

# Osteogenic potential of cell spheroids composed of varying ratios of gingiva-derived and bone marrow stem cells using concave microwells

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**Abstract.** The aim of the current study was to evaluate cell viability and osteogenic differentiation potential in cell spheroids composed of varying ratios of gingiva-derived and bone marrow stem cells cultured in concave microwells. Cell spheroids were established from bone marrow and gingiva-derived stem cells in ratios of 6:0 (Group 1), 2:1 (Group 2), 3:3 (Group 3), 1:2 (Group 4), and 0:6 (Group 5). On days 3 and 5, the viability of the cell spheroids was qualitatively analyzed using a calcein acetoxymethyl ester working solution and an ethidium homodimer-1 live/dead assay. On days 1, 3, 5 and 7, a quantitative cell viability analysis was performed using a Cell Counting Kit-8. Alkaline phosphatase activity assays were performed using a commercially available kit on day 7 to assess osteogenic differentiation. In addition, reverse transcription-quantitative polymerase chain reaction and western blot analysis were performed to evaluate runt-related transcription factor 2 (Runx2) and osteocalcin expression. The ratio of gingiva-derived to bone marrow stem cells did not affect the stem cell spheroid morphology. No significant changes in cell viability were noted among the different groups following incubation for 7 days. A consistent alkaline phosphatase activity was measured in co-cultured gingiva-derived and bone marrow stem cell spheroids of varying compositions. Runx2 and osteocalcin expression was increased when co-cultured compared with pure gingiva-derived or bone marrow stem cells. In conclusion, stem cell spheroids established by co-culturing maintained morphology, viability and a high osteogenic differentiation potential during the experimental period of 7 days. These spheroids containing human gingiva-derived and bone marrow stem cells may enhance the osteogenic differentiation potential. The use of multicell

spheroids may be a simple and effective strategy for improving stem cell therapy.

## Introduction

Mesenchymal stem cells are stromal cells that can differentiate into bone, fat and cartilage cells (1). These stem cells can be found in various tissues, including bone marrow and fat (2). A marrow-derived mesenchymal stem cell sheet with platelet-rich plasma can promote bone regeneration (3). Bone marrow is an attractive source for stem cells, but accessing stem cells from bone marrow can lead to pain and complications, including bleeding and infection (4). In the dental field, stem cells can be obtained from a variety of tissues, including periodontal ligaments and dental pulp (5,6). Gingiva has been demonstrated to serve as a feasible source for stem cells and obtaining gingival-derived stem cells solely requires local anesthesia, which is associated with less pain compared with bone marrow extractions (7).

Extensive research has been conducted on the use of stem cells for recovery and regeneration of teeth and surrounding tissues (8-12). The use of a three-dimensional cell culture has important effects on mimicking the native environment on intercellular interactions that regulate self-renewal and differentiation of stem cells (13). Two-dimensional cultures may provide a stiffer environment compared with the natural environment and this may lead to changes in cell function (14). Stem cell spheroids obtained from three-dimensional cultures have demonstrated increased differentiation abilities (15,16). Cell spheroids have the ability to reflect the paracrine effects of producing growth factors and metabolites (17).

The current study was performed using concave microwells to evaluate viability and osteogenic differentiation potential of cell spheroids established using varying ratios of gingiva-derived and bone marrow stem cells. The efficacy of cell stem spheroids was investigated by comparing viability and osteogenic differentiation ability of three-dimensional cell cultures composed of gingiva-derived and bone marrow stem cells compared with spheroids containing cells of a single origin.

## Materials and methods

*Formation of cell spheroids using human gingiva-derived and bone marrow stem cells.* Silicone elastomer-based concave

