

Fibroblast growth factor-4 maintains cellular viability while enhancing osteogenic differentiation of stem cell spheroids in part by regulating RUNX2 and BGLAP expression

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Received March 27, 2019; Accepted April 29, 2020

DOI: 10.3892/etm.2020.8951

Abstract. Fibroblast growth factors (FGFs) are growth factors that were initially identified as proteins that stimulate fibroblast proliferation. The aim of the present study was to examine the effects of FGF-4 on the morphology, cellular viability and osteogenic differentiation of stem cell spheroids. Stem cell spheroids were generated using concave microwells in the presence of FGF-4 at concentrations of 0, 50, 100 and 200 ng/ml. Cellular viability was qualitatively assessed by a fluorometric live/dead assay using a microscope and quantitatively determined by using Cell Counting Kit-8. Furthermore, alkaline phosphatase activity and calcium deposition were determined to assess osteogenic differentiation. Reverse transcription-quantitative PCR (RT-qPCR) was performed to evaluate the mRNA expression levels of Runt-related transcription factor 2 (RUNX2) and bone γ -carboxyglutamate protein (BGLAP). Spheroidal shapes were achieved in the microwells on day 1 and a significant increase in the spheroid diameter was observed in the 200 ng/ml FGF-4 group compared with the control group on day 1 ($P < 0.05$). The results regarding viability using Cell Counting Kit-8 in the presence of FGF-4 at 50, 100 and 200 ng/ml at day 1 were 98.0 ± 2.5 , 106.2 ± 17.6 and $99.5 \pm 6.0\%$, respectively, when normalized to the control group ($P > 0.05$). Furthermore, the alkaline phosphatase activity was significantly elevated in the 200 ng/ml group, when compared with the control group. The RT-qPCR results demonstrated that the mRNA expression levels of RUNX2 and BGLAP were significantly increased at 200 ng/ml. Therefore, the present results suggested that the application of FGF-4 maintained cellular viability while enhancing the osteogenic differentiation of stem cell spheroids, at least partially by regulating RUNX2 and BGLAP expression levels.

Introduction

Fibroblast growth factors (FGFs) are autocrine and paracrine growth factors that were initially identified as proteins and are able to stimulate fibroblast proliferation (1). However, previous studies have reported that, by binding to FGF receptors, FGFs are involved in multiple biological processes, including cellular proliferation, differentiation and tissue regeneration (2). Furthermore, FGFs have been applied to wounded tissues to examine its regenerative capability and results have revealed its healing potential (3). In this context, there have been several studies identifying the effect of FGFs on different types of stem cells (4,5); however, the role of FGFs remains elusive due to varied and contradictive results. It is speculated that FGFs have different effects depending on the developmental stages of stem cells and their origins (6).

FGF-4 is a member of the FGF family and is a highly mitogenic protein encoded by the FGF-4 gene. Similar to other members of the FGF family, with a high affinity to its receptor, FGF-4 affects the proliferation, differentiation and migration of numerous types of cell (7). Furthermore, FGF-4 has been tested for the clinical treatment of angina (8). FGF-4 gene therapy using adenoviral vector has also been applied for the treatment of chronic ischemic heart disease (9). In addition, FGF-4 has been reported to enhance cell survival following ionization radiation (10). A previous study examining the effect of FGF-4 on human bone marrow cells have indicated that it stimulates cell proliferation in a dose-dependent manner (11). However, the precise effects of FGF-4 on different types of stem cells are yet to be established.

Dental stem cells, including gingiva-derived stem cells, are considered to be promising candidates for restoring lost periodontal tissue (12). Furthermore, based on previous studies, FGF-4 may promote the proliferation of mesenchymal stem cells (11,13). It has also been reported that a 3-dimensional (3D) culture system may enhance the understanding of cell proliferation and differentiation in normal and pathologic environments (14). In addition, features of mesenchymal stem cells under a 3D system may be different from the 2D culture system (15), and the 3D spheroid system may be applied as a tool for tissue regeneration (16).

Therefore, it was hypothesized that the addition of FGF-4 may have specific effects on the viability and osteogenic differentiation of mesenchymal stem cells. Thus, the aim of

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Key words: cell survival, cell differentiation, cellular spheroids, fibroblast growth factors, gingival, osteogenesis, stem cells

the present study was to examine the effects of FGF-4 on the morphology, viability and osteogenesis of stem cell spheroids composed of gingiva-derived stem cells.

Materials and methods

Fabrication of stem cell spheroids. To create stem cell spheroids, commercially available concave microwells (cat. no. H389600; StemFIT 3D; Micro FIT Co., Ltd.) with a 600- μ m well diameter were used. A total of 4.5×10^5 stem cells were loaded into each well and cultured to evaluate the cell response. Ethics approval was obtained from the Institutional Review Board of Seoul St. Mary's Hospital (approval no. KC17SESI0290) and the participant provided written informed consent according to the Declaration of Helsinki. All of the experiments were performed according to the relevant guidelines, which are also specified in the Declaration of Helsinki.

