Role of FGF-2 on human bone marrow stem cells-induced collagen tissue regeneration

Dong-Soo Park

The Graduate School

Yonsei University

Department of Dental Science

Role of FGF-2 on human bone marrow stem cells-induced collagen tissue regeneration

Directed by Professor : Chang-Sung Kim

The Doctoral Dissertation
submitted to the Department of Dental Science,
and the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Dong-Soo Park

December 2013

This certifies that the Doctoral Dissertation of Dong-Soo Park is approved.

Thesis Supervisor : Chang-Sung Kim

Jung-Kiu Chai

Kyu-Sung Cho

Keun-Woo Lee

Kee-Deog Kim

The Graduate School
Yonsei University

December 2013

감사의 글

생소하며 익숙하지 않았던 한국의 대학원 생활을 하면서 이 논문이 완성되어 학위를 마치기까지 많은 지도와 격려를 아낌없이 해주신 모든 연세대학교 치과대학 교수님들과 임직원 여러분께 진심으로 감사의 말씀을 먼저 드립니다.

특히 미국에서 학교를 마치고 미군 군의관 생활만 하던 저를 연세대학원으로 이끌어 주시고 한국 대학원 생활을 알려주신 김희진 교수님께 먼저 감사의 말씀을 드립니다. 또한 부족함이 많은 저에게 끊임없는 연구지도와 함께 따뜻한 배려와 격려로 논문을 완성하는데 가장 중요한 역할로 이끌어 주신 김창성 교수님께 진심으로 감사 드립니다. 깊은 학문적인 지식과 자상하신 성품으로 늘 많은 관심과 조언을 아낌없이 해주시고 날카롭게 논문 지적을 해주시기도 하시며 저를 치주과로 이끌어 주신 조규성 병원장님, 항상 따뜻한 말과 교육의 정석을 가르쳐 주신 채중규 교수님, 인자하시면서도 부드럽고 정성스럽게 논문 수정을 도와주신 이근우 학장님, 늘 가까이서 제게 힘이 되어주시고 든든한 벽이 되어주시며 저의 많은 빈틈을 채워주신 김기덕 교수님, 그리고 늘 저를 따뜻하게 맞이해 주시며 많은 도움을 주신 최성호 교수님과 정의원 교수님께 머리 숙여 감사의 말씀을 드립니다.

또한 논문의 완성을 위해 늘 옆에서 가장 많은 도움을 주신 박정철 교수님께도 진심으로 감사를 드리며, 밖으로 드러나지 않으면서도 연구실에서 남 모르게 수고해 주신 여러 연구실 선생님들과 치주과 수련의 선생님들께도 진심 어린 깊은 고마움을 전합니다. 그리고 군생활을 하면서도 박사 학위과정 수업과 연구를 병행 할 수 있게끔 물심양면으로 격려와 배려를 아낌없이 해주신 미 8 군 치무 사령관이시며 친구인 COL Rock 과 COL Mott 대령께도 깊은 감사를 표합니다.

마지막으로 항상 사랑과 기도와 믿음으로 지켜봐 주시고 정도만을 걷게 도와주시는 양가 부모님들과 형제 자매들 그리고 제 동반자인 사랑하는 아내 미은과 제 인생의 버팀목이 되어주는 우리 아이들 지민, 영민, 혜민에게 이 논문을 바칩니다.

2013 년 저자 씀.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Role of FGF-2 on Human Bone Marrow Stem Cells-induced Collagen Tissue Regeneration

Dong-Soo Park, D.D.S., M.S.

Department of Dental Science
The Graduate School, Yonsei University

(Directed by Professor Chang-Sung Kim D.D.S., Ph.D.)

Objectives: Role of Bone Marrow Stem Cells (BMSCs) on collagen tissue regeneration, which is essential for wound healing process, might be modified by various biologic stimuli. The aim of this study was to assess the effects of fibroblast growth factor-2 (FGF-2) on collagen tissue regeneration by human BMSCs (hBMSCs) *in vitro* and *in vivo.*

Materials and Methods: hBMSCs were isolated from human vertebral bone marrow during a vertebral surgery. To confirm the stem cell population in the isolated cell group, characterization of cells used in this study was performed using well

established *in vitro* and *in vivo* assay models. The effect of FGF-2 (0, 2, 5, 20 ng/ml) treatment on biologic changes of characterization of hBMSCs was analyzed thoroughly. In order to investigate the effect of FGF-2 on the hBMSCs induced collagen tissue regeneration, *in vitro* insoluble/soluble collagen and corresponding hydroxyproline synthesis were assessed and real-time polymerase chain reaction (PCR) was performed using types of collagen and Lox (Lysyl oxidase) family genes. *In vivo* collagen formation was examined after hBMSCs were transplanted using an *in vivo* assay model for collagen tissue regeneration. After 8 weeks of healing, histologic and immunohistochemistry analysis were performed (n = 4).