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microwells (H389600; StemFIT 3D; MicroFIT, Seongnam, Korea) with 600  $\mu\text{m}$  diameter were used to prepare the stem cell spheroids. A total of  $1 \times 10^6$  cells at varying ratios were loaded into each well and cultured in osteogenic medium containing  $\alpha$ -minimal essential medium ( $\alpha$ -MEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) comprising 200 mM L-glutamine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 10 mM ascorbic acid 2-phosphate (Sigma-Aldrich; Merck KGaA), 100  $\mu\text{g}/\text{ml}$  streptomycin (Sigma-Aldrich; Merck KGaA), 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 2 mg/ml glycerophosphate disodium salt hydrate and 38  $\mu\text{g}/\text{ml}$  dexamethasone at 37°C in a humidified incubator with 5%  $\text{CO}_2$  and 95%  $\text{O}_2$  to evaluate cellular responses. The Catholic Institute of Cell Therapy (Seoul, Korea) provided bone marrow-derived mesenchymal stem cells (Catholic MASTER Cells). Isolation and propagation of bone marrow-derived mesenchymal stem cells were performed following previously reported method (18). In short, human bone marrow aspirates were obtained from the iliac crest of healthy male donors in their 40s in March 2012 after approval by the Institutional Review Board of Seoul St. Mary's Hospital was obtained. Bone marrow aspirate from each consented donor was collected and sent to the good manufacturing practice-compliant facility of Catholic Institute of Cell Therapy for the isolation, expansion and quality control. Human gingiva-derived stem cells were obtained as reported previously (19). Samples from a 91-year old female participant from Seoul St Mary's Hospital recruited in July 2013 were used in the current study. The gingival tissue was harvested during surgical procedure visiting Department of Periodontics, Seoul St Mary's Hospital. The participant was physically healthy without severe medical or psychological disease and written consent was obtained. The study was approved by the Institutional Review Board at Seoul St Mary's Hospital's College of Medicine and the Catholic University of Korea (KC17SESI0290; Seoul, Korea). All experiments were performed according to the Declaration of Helsinki.

Human bone marrow-derived mesenchymal and gingiva-derived stem cells were loaded at densities of  $1.2 \times 10^6$  cells/microwell and in ratios of 6:0 (Group 1), 2:1 (Group 2), 3:3 (Group 3), 1:2 (Group 4) and 0:6 (Group 5). Cells of each stock were added to the well and mixed in the well in case of Groups 2-4. The formation of spheroids and morphological changes were observed under an inverted microscope (Leica DM IRM; Leica Microsystems GmbH, Wetzlar, Germany).

**Cell viability.** Stem-cell spheroids were cultured in osteogenic medium at 37°C for 3-5 days.

A commercially available live/dead assay (LIVE/DEAD Cell Viability assays; Molecular Probes; Thermo Fisher Scientific, Inc.) for qualitative analysis of the stem cell spheroids was performed, following the manufacturer's instructions on days 3 and 5 of culturing. Spheroids were washed twice with growth medium and suspended in 1 ml  $\alpha$ -MEM, containing 2  $\mu\text{l}$  calcein acetoxyethyl ester working solution (50 mM) and 4  $\mu\text{l}$  ethidium homodimer-1 (2 mM) and cultured for 30 min at room temperature. In intact cells intracellular esterases produce green fluorescence by reacting with the nonfluorescent, cell-permeant calcein acetoxyethyl ester (20). The

ethidium homodimer can enter damaged cells and produce red fluorescence (21). Following staining, the spheroids were observed under a fluorescence microscope (magnification,  $\times 100$ ; Axiovert 200; Zeiss GmbH, Jena, Germany).

On days 1, 3, 5 and 7 of culturing, a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used for the quantitative analysis of cell viability according to manufacturer's instructions. Initially, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt was added and stem cell spheroids were cultured for 45 min at 37°C. The absorbance at 450 nm was measured using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

**Alkaline phosphatase activity assays.** Cell spheroids were obtained on day 7, following growth in culture plates with osteogenic media at 37°C. A commercially available kit (Alkaline Phosphatase Activity Colorimetric Assay kit; K412-500; BioVision, Inc., Milpitas, CA, USA) was used to evaluate alkaline phosphatase activity according to manufacturer's instructions. Cells ( $1.0 \times 10^4$ ) were suspended with assay buffer, sonicated and centrifuged at 13,000  $\times g$  for 3 min at 4°C to remove insoluble material. The supernatant was mixed with a p-nitrophenylphosphate substrate and incubated at 25°C for 40 min. The absorbance of the resultant p-nitrophenol was measured spectrophotometrically at 405 nm and the activity was calculated based on the absorbance.

**Total RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Samples were harvested on day 10 (12,000  $\times g$ ; 5 min; 4°C). A GeneJET RNA Purification kit (Thermo Fisher Scientific, Inc.) was used to isolate the total RNA according to the manufacturer's instructions. A total of 1 ng RNA was used as template for RT using the SuperiorScript II Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). Purity was determined spectrophotometrically (ND-2000; Thermo Fisher Scientific, Inc.) on day 10, with the ratios of absorbance measured at 260 and 280 nm.

qPCR was performed to detect mRNA expression using SYBR-Green Real-Time PCR Master mixes (Enzynomics Co., Ltd., Daejeon, Republic of Korea) according to the manufacturer's protocols. Primers were designed using GenBank (22). Primer sequences were as follows: Runt-related transcription factor 2 (Runx-2), forward 5'-AATGATGGTGTGACGCTGA-3' and reverse 5'-TTGATACGTGTGGGATGTGG-3'; osteocalcin, forward 5'-AGGGAGGTGTGTGAGCTCAA-3' and reverse 5'-CCGTAGAAGCGCCGATAGG-3'; and  $\beta$ -actin, forward 5'-TGGCACCCAGCACAATGAA-3' and reverse 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' following the following the manufacture's instructions. mRNA levels were normalized to  $\beta$ -actin and expressed as a fold change calculated using the  $2^{-\Delta\Delta C_q}$  method (23).