The tissue was obtained during the surgical procedures of dental implant second-stage surgery from a 75-year-old healthy female on August 2013. The epithelium of the gingival tissues was removed and cut into small pieces. Subsequently, digestion of the tissues was performed with 2 mg/ml collagenase IV (Sigma-Aldrich; Merck KGaA) and 1 mg/ml dispase (Sigma-Aldrich; Merck KGaA) (17). The cell suspension was filtered with a 70- μ m cell strainer (Thermo Fisher Scientific, Inc.) and seeded with α -minimum essential medium (MEM; Gibco; Thermo Fisher Scientific, Inc.) containing 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma-Aldrich; Merck KGaA). Cell spheroids were generated with gingiva-derived stem cells and were treated with FGF-4 (Prospec-Tany TechnoGene, Ltd.) at concentrations of 0, 50, 100 and 200 ng/ml at 37°C up to 14 days. The morphology of the cell spheroids was evaluated using an inverted light microscope (Leica DM IRM; Leica Microsystems GmbH). The diameter of the spheroids was measured by comparing the reference length on days 1, 3, 5 and 7 (14).

Determination of cellular viability. Stem cell spheroids were cultured in α -MEM (Gibco; Thermo Fisher Scientific, Inc.) and the cellular viability was qualitatively analyzed using a commercially available kit (Live/Dead assay; Molecular Probes; Thermo Fisher Scientific, Inc.) on day 3 (18). The spheroids were washed twice with the growth media and incubated at room temperature for 30 min after applying 2 μ l of 50 mM calcein acetoxymethyl ester and 4 μ l of 2 mM ethidium homodimer-1 (Thermo Fisher Scientific, Inc.). Subsequently, stem cell spheroids were observed using a fluorescence microscope at x200 magnification (Axiovert 200; Zeiss AG). The assay was based on the principle that the intact cells exhibit green fluorescence [excitation (ex)/emission (em) ~495/~515 nm], while cells with a compromised plasma membrane exhibit red fluorescence (ex/em ~495/~635 nm).

In addition, the number of viable cells was quantitatively examined using a commercially available kit (Cell Counting Kit-8; Dojindo Molecular Technologies, Inc.) on days 1, 3, 5 and 7 according to the manufacturer's instructions. The specific time-points were selected for analysis according to a previous study (19). Experiments were carried out in triplicate.

Flow cytometric analysis. The spheroids were detached to obtain a single-cell suspension prior to analysis. Stem cells were incubated with specific FITC-conjugated mouse monoclonal antibodies to human CD90 (cat. no. 11-0909-42; eBioscience; Thermo Fisher Scientific, Inc.) at 1 μ g/ml concentration, which is considered a marker for a variety of stem cells at day 1 (20). Quantification of stained cells was performed using a flow cytometer (FACSCanto II; BD Biosciences) and the FACSDiva software (v8.0.3; BD Biosciences).

Evaluation of osteogenic differentiation. A total of 4.5×10^5 cells were grown in each well with osteogenic media comprising α -MEM (Gibco; Thermo Fisher Scientific, Inc.), 38 μ g/ml dexamethasone, 2 mg/ml glycerophosphate disodium salt hydrate, 10 mM ascorbic acid 2-phosphate and 200 mM L-glutamine on days 3, 7, 10 and 14. Alkaline phosphatase activity was evaluated using a commercially available assay kit (cat. no. K412-500; BioVision, Inc.). The absorbance was measured at 405 nm after mixing a 5 mM p-nitrophenylphosphate substrate with cell lysates using assay buffer (cat. no. K412; BioVision, Inc.) and incubating it at 25°C for 40 min. Comparisons were made between the groups, as the same number of cells was loaded in each group. The assays were performed three times. Stem cell spheroids were stained with 2% Alizarin Red S at room temperature for 30 min after fixing the cell spheroids with 4% paraformaldehyde at room temperature for 15 min and washing them with deionized water twice on day 14 (18). The degree of osteogenesis was evaluated by measuring the relative intensity of Alizarin red S staining using an inverted light microscope at x100 magnification (Leica DM IRM; Leica Microsystems GmbH).

mRNA quantification by reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cell spheroids using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at day 8 (21,22). SuperScript II RTase (Invitrogen; Thermo Fisher Scientific, Inc.) was used for RT with total RNA according to the manufacturer's instructions at 42°C for 50 min. Complementary DNA of mRNA was amplified using primer pairs as follows: Runt-related transcription factor 2 (RUNX2) forward, 5'-CAGTTCCTCCCAAGCATTTCATCC-3' and reverse, 5'-AGG TGGCTGGATAGTGCATT-3'; bone γ -carboxyglutamate protein (BGLAP) forward, 5'-AATCCGGACTGTGACGAGTT-3' and reverse, 5'-CAGCAGAGCGACACCCTAGA-3'; and β -actin forward, 5'-AATGCTTCTAGGCGGACTATGA-3' and reverse, 5'-TTTCTGCGCAAGTTAGGTTTT-3'. RT-qPCR was performed on the StepOnePlus RT PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a SYBR-Green PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 30 sec at 59°C. The data were analyzed using the StepOne software v2.2.2 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The expression of each RNA was normalized to an endogenous control β -actin and was calculated using the $2^{-\Delta\Delta C_q}$ method (23). The experiments were performed three times.

