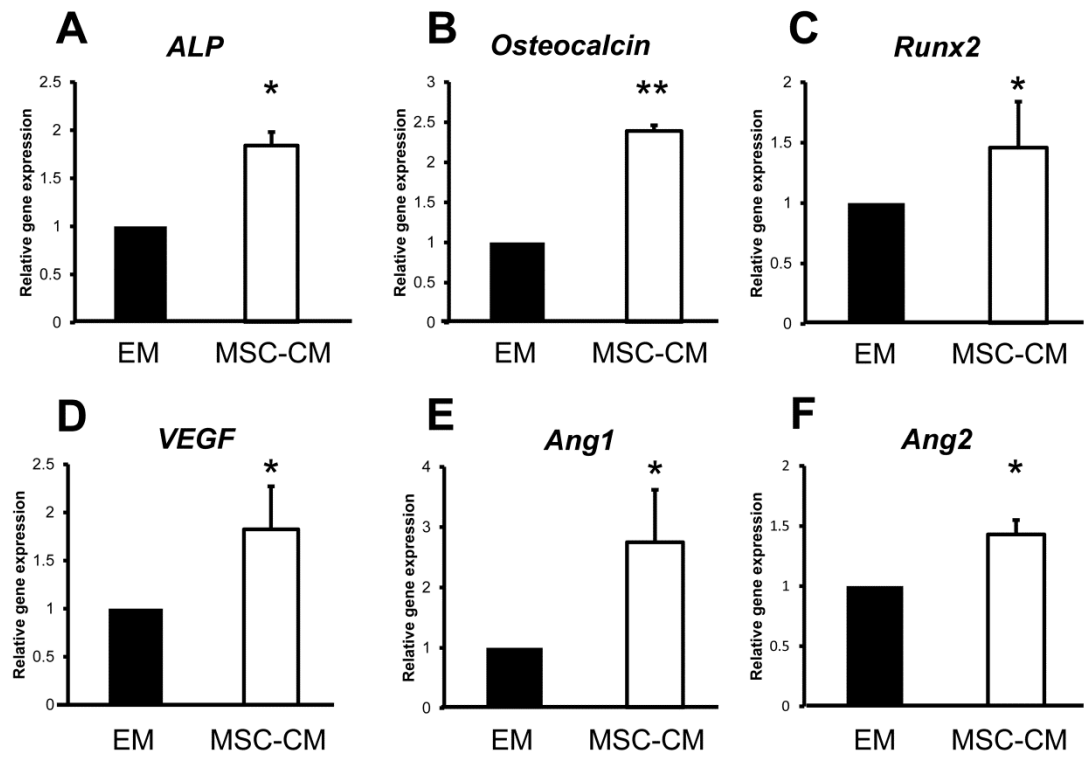
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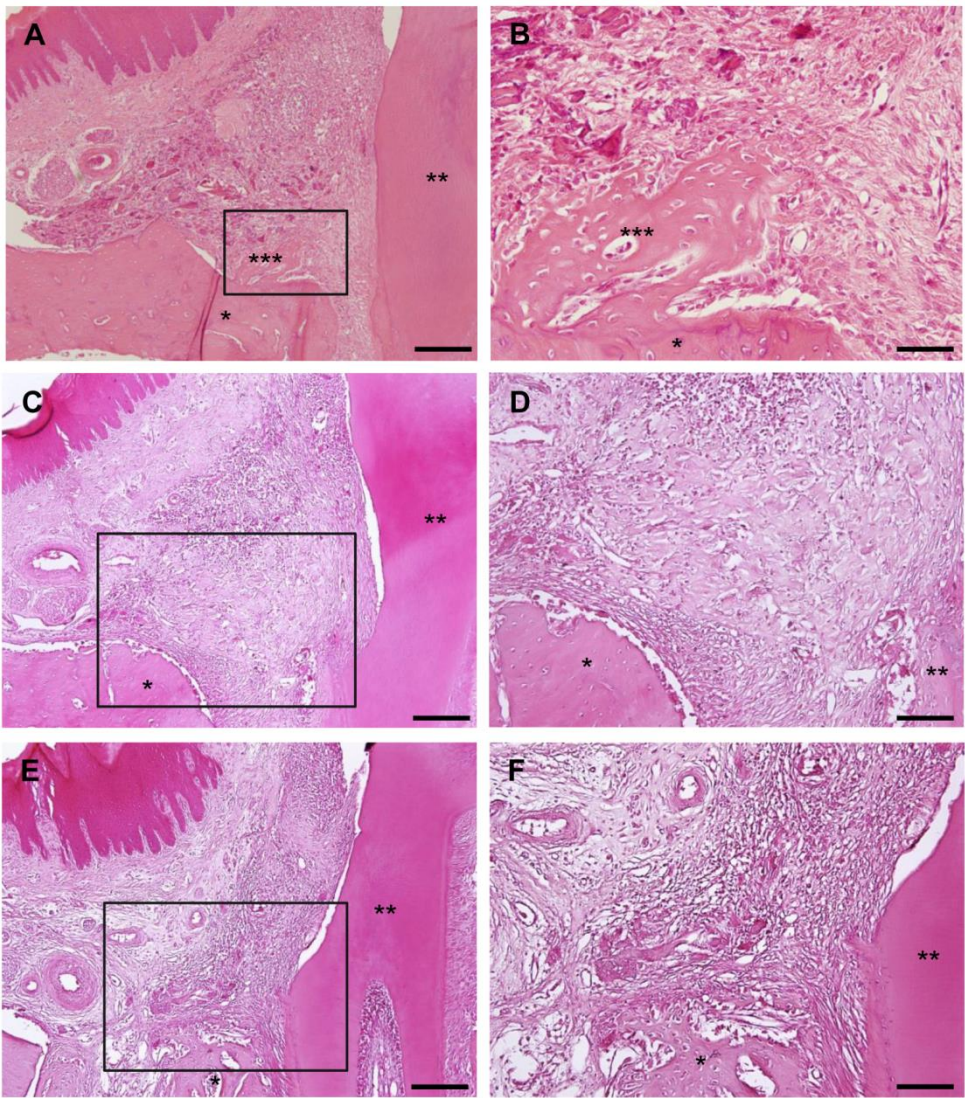
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