**Western blot analysis.** On day 7, samples were washed twice with ice-cold PBS and solubilized in a radioimmunoprecipitation lysis buffer (Thermo Fisher Scientific, Inc.) with protease inhibitors (PPI1015; Quartett Immunodiagnostika, Biotechnologie & Kosmetik Vertriebs GmbH, Berlin Germany) for 30 min at 4°C. Lysates were then centrifuged at 13,000  $\times g$  for 10 min at 4°C. SDS-PAGE (7% Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup>

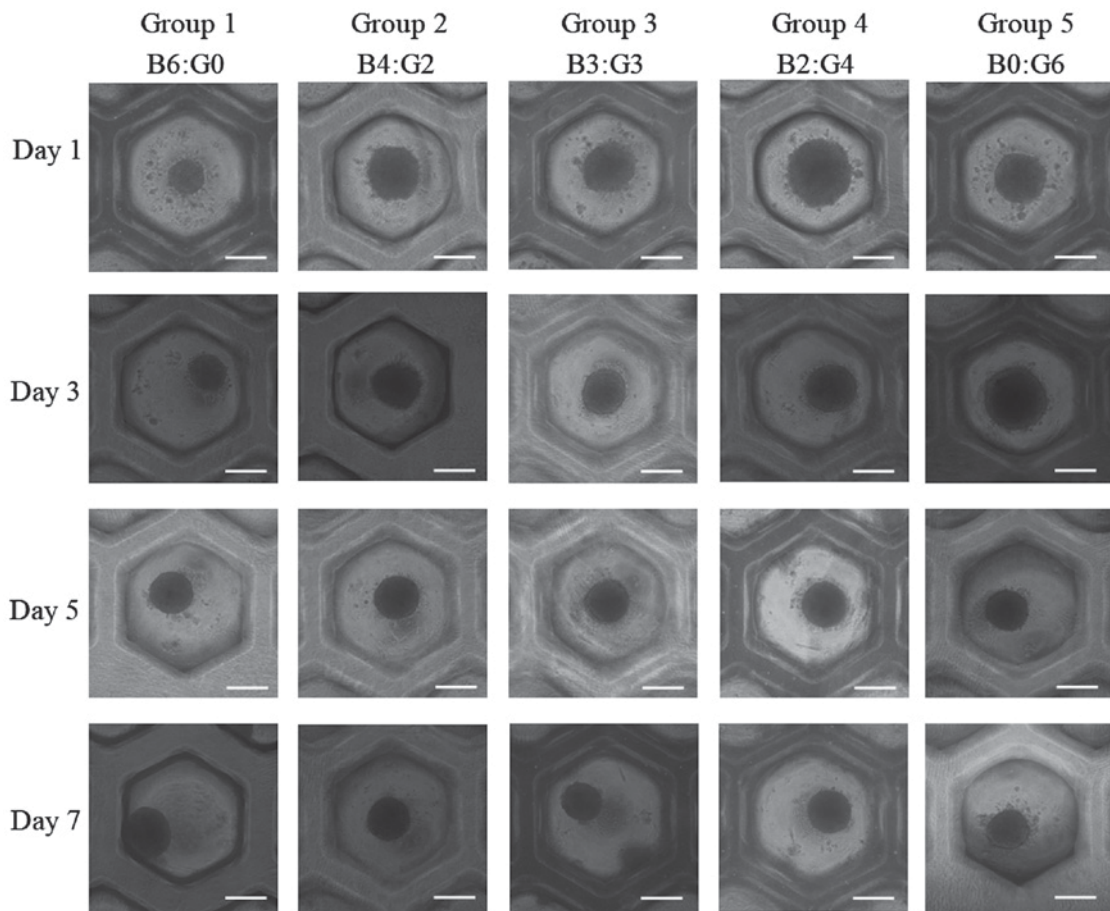


Figure 1. Morphology of gingiva-derived and bone marrow stem cells co-cultures. Cells were cultured in osteogenic media for 1, 3, 5 and 7 days (magnification, x100). Scale bar, 200  $\mu\text{m}$ . B, bone marrow stem cell; G, gingiva-derived stem cell.