Western blot analysis. Cells were lysed and extracted using lysis and extraction buffer (Pierce IP Lysis Buffer; cat.

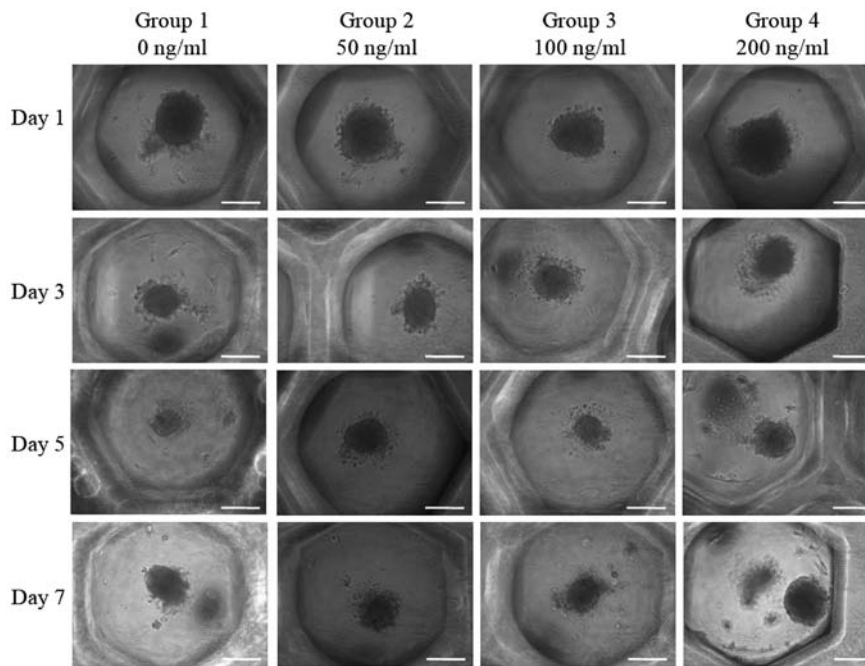


Figure 1. Commercially available concave microwells with 600- μm diameter were used to generate stem cell spheroids. Representative images revealing the morphology of the stem-cell spheroids at days 1, 3, 5 and 7 are provided (scale bar, 200 μm).

no. 87787; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols on day 7 (24). Protein in the whole-cell lysates was quantified using the bicinchoninic acid assay (Thermo Fisher Scientific, Inc.). A total of 10 μg /lane of protein samples were loaded on a 7.5% gel for collagen I and loaded on a 10% gel for RUNX2 and GAPDH experiments, respectively and then transferred to polyvinylidene difluoride membranes (Immun-Blot[®]; Bio-Rad Laboratories, Inc.) for immunoblotting. The membranes were blocked with 5% skim milk for 1 h at room temperature. The membranes were incubated with the following primary antibodies overnight at 4°C: Anti-collagen I (1:500; cat. no. ab6308; Abcam), anti-RUNX2 antibody (1:200; cat. no. ab76956; Abcam) and anti-GAPDH antibody (1:2,000; cat. no. ab9485; Abcam). After washing with TBS-0.1% Tween-20, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, goat anti-mouse immunoglobulin G (IgG; cat. no. ab205719; Abcam) and goat anti-rabbit IgG (cat. no. ab205718; Abcam) at 1:10,000 dilution for 2 h at room temperature. The immunoblot signals were visualized using horseradish peroxidase substrate (cat. no. WBKLS0100; Merck KGaA).

Statistical analysis. Values are expressed as the mean \pm standard deviation. A test of normality was performed to confirm the equality of variances in the samples. Differences among the groups were analyzed using one-way analysis of variance with Tukey's post-hoc test (SPSS 12 for Windows; IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Formation of cell spheroids with human gingiva-derived stem cells. Spheroids were well formed in each microwell on day 1 (Fig. 1). Furthermore, no noticeable morphological

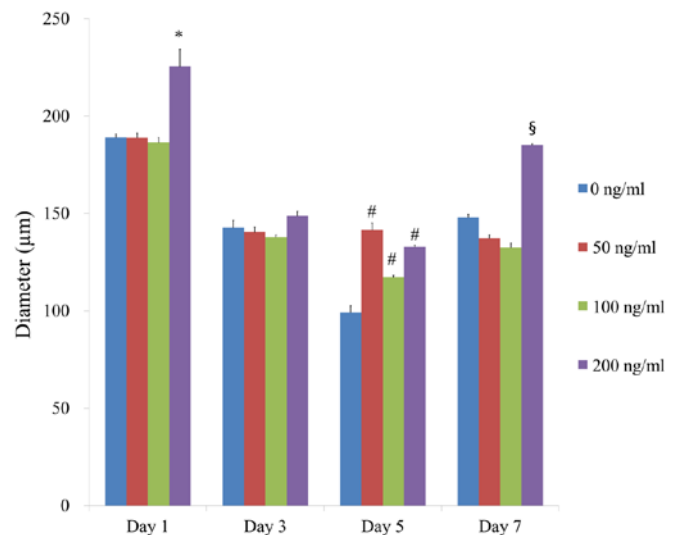


Figure 2. Diameter of the spheroids at days 1, 3, 5 and 7. Groups 1-4 were treated with fibroblast growth factor-4 at 0, 50, 100 and 200 ng/ml, respectively. * $P < 0.05$ vs. control at day 1; # $P < 0.05$ vs. control at day 5; § $P < 0.05$ vs. control at day 7.