Results: hBMSCs population with the characteristics of MSCs (Mesenchymal Stem Cells) was present in isolated cells from bone marrow of human vertebrae. Effect of FGF-2 on hBMSCs showed results consistent with previous reports in terms of CFE (Colony Forming Efficiency), proliferation, and *in vitro* differentiation. The amount of insoluble/soluble collagen production was also significantly enhanced in hBMSCs expanded with FGF-2 compared to hBMSCs without FGF-2. Although the changes of expression pattern of collagen-related mRNA were different dependent on the type of specific gene, FGF-2 decreased collagen type I expression of hBMSCs while it increased collagen type III. Expression of all Lox family gene was enhanced following FGF-2 treatment. The histologic and immunohistochemistry results revealed that the collagen formed *in vivo* by hBMSCs showed more relevant amount and well-organized structure in the FGF-2 treated hBMSCs at 8 weeks ($p < 0.05$)

Conclusion: FGF-2 facilitates the properties and collagen producing potency of hBMSCs, rendering them more suitable for collagen regeneration mediated wound healing by hBMSCs.

Key words: Fibroblast Growth Factor-2, human bone marrow stem cells, collagen tissue regeneration, wound healing, colony forming unit, proliferation, differentiation

Role of FGF-2 on Human Bone Marrow Stem Cells-induced Collagen Tissue Regeneration

Dong-Soo Park, D.D.S., M.S.

Department of Dental Science, The Graduate School,
Yonsei University

(Directed by Professor Chang-Sung Kim D.D.S., Ph.D.)

I. INTRODUCTION

It is well established that collagen plays a pivotal role for extracellular matrix formation and its regeneration is deeply involved in wound healing process that directs the clinical outcomes. Recently it was shown that gene expression of collagen type I and corresponding hydroxyproline, which is known to be an indicator of collagen formation, were significantly reduced in the animals showing delayed wound healing, and the importance of collagen was emphasized [1]. In terms of clinical consideration, insufficient formation of collagen tissue can result in various

diseases or complications including osteogenesis imperfecta or facial wrinkles, while excessive collagen tissue formation can induce hypertrophic or keloid scar formation. Therefore, understanding the underlying mechanism of collagen regeneration and developing the technique to control the collagen tissue regeneration can provide the future therapeutic approaches, and it is suggested that it would be an attractive technique or process for clinicians, especially for cosmetic purposes [2, 3].

Despite local resident fibroblasts [4] or recruited blood-borne 'fibrocytes' [5] play the main role of collagen tissue regeneration in the wound healing process, bone marrow stem cells (BMSCs) also participate in wound healing in direct and indirect manners [6-10]. Previous studies have demonstrated that the migrated BMSCs affect collagen tissue regeneration by local resident fibroblast through paracrine effect, and prevent scar formation by suppressing differentiation of myofibroblasts. BMSCs are also known to attenuate the inflammatory phase of the healing putative tissue cells such as endothelial cells, keratinocytes, pericytes [11-13]. Also BMSCs affect collagen tissue regeneration by local resident fibroblasts through paracrine effect and promote healing by forming neovasculature and enhancing collagen formation [6, 7, 14] after differentiating into subpopulations of fibroblasts in the wound site [7]. Therefore, BMSCs transplanted wound site is reported to increase collagen deposition [15], form more active granulation tissue, and stimulate faster wound healing [12, 16]. Up to now, subcutaneous wound site is considered to be one of the

main homing target sites of systemically infused bone marrow stem cells [17].

Previously, the authors have extrapolated the potential modulator of various stem cells in collagen tissue regeneration, and evaluated the impact of local micro-environment such as chitosan[18], or biologic stimuli such as bone morphogenetic protein [19] or fibroblast growth factor-2 (FGF-2) [20]. Above all, FGF-2 is one of the most popular cytokines that is being investigated for application in the fields of cell culture and tissue engineering [21, 22], and its clinical applications have been the topic of recent studies [23-26]. Previous studies demonstrated a positive effect of FGF-2 on biologic activity of stem cells, contributing to the preservation of the multipotency of human BMSCs (hBMSCs) in an undifferentiated state [21, 27]. The stimulatory effect of FGF-2 on osteogenic potential of human mesenchymal stem cells has been well investigated [28], however there was little, if any, study investigating the impact of FGF-2 on collagen tissue regeneration by hBMSCs despite that FGF-2 largely regulate the behavior of cells which are deeply related to wound healing.

Therefore, the aim of this study was to assess the effects of FGF-2 on collagen tissue regeneration by hBMSCs *in vitro* and *in vivo.* The results of this study may provide potential application of hBMSCs and FGF-2 in clinics.

II. MATERIALS AND METHODS

1. Human Bone Marrow Stem Cells isolation from human vertebral body and culture

Fresh human bone marrows (BMs) were harvested from the vertebrae of 5 volunteers (1 male, 4 females, median age 46 years old, range 41-58) during orthopedic vertebral surgery (Naeun Hospital, Seoul, Korea). The patients were given the informed consent using guidelines approved by the Institutional Review Board, College of Dentistry, Yonsei University, Seoul, Korea. From BM aspirates, mono nuclear cells fraction for mesenchymal stem cells (MSCs) isolation was obtained using conventional density gradient centrifugation with density gradient solution (Ficoll-Paque^{TM} Plus; GE Healthcare Bio-Sciences, Sweden). The resulting cells were seeded at 10-20 x 10^6 / 20 ml media on T75 culture flask with a growth medium of α-MEM (Gibco) containing 10% fetal bovine serum (FBS; Gibco), 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). The cultures were incubated at 37℃ in 5% CO_2. Colony formation from the culture was observed under the microscope (CK40, Olympus Optical, Tokyo, Japan). After 2 weeks of culture, adherent MSC-like cells were obtained. For the purpose of the present study, the cells were further expanded under the addition of specific concentration of FGF-2 (Genoss, Seoul, Korea). The concentration of FGF-2 was divided into 4 preliminary

experimental groups with 0, 2, 5, and 20 ng/ml concentration each and cell expansion was performed respectively. Cells at second to eighth passage were used for the study.