Precast Gels; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to separate the samples (15  $\mu\text{g}$ ). Protein concentration was determined as previously reported (24). Samples were transferred to polyvinylidene difluoride membranes (IB24002, Immun-Blot<sup>®</sup>; Bio-Rad, Laboratories, Inc.) using a transfer apparatus (iBlot<sup>®</sup> 2 Transfer Stacks, Bio-Rad Laboratories, Inc.), prior to blocking with 5% skim milk at room temperature for 1 h, incubation with corresponding antibodies (primary antibodies, 2 h at 4°C; secondary antibodies, 2 h at room temperature) and enhanced chemiluminescent detection (Chemiluminescent HRP Substrate; Immobilon<sup>™</sup> Western; Merck KGaA). Primary antibodies against Runx2 (ab76956; Abcam, Cambridge, MA, USA), osteocalcin (ab13418; Abcam) and GAPDH (ab9485; Abcam) at 1:300 dilution were used in the analysis. Horseradish peroxidase-conjugated secondary antibodies, goat anti-mouse IgG F(ab')<sub>2</sub> (ADI-SAB-100-J; Enzo Life Sciences, Inc., Farmingdale, NY, USA) and goat anti-rabbit IgG (ADI-SAB-300-J; Enzo Life Sciences, Inc.) were incubated at 1:10,000 dilution for 2 h at room temperature. The quantitative analysis of the protein expression for Runx2, osteocalcin and GAPDH was conducted using image processing and analysis software (ImageJ v1.8.0\_112; National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** Data are represented as the mean  $\pm$  standard deviation (n=3). Using commercially available software (SPSS 12; SPSS Inc., Chicago, IL, USA), tests of normality,

one-way analysis of variance with a post hoc Tukey's tests and Kruskal-Wallis tests were performed to determine the differences among the groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*Stem cell spheroids maintain cell morphology and viability at varying gingiva to bone marrow cell ratios.* Stem cell spheroids were established in silicone elastomer-based concave microwells (Fig. 1). There were no differences in shape among the groups. Morphological results for days 3, 5 and 7 are presented in Fig. 1. The shape of the spheroids was similar over time and no noticeable changes were observed at longer incubation times.

Cell viability was determined using a fluorescence-based live/dead assay. The results are presented in Figs. 2 and 3. On day 3, a majority of the cells in the spheroids emitted green fluorescence, indicating live cells and a round shape was observed (Fig. 2) (25). An increase in red fluorescence was noted at longer incubation times, starting on day 5 (Fig. 3). CCK-8 assay results for days 1, 3, 5 and 7 are presented in Fig. 4. No significant differences in cell viability were noted among the five groups at the same time points. Decreases in cell viability were observed at longer incubation times.

*Spheroids with varying gingiva to bone marrow cell ratios exhibit similar alkaline phosphatase activities.* Alkaline