changes of the cell spheroids were observed with the addition of FGF-4 at the concentrations of 50, 100 and 200 ng/ml. Images revealing the morphology of the spheres of days 1, 3, 5 and 7 are presented in Fig. 1. It was indicated that there were no noticeable changes at the longer incubation times.

The average spheroid diameters at day 1, 3, 5 and 7 in the presence of FGF-4 at 0, 50, 100 and 200 ng/ml were presented in Fig. 2. A statistically significant increase was identified with FGF-4 at 200 ng/ml compared with the control at day 1 ($P < 0.05$). Addition of FGF-4 led to the increase of the diameter at 50, 100 and 200 ng/ml compared with the control at day 5 ($P < 0.05$). Furthermore, a statistically significant increase was

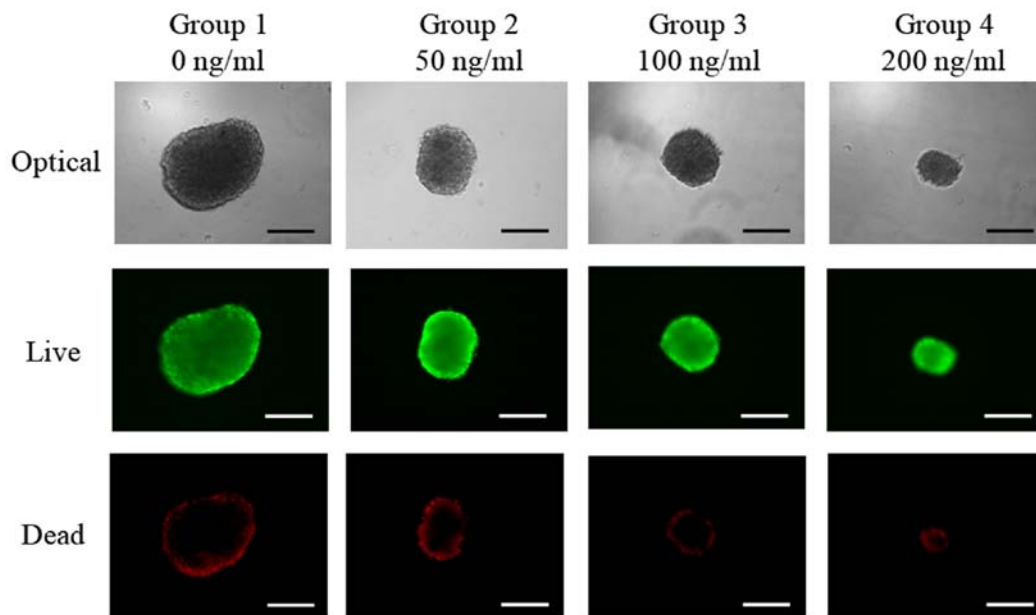


Figure 3. Cellular viability was qualitatively analyzed using a Live/Dead assay. Optical, live and dead cell images of stem cell spheroids at day 3 are provided (scale bar, 100 μ m). Groups 1-4 were treated with fibroblast growth factor-4 at 0, 50, 100 and 200 ng/ml, respectively.

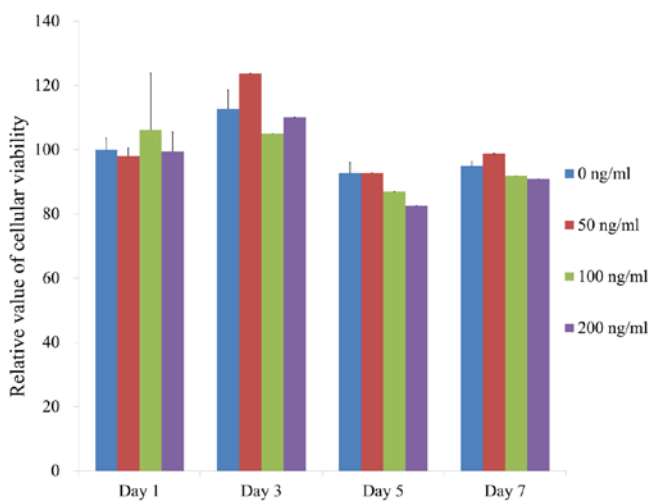


Figure 4. Cellular viability determined using a Cell Counting Kit-8 assay on days 1, 3, 5 and 7. The effect of FGF-4 at 0, 50, 100 and 200 ng/ml at day 1 on the number of viable cells was quantified as 100.0 ± 3.5 , 98.0 ± 2.5 , 106.2 ± 17.6 and $99.5 \pm 6.0\%$, respectively ($P > 0.05$). Groups 1-4 were treated with FGF-4 at 0, 50, 100 and 200 ng/ml, respectively. FGF-4, fibroblast growth factor-4.

demonstrated in the group treated with FGF-4 at 200 ng/ml compared with the control at day 7 ($P < 0.05$).