2. Colony-forming unit assay

Cells were plated 1 x 10^2 cells/well onto 6 well plate with growth media, and 14 days thereafter the dishes were fixed with 10% neutral buffered formalin and then stained with crystal violet (Sigma-Aldrich). The number and size of colony formation unit at each passage were calculated under the microscopic observation. (CK40, Olympus Optical)

3. Assessment of proliferation (MTT) and doubling time

Proliferation rate of each group of hBMSCs was assessed using the MTT assay[18]. Cell (1 x 10^3) were plated on 48-well plates and cultured, and the MTT assay at specific time points was performed. After incubation with 10% MTT solution (5 mg/ml; Amresco, Solon, Ohio, USA) and culture media at 37℃, 5% CO_2 for 2h, the supernatant was discarded, and dimethylsulfoxide (DMSO; Amresco) was added to each well. The absorbance was measured at 540 nm using a microplate spectrophotometer [Benchmark Plus enzyme-linked immunosorbent assay (ELISA) reader, Bio-Red, Hercules, USA]. Doubling time was calculated with Td=(t2-

t1)*log(2)/log(q2/q1) [Td=doubling time, q1= growing quantity at time t1, q2= growing quantity at time t2].

4. Expression of surface marker using FACS

In order to further characterize acquired hBMSCs, cell-surface-marker characterization of isolated cells were performed using flow cytometry analysis, a procedure for which is described elsewhere [29]. After the cells were harvested and transferred to a 1.7 ml tube (Oxygen, Union City, CA, USA), they were fixed by adding 4% paraformaldehyde for 15 min. The cells were then incubated with 3% bovine serum albumin and primary antibodies were raised against CD105, CD73, CD90, CD44, CD29, CD14, CD34, and CD45 (CD14: IM0645U, Beckman coulter; CD29: IM0791U; CD34: IM1870; CD90: IM1839U; CD146: 560846, BD Pharmingen; CD44: 555479; CD45: A07783, Beckman; CD105: A07414; CD73: 550257, BD; STRO-1: SC-47733 PE, Santa Cruz) for 1 h. The cells were washed with washing buffer, and the secondary antibody (to which fluorescein isothiocyanate (FITC) was attached) was added for 45 min at room temperature. The cells were washed three times and observed with a flow cytometer (FACS Calibur, BD Biosciences, Franklin lakes, NJ, USA).

***In vitro* induction of adipogenic, osteogenic, and chondrogenic differentiation**

Adipogenic, osteogenic, and chondrogenic differentiations were induced after

cells were cultured. Cells (P4) were seeded into 6–well plates at 1×10^5 cells/well, and cultured until they reached subconfluence. The culture medium for adipogenic differentiation comprised α-MEM containing 10% FBS, 2 mM L-glutamine, 100 μM L-ascorbic acid 2-phosphate, 500 μM isobutyl methyl xanthine (Sigma-Aldrich), 60 μM indomethacin (Sigma-Aldrich), 0.5 μM hydrocortisone (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin, and was refreshed at 3 day interval. After 3 weeks of induction, the cells were stained with Oil Red O stain for lipid droplets. The culture medium for osteogenic differentiation comprised α-MEM (Gibco) containing 10% FBS (Gibco), 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 2 mM L-glutamine (Gibco), 10^{-8} M dexamethasone (Sigma-Aldrich), 1.8 mM KH_2PO_4 (Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), and was refreshed at 3 day intervals. After 2-3 weeks of induction, mineralized nodule formation as an osteogenic differentiation was confirmed by staining with Alizarin red. Chondrogenic differentiation was induced after cells were cultured. cells (P4) were seeded into 15 ml tube at 4×10^5 cells/tube, and cultured pellet for 2-3 days. The culture medium for chondrogenic differentiation comprised α-MEM (Gibco) containing 10% FBS (Gibco), 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 2 mM L-glutamine (Gibco), 1% ITS (BD), 0.1 μM dexamethasone (Sigma-Aldrich), 172 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 40 μg/ml proline (Sigma-Aldrich), 10 ng/ml TGF-ß3 (R&D), 100 U/ml

penicillin and 100 μg/ml streptomycin (Gibco), and was refreshed at 3 day intervals. After 3 weeks of induction, pellets were left free floating for 21 days and fixed with 10% neutral buffered formalin. Paraffin embedded and 5μM section were sectioned and stained with Toluidine blue to assess chondrogenic differentiation.