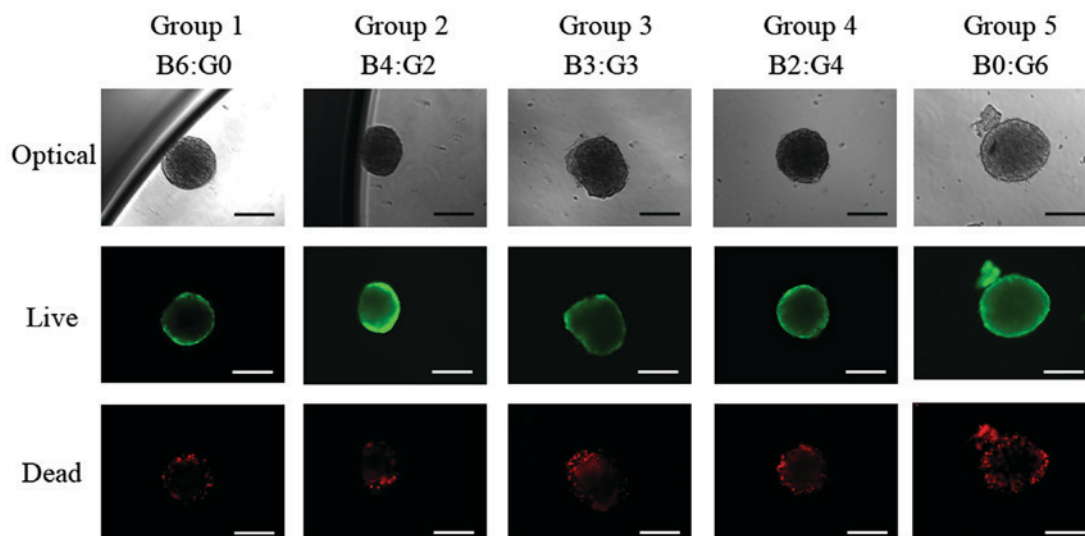


Figure 2. Qualitative analysis of cell viability on day 3. Optical microscope image and live and dead cell images were captured using a confocal microscope (magnification,  $\times 100$ ). Scale bar,  $200 \mu\text{m}$ . B, bone marrow stem cell; G, gingiva-derived stem cell.

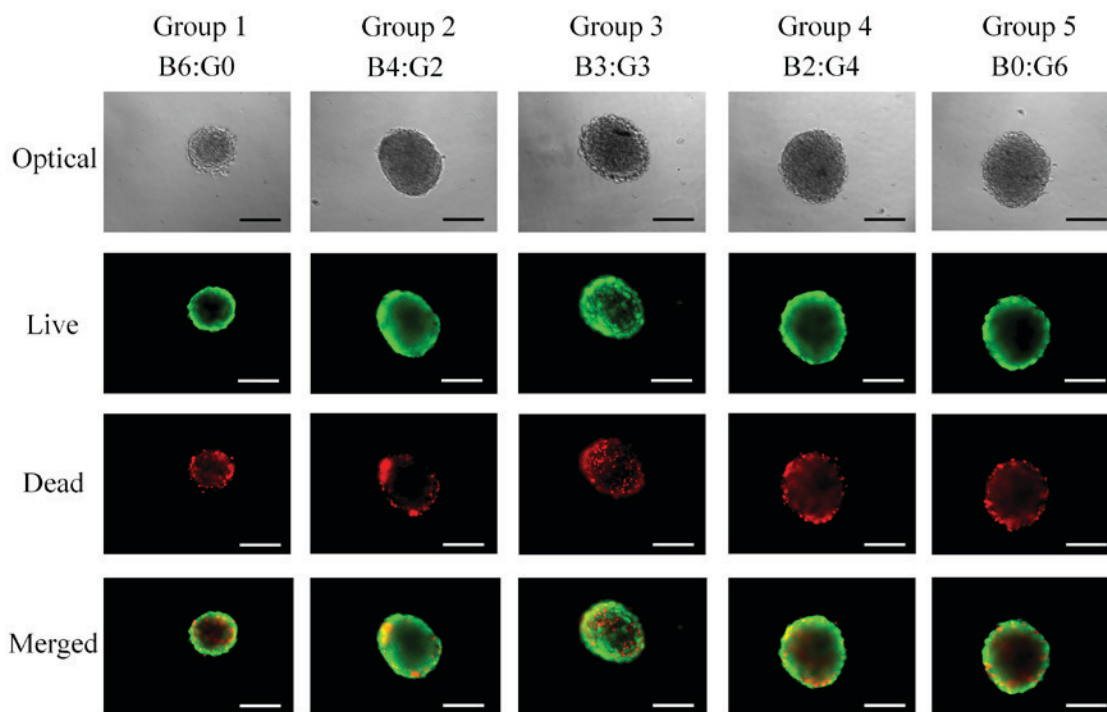


Figure 3. Qualitative analysis of cell viability on day 5. Optical microscope image and live and dead cell images were captured using a confocal microscope (magnification,  $\times 100$ ). Scale bar,  $200 \mu\text{m}$ . B, bone marrow stem cell; G, gingiva-derived stem cell.

phosphatase activities determined at day 7 of culturing are presented in Fig. 5. The determined relative values were  $100.0 \pm 9.0$ ,  $105.2 \pm 1.4$ ,  $105.5 \pm 1.5$ ,  $101.6 \pm 0.7$  and  $85.5 \pm 3.5\%$  for Groups 1, 2, 3, 4 and 5, respectively. A statistically significant difference between Groups 1-4 and Group 5 was determined ( $P < 0.05$ ).

*Increasing amounts of gingiva cells in spheroid formation decrease Runx2 and osteocalcin mRNA expression.* On day 10, mRNA expression levels of Runx2 and osteocalcin were determined using RT-qPCR (Fig. 6A and B, respectively).