Determination of cellular viability. Qualitative results regarding the viability of cell spheroids were obtained using a Live/Dead kit assay at day 3 (Fig. 3). In all cases, most of the cells in the cell spheroids emitted green fluorescence. Red fluorescence was partly seen on the boundary of the spheroids. Furthermore, the quantitative results for cellular viability on days 1, 3, 5 and 7 are provided in Fig. 4. The effect of FGF-4 at 0, 50, 100 and 200 ng/ml at day 1 on the number of viable cells was quantified as 100.0 ± 3.5 , 98.0 ± 2.5 , 106.2 ± 17.6 and $99.5 \pm 6.0\%$, respectively. The results indicated that there were no significant differences among the groups on day 1 ($P > 0.05$).

Furthermore, no significant differences were identified between the groups with longer incubation times ($P > 0.05$).

Expression of stem cell markers. The expression of the CD90 surface marker was observed on day 1 (Fig. 5); the percentages of CD90 were 88.1% for the untreated control (0 ng/ml), 41.1% for the 50 ng/ml group, 64.7% for the 100 ng/ml group and 76.3% for the 200 ng/ml group.

Increase of alkaline phosphatase activity assay and Alizarin Red S staining with addition of FGF-4. The results of the alkaline phosphatase activity assay at days 3, 7, 10 and 14 are presented in Fig. 6. The relative value for alkaline phosphatase activity at day 7 for the groups treated with FGF-4 at 50, 100 and 200 ng/ml were 111.3 ± 1.1 , 102.5 ± 1.2 and $106.2 \pm 3.3\%$, respectively, when the control group was considered 100% ($100.0 \pm 2.3\%$). In addition, the group treated with FGF-4 at 200 ng/ml had a significantly higher value compared with that of the control group at day 7 ($P < 0.05$).

The results of the Alizarin Red S staining assay to detect mineralization at day 14 are provided in Fig. 7A. It was observed that mineralized extracellular deposits were present in each group. Furthermore, the results of the quantitative analysis of Alizarin Red S staining indicated increasing trends with increasing concentrations of FGF-4, however, this was not statistically significant ($P > 0.05$; Fig. 7B).

Increases of mRNA expression by RT-qPCR and protein expression by Western blot analysis. The results of the RT-qPCR analysis suggested that the mRNA expression of RUNX2 was 100.0 ± 12.0 , 106.2 ± 7.2 , 65.3 ± 2.7 and $224.6 \pm 17.1\%$ for the groups treated with FGF-4 at 0, 50, 100 and 200 ng/ml, respectively. It was demonstrated that the application of 100 ng/ml FGF-4 decreased RUNX2 expression but 200 ng/ml FGF-4 caused a significant increase in RUNX2 expression (Fig. 8A).

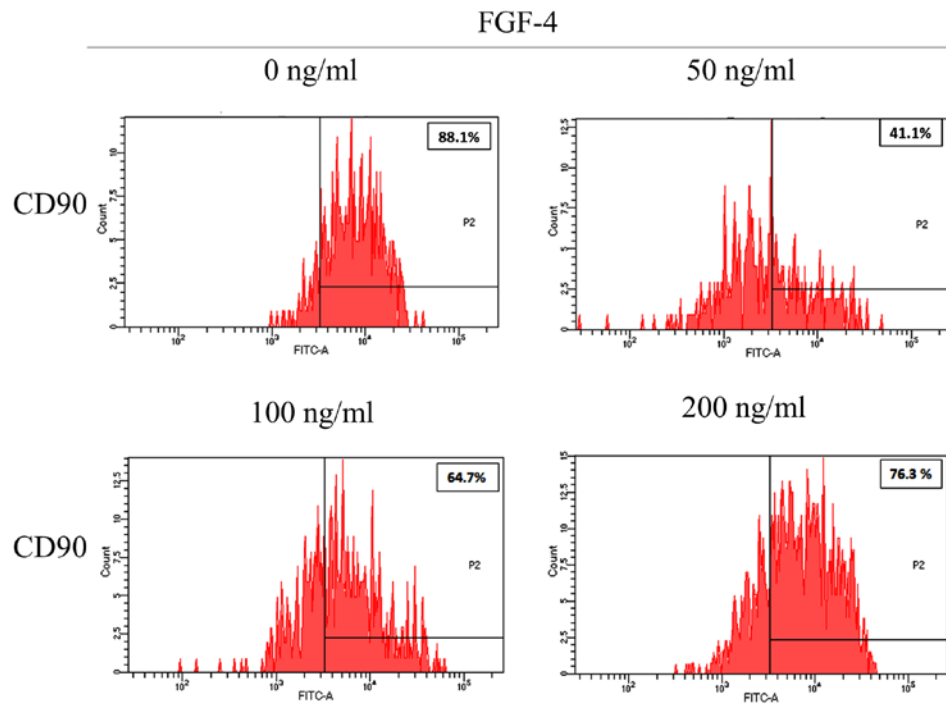


Figure 5. Evaluation of stem cell surface marker expression using CD90 cultured in growth media on day 1. The percentages of CD90 cells were 99.0% for the untreated control (0 ng/ml), 93.5% for the 50 ng/ml group, 99.1% for the 100 ng/ml group and 99.9% for the 200 ng/ml group. Groups 1-4 were treated with fibroblast growth factor-4 at 0, 50, 100 and 200 ng/ml, respectively.