To further confirm each differentiation, quantitative real-time polymerase chain reaction (PCR) was performed. Cells were harvested using Trizol® (Invitrogen), and total cell RNA was prepared. Isolated total RNA was used as a template for the synthesis of cDNA with OligodT primer (Maxime RT Premix®, iNtRon Biotechnology). The subsequent PCR amplification utilized the SYBR® real-time PCR kit (Premix Ex Taq™, Takara Bio), ABI7300 real-time PCR system and software (Applied Biosystems), and specific primers for each specific differentiation (Table 1) under the following conditions: an initial step at 50℃ for 2 min and 95℃ for 10min, followed by 3 steps of denaturation at 95℃ for 15s, annealing at 60℃ for 30s, and extension at 72℃. The relative levels of mRNA expression were quantified by comparison with the internal standard (β-actin). The amount of gene expression at real-time PCR was computed from the target threshold cycle (Ct) values and β-actin Ct values by means of the standard curve method: ΔCt= Ct of collagen type I ,II, III, IV or V- Ct of β-actin; ΔΔCt=ΔCt of target gene – ΔCt of control; normalized amount of gene expression = $2^{-\Delta\Delta Ct}$. Each PCR was performed in triplicate with the same total RNA.

***In vivo* regenerative potential of hBMSCs using ectopic transplantation model**

In vivo transplantation was performed to evaluate the regenerative capacity of hBMSCs. Hydroxyapatite/tricalcium phosphate (HA/TCP)-based scaffolds were used for the cell carrier. For a single transplantation, $6x10^6$ cells were loaded with 80 mg of HA/TCP ceramics powder (Biomatlante, Vigneux, France) and then transplanted into subcutaneous pockets on the dorsal surface of immunocompromised mice (*n*=4). All animal experiments were carried out in accordance with the Guidelines and Regulations for the Use and Care of Animals of the Animal Care Committee of Medical College, Yonsei University. The mice were euthanized after 8 weeks, the implants were harvested and fixed in 4% PFA for 3 days. For histological analysis, the sections (serially sectioned at 6 μm) were stained with hematoxylin-eosin and masson's trichrome staining, and immunohistochemical staining was performed.

5. *In vitro* collagen and insoluble synthesis assay (Sircol™ collagen assay)

Total soluble and insoluble collagen in the culture was measured using the Sircol collagen assay kit (Biocolor Ltd). To compare the fibroblastic potentials of hBMSCs with hPDLSCs were also cultured.

After 7 days, cell supernatants of hBMSCs and hPDLSCs were collected by centrifugation at 260g for 5min. A 100-μl aliquot of the supernatant was added to 1 ml of the dye reagent provided with the kit, and the solution was incubated for 30 min at room temperature. After centrifugation at 9300g for 10 min, the separated suspensions were discarded and the remnant pellets were dissolved in 1 ml of the

alkali regent provided with the kit. After ensuring thorough dissolution, the same aliquots were plated on 96-well plates and relative absorbance was at 540 nm.

hBMSCs and hPDLSCs were cultured with or without 0.3 mg/ml WSC in culture medium for 7 days. The samples were collected of 500 µl unused cell medium and added 500 µl of a pepsin (Sigma-Aldrich) concentration of 0.1 mg/ml 0.5 M acetic acid (Sigma-Aldrich). And then they were incubated overnight at 4℃. The samples were added 100 µl Acid Neutralizing Reagent to 1.0 ml acid extracts and each tube add cold Isolation & Concentration Reagent (200 µl/tube). The tubes were placed filled rack into a container half filled with an ice-water mix and incubated overnight at 0 to 4℃. After centrifugation at 9300g for 10min the separated suspensions were discarded and the remnant pellets were dissolved in 1 ml of the dye reagent provided with the kit, and proceed with the assay as described the soluble collagen assay.

6. The analysis of hydroxyproline in soluble and insoluble collagen

Hydroxyproline in both supernatant of culture and collagenous extracellular matrix accumulated on the culture dishes was quantified using the Reddy–Enwemekas method with slight modifications.

After 7 days, cell supernatants of hBMSCs and hPDLSCs were collected by centrifugation at 260g for 5min. A 200-µl aliquot of the supernatant was placed in a

2 ml clear wide opening crimp (Agilent Technologies, USA), and closed by 11mm crimp cap (Agilent Technologies, USA). The cells were collected of 100 µl dH_2O and added 100 µl concentrated HCL (Duksan Pure Chemicals Co., Ltd, Korea) in a 2 ml clear wide opening crimp and closed by 11 mm crimp cap. The samples were hydrolyzed by autoclaving at 120℃ for 20 min. After cooling, the 10 µl aliquots were plated on 96-well plates and evaporated to dryness under vacuum. Total soluble and insoluble collagen in the culture was measured using the Hydroxyproline assay kit (Biovision Incorporated, CA, USA). After vaporization, a 100 µl chloramin-T Reagent was added to each sample and then it was left standing for 5 min at room temperature. Chloramin-T Reagent used here was prepared by 6 µl chloramine-T concentrate of 94 µl Oxidation buffer provided with the kit, and mixed well. The prepared 100 µl of DMAB reagent, that was 50 µl DMAB concentrated to 50 µl of Perchloric acid/Isopropanol solution provided with the kit, and mixed well, was added to each well and incubated for 90 min at 60℃. The optical density of the final solution was measured at 560 nm using a microplate spectrophotometer [Benchmark Plus enzyme-linked immunosorbent assay (ELISA) reader, Bio-Red, Hercules, USA].