Relative Runx2 expression for Groups 1, 2, 3, 4 and 5 were  $100.0 \pm 5.1$ ,  $55.3 \pm 3.9$ ,  $41.3 \pm 1.3$ ,  $34.0 \pm 1.3$  and  $37.8 \pm 1.3\%$ , respectively ( $P < 0.05$ ; Fig. 6A). Osteocalcin expression in Groups 2, 3, 4 and 5 was significantly decreased compared with Group 1 ( $P < 0.05$ ; Fig. 6B).

*Increasing amounts of gingiva cells in spheroid formation increase Runx2 and osteocalcin protein expression.* Western blot analysis was performed to detect Runx2, osteocalcin and GAPDH protein expression levels (Fig. 7). On day 7, Runx2 expression following normalization in Groups 1, 2, 3, 4 and

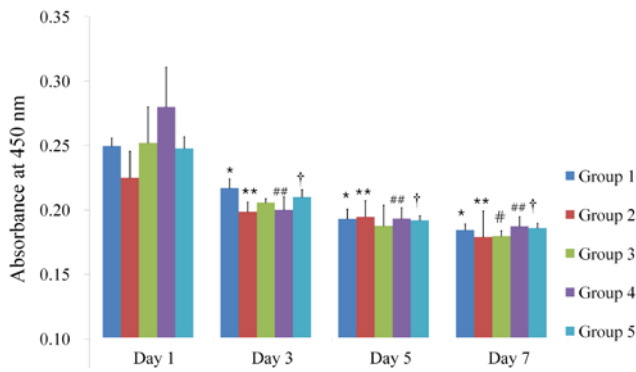


Figure 4. Cell viability of gingiva-derived and bone marrow stem cells co-cultures on day 1, 3, 5 and 7. Cell viability was determined using a Cell Counting Kit-8. \* $P < 0.05$  vs. Group 1 on day 1; \*\* $P < 0.05$  vs. Group 2 on day 1; # $P < 0.05$  vs. Group 3 on day 1; ## $P < 0.05$  vs. Group 4 on day 1; † $P < 0.05$  vs. Group 5 on day 1.

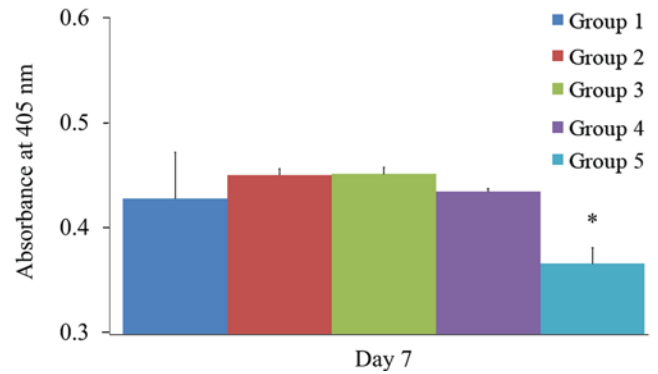


Figure 5. Alkaline phosphatase activity of gingiva-derived and bone marrow stem cells co-cultures on day 7. Relative values were 100.0 ± 9.0, 105.2 ± 1.4, 105.5 ± 1.5, 101.6 ± 0.7 and 85.5 ± 3.5% for Groups 1, 2, 3, 4 and 5, respectively. \* $P < 0.05$  vs. Group 1.

5 were 100.0 ± 9.5, 306.9 ± 14.5, 292.8 ± 17.3, 410.9 ± 32.9 and 371.1 ± 43.4% (Fig. 7B). No significant differences in Runx2 expression among the groups were determined. Osteocalcin protein expressions on day 7 following normalization for Groups 1, 2, 3, 4 and 5 were 100.0 ± 17.0, 144.6 ± 20.3, 142.9 ± 28.4, 245.1 ± 44.7 and 186.6 ± 22.8%, respectively (Fig. 7C). No significant differences in osteocalcin expression among the groups were observed (Fig. 7C).

## Discussion

The current study evaluated cell viability and osteogenic differentiation potential of cell spheroids composed of various ratios of gingiva-derived and bone marrow stem cells using concave microwells. It suggested that spheroids containing these cell types exhibit a high osteogenic differentiation potential.

In the present study, gingiva-derived and bone marrow stem cells were considered as part of a possible stem cell therapy involving allogeneic stem cell transplantation. Stem cells have the ability to self-renew and differentiate into various types of cells, including osteoblasts (26). Bone marrow-derived stem cells are regarded as a potential source for cell therapy (27). Human gingiva may serve as an alternative source for stem cells, as large numbers of functionally competent stem cells can be obtained from the gingiva in a short time, which may indicate an advantage for tissue engineering purposes (28). It has previously been indicated that gingiva-derived stem cell spheroids exhibited increased osteogenic potential compared with two-dimensional cultures, including increased level of alkaline phosphatase activity and mineralization (20). Stem cell spheroids in the current study were obtained through co-culturing of human gingiva-derived and bone marrow stem cells and results suggested an enhanced osteogenic differentiation potential. This cell delivery method may be a simple and effective strategy for improving stem cell therapy.