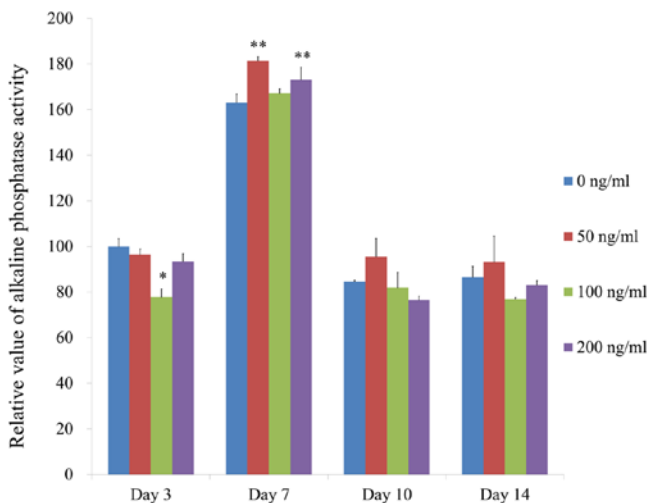


Figure 6. Alkaline phosphatase activity on days 3, 7, 10 and 14. * $P < 0.05$ vs. group 1 at day 3. ** $P < 0.05$ vs. group 1 at day 7. Groups 1-4 were treated with fibroblast growth factor-4 at 0, 50, 100 and 200 ng/ml, respectively.

The RT-qPCR results also indicated that the mRNA expression of BGLA was 100.0 ± 4.8 , 135.6 ± 16.6 , 86.8 ± 21.2 and $293.3 \pm 43.7\%$ in the groups treated with FGF-4 at 0, 50, 100 and 200 ng/ml, respectively. Of note, application of 200 ng/ml FGF-4 produced a significant increase in BGLA expression (Fig. 8B).

Western blot analysis was performed to detect the expression of certain proteins following treatment with FGF-4 at day 7 (Fig. 8C). The relative expressions of collagen I expression of 0, 50, 100 and 200 ng/ml groups after normalization were 100.0, 94.9, 98.7 and 152.1%, respectively. It was indicated that the expression of collagen I increased with the

addition of FGF-4. Furthermore, the relative expressions of RUNX2 expression of 0, 50, 100 and 200 ng/ml groups after normalization were 100.0, 100.6, 101.0 and 118.3%, respectively. Addition of FGF-4 enhanced the expression of RUNX2.

Discussion

In the present study, the effects of FGF-4 on cellular viability and osteogenic differentiation were investigated using cell spheroids of stem cells. It was indicated that the application of 200 ng/ml FGF-4 increased alkaline phosphatase activity and the expression levels RUNX2 and BGLA.

Mesenchymal stem cells are well-known for their pluripotent nature (25); these cells are able to differentiate into tissues of mesodermal origin, including tendons, bone, cartilage, ligaments, muscles and neurons (26). It has also been reported that mesenchymal stem cells may be isolated from human gingival tissue (17). Gingiva may be a desirable source of mesenchymal stem cell, as the harvesting procedure is relatively less invasive and tissue may be harvested during common dental treatments, including tooth extraction or gingivectomy (27). Furthermore, gingiva-derived mesenchymal stem cells grow faster than bone marrow mesenchymal stem cells and exhibit a stable morphology without losing their features of mesenchymal stem cells (28). Similar to other types of mesenchymal stem cell, gingiva-derived stem cells have demonstrated a significant osteogenic capability (29).

Mesenchymal stem cells have been reported to enhance bone regeneration by exerting auto or paracrine effects via the secretion of growth factors or direct differentiation into bone cells (30). Previous studies have revealed that mesenchymal stem cells are generally used with scaffolds (31,32). Furthermore, due to its osteogenic potential, a large number of