7. Expression of collagen and LOX family related gene

Based on the results of collagen and corresponding hydroxyproline assay,

hBMSCs expanded with 5 ng/ml of FGF-2 was selected for further experiments including quantitative real-time PCR and *in vivo* transplantation for collagen tissue regeneration. The finding of increased ECM synthesis prompted the observation of the changes in collagen synthesis at mRNA and protein levels. The quantitative real-time polymerase chain reaction (PCR) analysis was performed for types of collagens including collagen type Ⅰ, Ⅱ, Ⅲ, Ⅳ and Ⅴ according to aforementioned procedures. In addition, the gene expressions of lysyl oxidase (LOX), lysyl oxidase-like (LOXL)1, LOXL2, LOXL3 and LOXL4 were detected. Information for each specific primer is listed on the Table 1.

***In vivo* collagen synthesis assay using *in vivo* ectopic transplantation model.**

In vivo transplantation was performed to evaluate the collagen synthesis capacity of hBMSCs. Since the use of an appropriate carrier is important to the potential of hBMSCs, we selected a hyaluronic acid (HA) component since this is one of the most common components used in biomedical surgeries, and our previous study showed that MSCs successfully induced collagen tissue regeneration when they were transplanted with HA-based scaffolds. For a single transplantation, hBMSCs (6×10^6cells) expanded with FGF-2 treatment (5 ng/ml) or not were mixed with 0.15 ml of HA filler (ReDexis, Prollenium Medical Technologies), and then injected into subcutaneous pockets on the dorsal surface of immunocompromised mice (n=4). All animal experiments were carried out in accordance with the Guidelines and

Regulations for the Use and Care of Animals of the Animal Care Committee of Medical College, Yonsei University. The mice were euthanized after 8 weeks, and the implants were harvested. After being weighed on a balance (Adventurer, Ohaus Corp.) and fixed in 4% PFA for 3 days, they were washed with PBS before embedding in Tissue-Tek O.C.T. Compound (Sakura-Finetek, Japan) for frozen section. For histological analysis, the sections (serially sectioned at 4 mm) were stained with hematoxylin-eosin and masson's trichrome staining. Histological analyses were performed using light microscopy (BX41, Olympus Optical). For histometric analysis of collagenous ECM formation, computer-assisted histometric measurements were acquired with an automated image-analysis system (Image-Pro Plus).

8. Immunohistochemistry analysis

In brief, after routine deparaffinization and rehydration, the antigen was retrieved using a microwave-based technique for 30 min in citrate buffer (pH 9.0). The endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min. The sections were incubated for 30 min at room temperature with primary antibodies: human-specific mitochondrial ribosomal protein (ab74285, Abcam) diluted 1:50, hydroxyproline (bs-7512R, Bioss) diluted 1:500, collagen type I (ab7778, Abcam) diluted 1:200, and collagen type III (ab292, Abcam) diluted 1:1000. After washing three times, immunodetection was performed using a commercially available kit (EnVision™Detection System, DakoREAL™) according to the manufacturer's

instructions. Slides were then counterstained with hematoxylin.

9. Statistical analysis

All *in vitro* experiments were performed three times. Mean and standard deviations (SD) were calculated. Unpaired t-testing was used to analyze the differences between two groups. For multiple analysis, ANOVA test followed by (ANOVA repeated measure) Scheffé's comparison was used. Differences were considered statistically significant at $P < 0.05$.

III. RESULTS

1. The characterization of BMSCs from fresh bone marrow of human vertebral body

Putative cells were isolated and cultured from fresh bone marrow of human vertebral body; their characteristics as mesenchymal stem cells were investigated using various assay models such as CFE, expression of surface markers, and multipotency *in vitro* and *in vivo*.

Adherent cells with a thin and long fibroblastic spindle morphology was observed 1-3 days after seeding. The ability to form CFU was demonstrated when they were plated at low density. In general, the cells presented as a heterogeneous population between the colonies. hBMSCs used in this study showed approximately 10% of CFE over passages (Figure 1A). The size and quantity were measured according to each passage, and the latter passages showed a tendency to gradually decrease in numbers even though the potency to form CFU were continuously well maintained. Cell proliferation also showed good results, but the latter passages tended to show a slight decrease (Figure 1B) whereas doubling time showed initial decrease but gradual increase in latter passages (Figure 1C). FACS assay confirmed that the cells used in this study were consistently positive for the presence of CD105 (99.89%), CD73 (99.66%), CD90 (99.11%), CD44 (98.05%), and CD29 (85.46%), and negative for CD14 (0.97%), CD34 (0.84%), or CD45 (1.08%) (Figure 1D). *In vitro*

differentiation model showed that hBMSCs successfully differentiated into osteogenic, adipogenic and chondrogenic lineages depending under the specific differentiation stimuli (Figure 1E). Each differentiation was confirmed by staining with Alizarin red, Oil red O and Toluidine blue, respectively. Real-time PCR confirmed that the isolated cells provoked *in vitro* multipotency. They expressed enhanced level of osteogenic markers of osteocalcin and Runx2, adipogenic markers of LPL and PPARγ -2, and chondrogenic markers of collagen 2A1 and SOX9 (Figure 1F).