Co-culturing of these types of cells has been reported in previous research and was demonstrated to result in enhanced functionality (14,20). Co-culturing of stem cells with cell types exhibiting desired functionalities is an effective method for promoting the differentiation of stem

cells (29). Implantation of different types of cells into a test object has been widely applied in tissue engineering in recent years (30). The co-culture of endothelial progenitor cells and mesenchymal stem cells exhibited a synergistic effect in new-vessel formation and it is a potential osteogenic construct for *in vivo* applications (31). In a previous study, cell spheroids co-cultured from gingiva-derived stem cells and osteoprecursor cells maintained shape, viability, ability to self-renew and osteogenic differentiation potentials (20). A co-culture of adipose-derived stem cells and chondrocytes has been applied in regenerative therapy for treatment of cartilage defects (32).

Cross-talk between mesenchymal stem cells and endothelial progenitor cells occurs through direct cell contact and paracrine effects (33,34). The incubation of endothelial progenitor cells with mesenchymal stem cell supernatants resulted in significantly higher cell viability compared with the controls cultivated in endothelial cell medium (35). Additionally, endothelial progenitor cells stimulated mesenchymal stem cell proliferation and mesenchymal stem cells promoted endothelial progenitor cell survival (36).

Cell viability is considered when evaluating the toxicity of chemicals (20). Protein assays may provide inaccurate measurement of cell viability, as they determine the protein content of the viable cells, which were retained following the removal of dead cells (37). A trypan blue assay may be used to assess cell viability, as it stains dead cells and calculations are based on unstained cells (38). The [<sup>51</sup>Cr-uptake] assay is a sensitive and reliable method for quantifying cell viability and cell death, as it evaluates the ability of viable cells to take up isotope-labeled sodium chromate (39). Furthermore, DNA synthesis may be used for the evaluation of cell viability via tritiated-thymidine and bromodeoxyuridine analysis (40). In the current study, cell viability was evaluated using the CCK-8 assay. This assay is based on dehydrogenase activity and involves a majority of high-sensitivity dehydrogenases found in cells and no significant differences in cell viability were noted among the groups at the same time points (41).

Western blot analysis was performed to evaluate Runx2 and osteocalcin protein expression in each group consisting of varying ratios of gingiva-derived and bone marrow