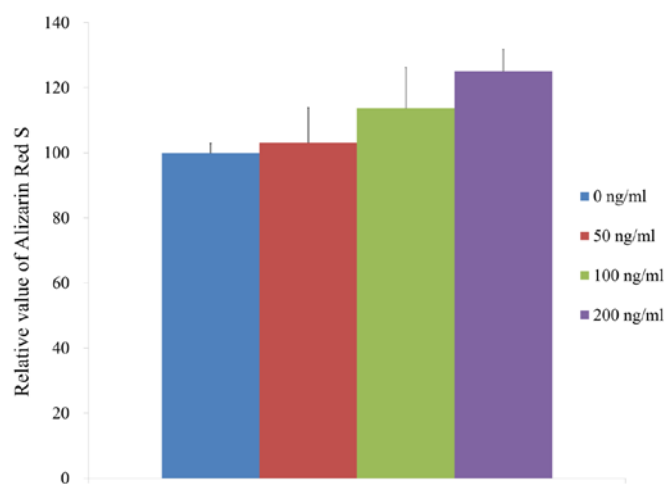
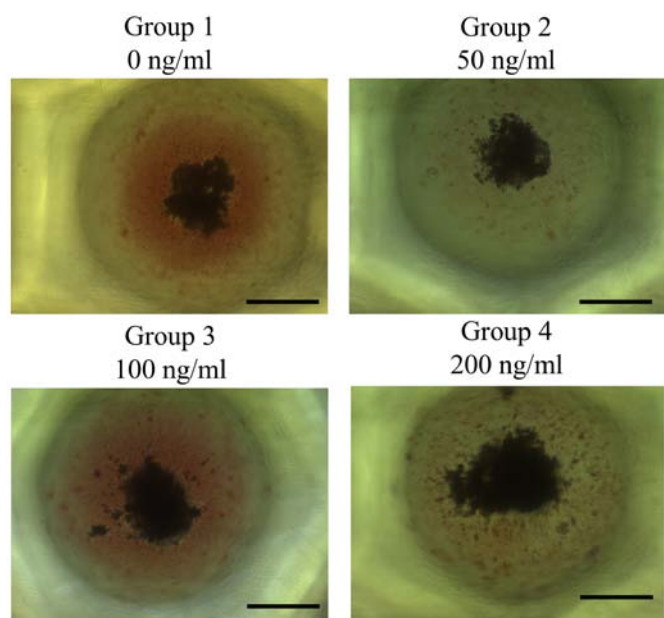


Figure 7. Results of Alizarin Red S staining at day 14. (A) Microscopic observation (scale bar, 200 μ m). (B) Quantitative analysis of Alizarin Red S staining. Groups 1-4 were treated with fibroblast growth factor-4 at 0, 50, 100 and 200 ng/ml, respectively.

dent studies have been performed based on the mesenchymal stem cell-loaded hydroxyapatite/ β -tricalcium phosphate scaffold (31,33). If it becomes possible to ensure that the actions of mesenchymal stem cells are predictable and manageable, regeneration of alveolar bone damaged by periodontal disease may become increasingly feasible.

In recent years, 3D structures have gained increased interest (34). Cellular features on 2D *in vitro* cultures have been improved to mimic physiological conditions *in vivo* by applying 3D cultures (35). Furthermore, 3D cultures of adult human liver stem cells produced islet-like structures and were able to reverse hyperglycemia in mice with severe diabetes combined with immunodeficiency (34). In addition, 3D spheroid cultures allow for the fabrication of bone marrow mesenchymal cells, which retain osteogenic differentiation potential over a monolayer culture of bone marrow mesenchymal cells without the requirement to use chemicals or hormonal modulation (36). Spheroids of mesenchymal stem cells also expressed higher

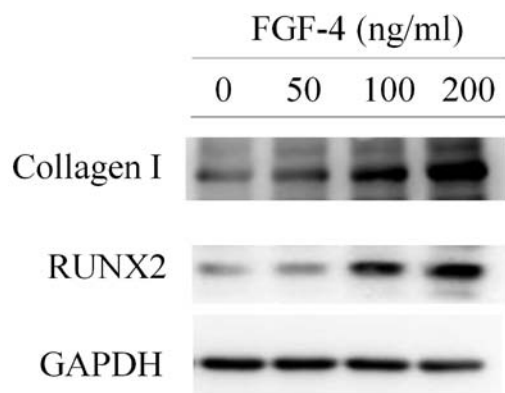
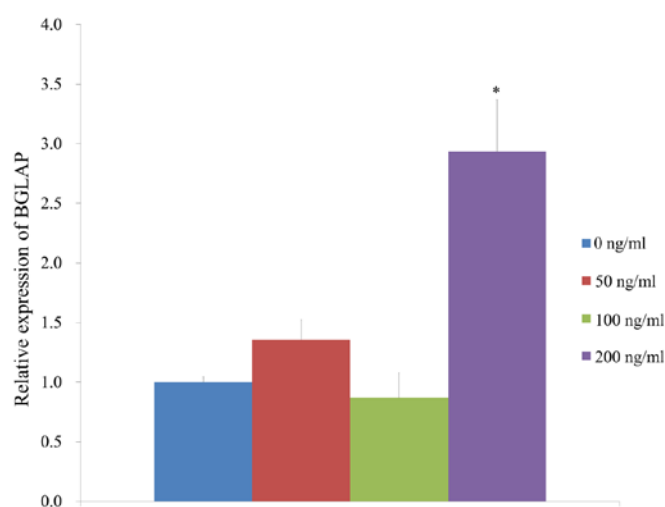
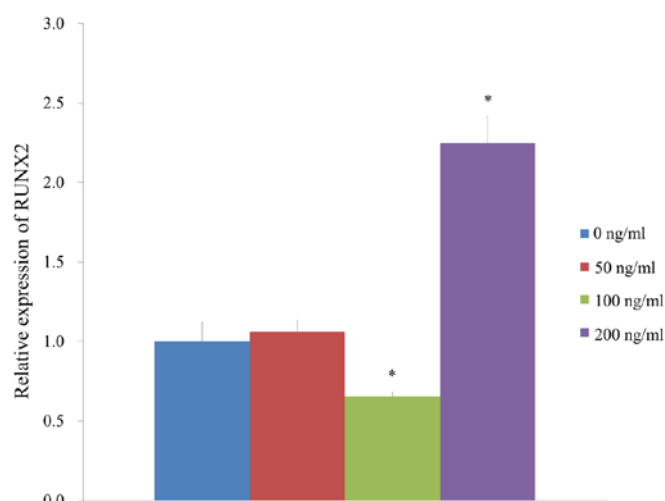