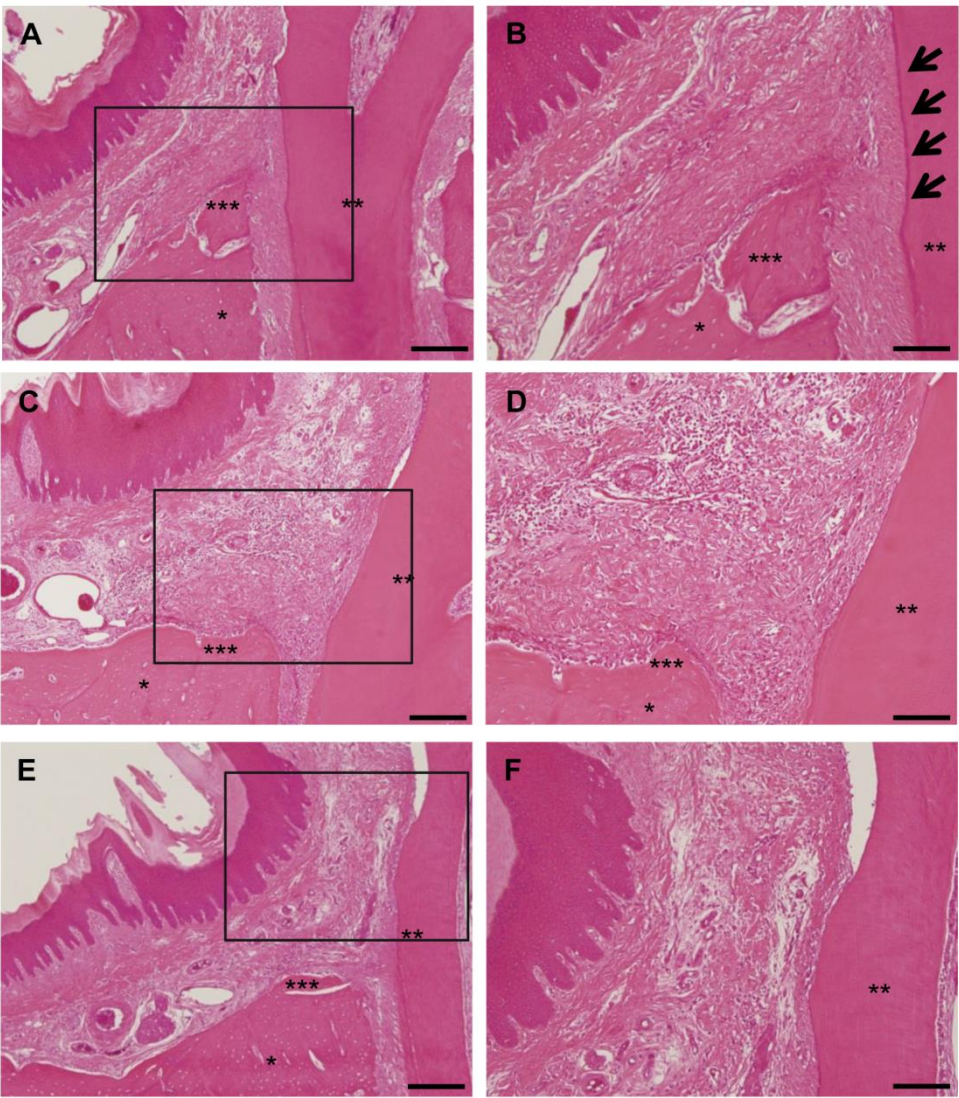
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829 Fig5



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