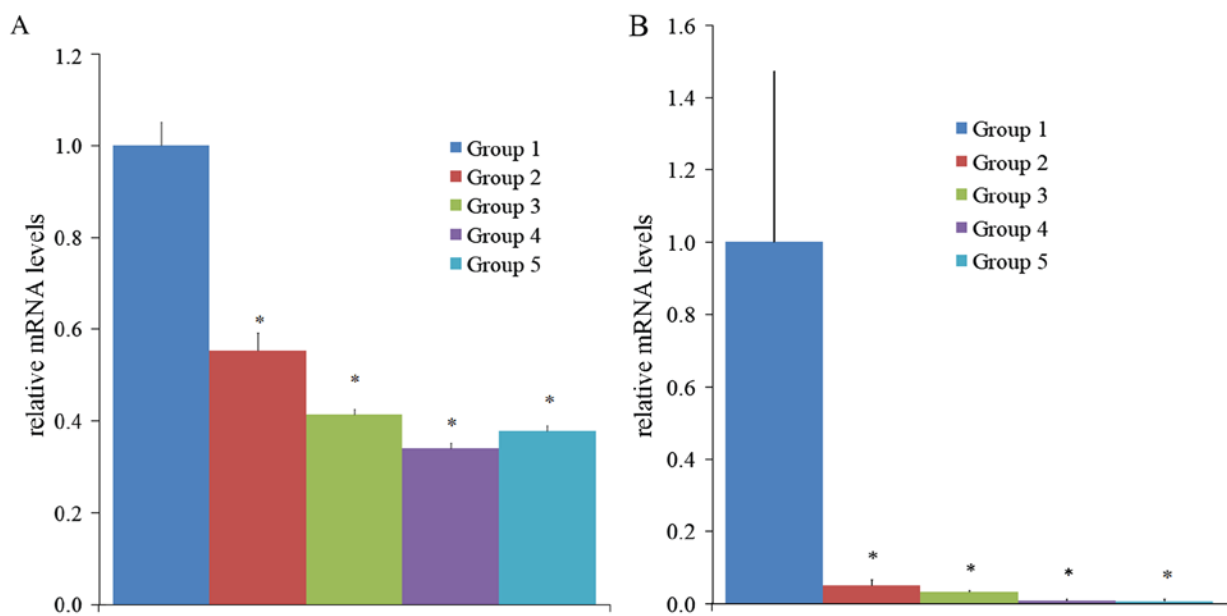


Figure 6. Runx2 and osteocalcin mRNA levels in gingiva-derived and bone marrow stem cells co-cultures determined by reverse transcription-quantitative polymerase chain reaction. Quantitative results for (A) Runx2 and (B) osteocalcin mRNA expression on day 10. \* $P < 0.05$  vs. Group 1.

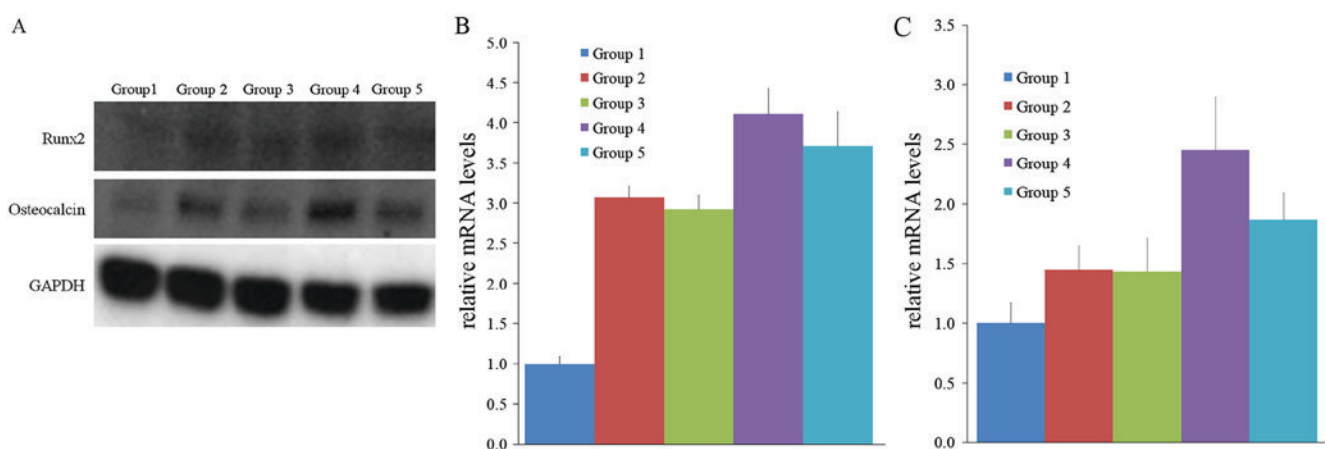


Figure 7. Runx2 and osteocalcin protein expression in gingiva-derived and bone marrow stem cells co-cultures on day 7. (A) Western blot for Runx2, osteocalcin and GAPDH expression on day 7. Quantitative analysis of (B) Runx2 and (C) osteocalcin protein expression normalized to GAPDH. Runx2, runt-related transcription factor 2.

stem cells and to gain insight into potential mechanisms of osteogenic differentiation. Runx2 is closely associated with the osteoblast phenotype evaluating the osteogenic potential of stem cells (42). Osteocalcin, a bone-specific protein produced by osteoblasts, is regarded as a maturation marker for osteogenesis (43). Additionally, osteocalcin has been suggested as an early marker for *in vitro* osteogenesis in stem cells (44). Co-culturing of gingiva-derived and bone marrow stem cells exhibited a high osteogenic differentiation potential when compared with gingiva-derived stem cell only group.

Stem-cell spheroids, which comprised various ratios of gingiva-derived and bone marrow stem cells, maintained morphology, viability and osteogenic differentiation potential during the experimental period. In conclusion, multicell spheroids may be a simple and effective strategy for improving stem cell therapy.

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## Availability of data and materials

All data generated or analyzed during the present study are included in the published article.

### Authors' contributions

JT, HyunaL, HyunJL, YK and JP collaborated to design the study, data access and analysis, performance of experiments and writing the manuscript. All authors reviewed the manuscript.

### Ethics approval and consent to participate

Ethics approval was obtained from the Institutional Review Board at Seoul St Mary's Hospital, College of Medicine and The Catholic University of Korea (KC17SESI0290). Written informed consent was obtained from all participants.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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