When they were transplanted into the *in vivo* ectopic transplantation model with cell carriers of HA/TCP, they induced ectopic tissue regeneration including bone and bone marrow-like tissues. Immunohistochemical assay using hMito showed that regenerated tissue originated from transplanted hBMSCs (Figure 1G). Based on the results, it could be concluded that hBMSCs were successfully isolated and expanded from fresh bone marrow of human vertebral body during the surgery.

2. Effect of FGF-2 treatment on the biologic activity of hBMSCs

Before the evaluation of BMSCs-induced collagen tissue formation enhanced by FGF-2, the effect of FGF-2 on the basic behaviors of hBMSCs was investigated beforehand. Therefore, hBMSCs were expanded with the different dosages of FGF-2 treatment and they showed morphological changes; smaller in size and less flattened shape compared to the cells without FGF-2, which was in accordance with previous reports (Figure 2A)[20]. The results of MTT test at 1, 3, and 5 days revealed that FGF-

2 treatment significantly enhanced the proliferation in a dose dependent manner ($p<0.05$), and hBMSCs expanded under 20 ng/ml showed the highest proliferation rate ($p<0.05$) (Figure 2B). In CFE test under the FGF-2 influence, the number of colonies was increased and the colony size appeared significantly greater in a dose dependent manner ($p<0.05$) (Figure 2C). Maximum influence was provoked in the hBMSCs expanded with 20 ng/ml of FGF-2. However, 5 ng/ml of FGF-2 also showed relevant enhancement of CFU formation capacity (approximately 2.5 times of colony size and 1.5 times of number respectively compared to hBMSCs without FGF-2.)

Treatment of FGF-2 on hBMSCs also modified their differential potency. In the present study, osteogenic, adipogenic and chondrogenic differentiation were assessed (Figure 2D). Similar to previous studies[30], osteogenic differentiation determined by mineralized nodule formation was significantly decreased in a dose dependent manner. Assay for mRNA expressions of Runx-2 and osteocalcin using quantitative real-time PCR decreased in a similar pattern to what we observed *in vitro* mineralized nodule formation (Figure 1F). Activity of lipid droplet formation was significantly enhanced in the FGF-2 expanded group. Taken together with the results from real-time PCR for LPL and PPARr2, which are master genes for adipogenic differentiation (Figure 1F), it can be concluded that FGF-2 treatment enhance the adipogenic differentiation of hBMSCs. The result of chondrogenic differentiation was in line with previous studies [21, 27] showing that chondrogenic differentiation was enhanced by FGF-2. Expression of collagen type 2A1 and SOX 9 were significantly

increased in the hBMSCs expanded with FGF-2. FGF-2 treatment not only provoked the formation of collagenous ECM, but increased the size of pellets, suggesting the activity of ECM formation can be significantly modified by FGF-2 (Figure 1F). The changes by FGF-2 were shown in a dose dependent manner. Collectively, results from *in vitro* differentiation experiments showed the different patterns from each other among the tested differentiation lineages.

3. Enhancement of hBMSCs induced collagen synthesis by FGF-2 treatment.

The effect of FGF-2 on collagen regeneration was evaluated using hBMSCs expanded by the aforementioned concentrations of FGF-2. Soluble and insoluble collagen production were measured by Sircol assay kit. Detected soluble collagen in a culture media gradually decreased while insoluble collagen deposit on the culture dishes was increased as the concentration of FGF-2 increased (Figure 3A). The content of hydroxyproline, which is a major component of collagen, can be used to assess the amount of collagen. At the 20 ng/ml of FGF-2 treatment, expression of insoluble collagen deposition was shown in the greatest amounts, whereas soluble collagen production was shown the least (Figure 3A). The determination of hydroxyproline content showed the similar pattern to those of soluble and insoluble collagen. These results suggest that collagen deposition is regulated by the reciprocal balance of soluble and insoluble collagen. Based on these results, 5 ng/ml of FGF-2 was selected for the further experiments, because the behaviors of hBMSCs were

efficiently enhanced as low as 5 ng/ml of FGF-2, while collagen production was not significantly different between 5 and 20 ng/ml.

To better understand the effect of FGF-2 on collagen synthesis by hBMSCs, collagen formation related gene expressions were closely examined. According to the collagen type, the gene expressions showed significant changes by FGF-2, but the pattern of increase or decrease was apparently different. Interestingly, mRNA expression of collagen type I, IV, and V were decreased, while mRNA expression of collagen type II and III were significantly up-regulated (Figure 3C). Collagen type I is the major type of collagen that constitutes ECM of mineralized tissue, and it is the major type of scar-inducing collagen when it is excessively formed. Also, down regulation of collagen type I might be responsible for the decrease in mineralized nodule formation in the present study. Accumulation of collagen type III, which is another major collagen type in ECM that is not formed by resident local fibroblast, was also noted. Therefore, together with enhancement of collagen type II, which is in accordance with previous studies [21, 27], it can be assumed that healing characteristics of hBMSCs has been improved by treatment with FGF-2.