Figure 8. Reverse transcription-quantitative PCR and Western blot analysis. (A) Expression of RUNX2. (B) Expression of BGLAP. (C) Western blot analysis to evaluate the expression levels of collagen I, RUNX2 and GAPDH. * $P < 0.05$ vs. control. RUNX2, Runt-related transcription factor-2; BGLAP, bone γ -carboxyglutamate protein; FGF-4, fibroblast growth factor-4.

transcription factors that regulate stemness compared with monolayer cultures, along with higher alkaline phosphatase activity and enhanced expression of osteogenesis-associated genes (37). In another previous study, encapsulation of stem cell microspheroids was performed using gelatin-based hydrogels and it was demonstrated to have promising potential for bone or cartilage tissue engineering (38).

The present results suggested that significant effects were achieved with 200 ng/ml FGF-4. The physiological concentration of FGFs in humans may vary but the serum concentration of FGF may be 10-100 pg/ml (39). In a previous study, FGF-4 was applied at a range of concentrations ≥ 100 ng/ml for cell culture and at 0.03, 0.1 and 0.3 mg/kg for *in vivo* experiments (40). In another study, FGF-4 was prepared at a concentration of 0.1 mg/ml, and it was subcutaneously injected into rodent models at a dose of 0.1 mg/kg (41). Another study reported on injection of 10 μ g FGF-4 in an altelocollagen carrier or the carrier alone into the intended implant sites and it was revealed that a local single injection of FGF-4 stimulates bone formation around titanium implants in bone (42). Furthermore, FGF-4 produces synergistic effects in ectopic bone formation, which is induced by bone morphogenetic protein-2 (41). However, it should be noted that the optimal effective concentration of FGF-4 may differ due to differences in cell types, stage and passage of the cells, system model and duration of the culture (24,31). Thus, the observations of the present study may apply only to cells on the spheroid surface, but not for cells on the inside.

The present results indicated that cellular viability was maintained in the presence of FGF-4, while osteogenic differentiation of stem cell spheroids was enhanced, at least partially by regulation of RUNX2 and BGLAP expression. In a previous study, RUNX2 and BGLAP were selected as markers for osteogenesis (38). RUNX2 is a molecular biomarker for osteoblastic differentiation and is able to induce the expression and synthesis of BGLAP (43). Furthermore, BGLAP is considered one of the most specific markers of mature osteoblasts (22). Collagen I is considered as an osteogenic marker and induction of osteogenic supplements led to activation of collagen I expression (44). In a previous study, evaluation of mesenchymal stem cells directed toward osteogenic differentiation was performed by RNA extraction and PCR analysis of RUNX2 and BGLAP (45). However, there are limitations in the present study. The tissue was obtained from an individual of old age and this may have influenced the results (46). It appears that only the cells on the surface of the spheroids were detectable using the live/dead assay.

In conclusion, the present results suggested that the application of FGF-4 maintained cellular viability while enhancing the osteogenic differentiation of stem cell spheroids, at least partially by regulating RUNX2 and BGLAP expression levels.

Acknowledgements

We would like to thank Ms Minji Kim (College of Dentistry, Chosun University, Gwangju 61452, Republic of Korea) for performing the experiments and providing technical assistance.

Funding

This work was supported by the National Research Foundation of Korea grant funded by the Korean government (Ministry of Science and ICT; grant no. 2020R1A2C4001624). The research was also funded by the Research Fund of Seoul St. Mary's Hospital, The Catholic University of Korea (Seoul, Korea).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JS, JYT, SKM, YK and JBP collaborated to design the study; JS, JYT, SKM, YK and JBP were responsible for data access and analysis; JS, JYT, SKM, YK and JBP performed the experiments; JS, JYT, SKM, YK and JBP wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Approval was obtained from the Institutional Review Board at Seoul St Mary's Hospital, College of Medicine, The Catholic University of Korea (approval no. KC17SESI0290). Informed consent was obtained from the participant as specified in the Declaration of Helsinki, and all of the experiments were performed according to the relevant guidelines as specified in the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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