The expression of Lox family genes increased upon the treatment of FGF-2 (Figure 3C). In general, Lox family genes are related to collagen structural integrity, and they are involved in a more stable and mature type of collagen tissue formation[18]. Based on the results from real time PCR, it could be suggested that collagen deposition with structural integrity could be induced by hBMSCs with FGF

treatment.

4. Enhancement of *in vivo* collagen tissue formation by hBMSCs expanded with FGF-2

The authors postulated that the application of FGF-2 on hBMSCs will improve tissue regeneration capacity and increase the formation of collagen tissue with structural integrity in *in vivo*. The *in vivo* ectopic regeneration model is one of straightforward experiment models to analyze the histological results of regenerative potential by hBMSCs transplanted with hyaluronic acid as a cell carrier. It has been well described that multipotency of stem cells can be influenced by the biologic stimuli or micro environmental. For instance, when it is transplanted with hyaluronic acid, collagen producing fibroblasts will be highly induced rather than osteoblasts or chondroblasts, whereas they differentiate into mineralized tissue producing osteoblasts or chondroblasts with HA/TCP carriers [18, 31]. After 8 weeks of transplantation, hBMSCs formed collagenous ECM with hyaluronic acid as shown in figure 4. The measurement of collagenous ECM revealed that hBMSCs significantly greater ECM when treated by FGF-2 (Figure 4A and 4B). In histology, there was no sign of inflammatory reaction. The results from *in vivo* transplantation experiments suggested that hBMSCs treated with FGF-2 and hyaluronic acid could be potentially applied in clinical indication for collagen regeneration.

In addition to quantitative analysis for collagen synthesis, immunohistochemial

analysis was performed for qualitative analysis (Figure 4C). The hMito that was darkly stained in both hBMSCs and hBMSCs/FGF-2 group showed that these tissues originated from the newly transplanted human cells. More cells appeared positively stained in hBMSCs treated FGF-2 group and it can be suspected that FGF-2 affected the proliferation of transplanted hBMSCs. Hydroxyproline was expressed along the newly formed collagen tissue. However, the intensity of staining was stronger in the hBMSCs/FGF-2 group. The newly formed collagen tissue showed distinct positive response to type I and III collagens, which was one of the hBMSCs-induced healing characteristics. The amount of expression of collagen type I and III showed similar patterns to that of hydroxyproline. Expression of collagen type I was more intense compared to that of collagen type III, which coincides with previous studies showing the ratio of these two types of collagen in normal and healing tissue[32].

IV. DISCUSSION

In this study, the hBMSCs were isolated from vertebral bodies during surgery, and they presented with full stem cell-like characteristics including adherent characteristics, expression of MSC-specific surface markers, and *in vitro* and *in vivo* multipotency which are consistent results of previous reports using different parts of the body as a stem cell source [20, 33]. In addition to their superior potency as stem cells, hBMSCs acquired from vertebral body have several advantages; utilization of bone marrow which are discarded during surgery as medical waste, using the same surgical site to avoid forming a secondary surgical wound site, easy to obtain bone marrow through a non-invasive aspiration technique with minimal ethical issue. Thus, bone marrow from the vertebral body may be the alternative and promising source for hBMSCs with high therapeutic value.

One of the most important characteristics hBMSCs is that it facilitates the wound healing effect [12]. It is well established that they facilitate wound healing though a paracrine effect on local resident cells when they migrate into the specific wound site by homing mechanism. In addition, they are directly involved in functional tissue healing via differentiation into a variety of tissue appendage cells[11-13]. They also enhance collagen tissue production and promote wound healing[15]. Therefore, bone marrow–derived MSCs, when they can be systemically administered, are effective in improving the rate and quality of extracellular matrix

deposition and increasing the tensile strength of incisional wounds. Furthermore, they also facilitate wound healing in the complicated healing environments such as in diabetes mellitus[13]

In this study, FGF-2 was used as biologic stimuli to improve wound healing effect of hBMSCs. FGF-2 has been considered to be an highly effective modulator MSCs, because it has been shown by many to affect the proliferation, migration, differentiation, and self-renewal potential of stem cells thus attributable to the preservation of the multipotentiality of MSCs in an undifferentiated state [21, 27, 34-36] . The result of our study on hBMSCs expanded with FGF-2 treatment is also in line with the previous studies in regard to CFE, proliferation, migration, and *in vivo* regeneration.

Collagenous extracellular matrix regeneration is another major concern for the both of soft and hard tissue reconstructive surgery. The insoluble collagen exhibited a greater compression strength and lower degradation rate than the soluble form despite sharing similar cross-linking structures between each other[18]. Interestingly, the results of our study showed that hBMSCs expanded with FGF-2 produced a significant amount of insoluble collagen formation while the quantity of soluble collagen formation was decreased. In addition, changes in collagen-related gene expression was observed in hBMSCs expanded by FGF treatment. Gene expression of collagen type II and III were significantly enhanced, while other types of collagen were down-regulated. Gene expression of LOX family which is essential for

stabilization and maturation of collagen fibrils [37, 38], showed marked up-regulation when hBMSCs was expanded by FGF-2. LOX and LOX like ligands (LOXL) are deeply involved in the formation of cross-linked collagen tissue, which is important for the tensile strength, structural integrity, and function of connective tissues [38, 39]. Increase in LOX activity is closely associated with enhanced accumulation of insoluble collagen with well cross linked structure[40]. On the contrary, reduction in LOX activity may have a critical effect on the pathogenesis of collagen tissue disorders including pulmonary disease, skin, and vascular defects[41-43]. For example, when collagen tissue was analyzed in a transgenic Lox-/- mouse, the arrangement of individual collagen fibers showed mostly disperse, loose and poorly-ordered forms compared to normal tissue although no obvious differences were detected in the types of collagen[43]. Our results propose that hBMSCs expanded by FGF-2 might enhance deposition of insoluble collagen with a more mature and stable type of structure through the enhancement of Lox family gene expression and following enhancement of crosslinking of collagens.

Concerning collagen tissue regeneration and maturation, the authors have previously investigated the *in vivo* healing cascade of collagen tissue by human periodontal ligament stem cells (hPDLSCs) in a time course [32]. When hPDLSCs were transplanted with a cell carrier into the ectopic transplantation model, they produced collagen matrix after active proliferative phase. At the initial stage of healing within 1 week, amorphous collagen matrix was formed, and both collagen

type I and III were expressed strongly. However, type III collagen expression was weakened in the later stage of maturation, and collagen tissue orientation stage, and this type of healing appeared to be specific in MSC mediated tissue regeneration.

Collagen type I is predominant in subcutaneous ECM and exceeds collagen type III by a ratio of 4:1. During wound healing, this ratio decreases to 2:1 because of an early increase in the deposition of collagen type III. Collagen type III is considered to be particularly important for normal subcutaneous wound healing because the expression of collagen type III markedly increases in the early phase of wound healing. Interestingly, collagen type III was produced by cells derived from the bone marrow, but not local resident fibroblasts[7], suggesting an indispensable role for bone marrow-derived cells in early wound healing. In the present study, gene expression of collagen type III was markedly enhanced by FGF-2 treatment while collagen type I was reduced. This shows that FGF-2 treatment enhances the specific characteristics of hBMSCs mediated healing. Also collagen type III is relatively insoluble in experimental setting[44], and this is consistent with the highly upregulated expression of mRNA and actual protein production revealed in Sircol Assay.

Now, we can speculate the distinct characteristic of insoluble collagen tissue that was formed and deposited by hBMSCs expanded with FGF-2. Type I collagen is formed by the dermal resident fibroblast in soft tissue healing[45], and excessive production of type I collagen is the hallmark of scar formation[46, 47]. Clinically, this

leads to scar formation, an unsatisfactory healing outcome. As this study proposed, effectiveness of decreased collagen type I by using hBMSCs expanded with FGF-2 may contribute to suppressing the differentiation of myofibroblast which produces α SMA (α Smooth Muscle Actin) while promoting the distinctive characteristic of hBMSCs; wound healing with minimal scar formation. Despite a need for additional research, application of this study clinically, may lead to improved clinical wound healing.

Collagen type I and III are the major types of collagen that constitute the collagenous ECM. Even though FGF-2 treatment decreased expression of collagen type I in this study, another essential collagen type II was also increased. They improved cellular attachment, migration, and proliferation that were directly related to *in vivo* tissue healing. In addition, the expression of Lox family gene related to collagen structural integrity and maturation were enhanced by FGF-2. In the analysis of the *in vivo* results, all these effects must be considered. The final *in vivo* results were determined by the effect of the net results of all these factors. We suggest that the therapeutic approach proposed in this study might provoke great potential in collagen tissue regeneration mediated wound healing.

V. CONCLUSION

The results of this study suggest that hBMSCs largely participate in collagen tissue formation *in vitro* or *in vivo*, and the collagen producing potency may be greatly enhanced with FGF-2 treatment. hBMSCs expanded with FGF-2 significantly reduced *in vitro* soluble collagen formation and promoted insoluble collagen deposition compared to hBMSCs expanded without FGF-2. The *in vivo* study results indicate that an addition of 5 ng/ml of FGF-2 was significantly enough to produce collagen tissue regeneration, and related gene expressions were similarly enhanced. The results from *in vivo* ectopic transplantation model also have demonstrated that application of 5 ng/ml of FGF-2 can induce significant collagenous ECM formation and related gene expression. Collectively, the results of this study strongly imply that the potential utilization of FGF-2 in the medical or dental fields where collagen tissue regeneration can benefit the treatment. Specifically, the therapeutic approach proposed in this study may be applied to the treatment of subcutaneous depressions, wrinkle repair, and soft tissue augmentation as well as wound dressing for the surgeries.

Collectively, *in vitro* and *in vivo* results demonstrated that FGF-2 regulated the cellular properties of hBMSCs, and enhanced the quantity and quality of collagen, rendering the collagen production more suitable for clinical regeneration mediated

by hBMSCs. The results from this study indicate that modulation of hBMSCs by FGF-2 may provide clinical benefits in medical as well as dental fields.

REFERENCES

[1] Ishida Y, Kimura A, Kuninaka Y, Inui M, Matsushima K, Mukaida N, et al. Pivotal role of the CCL5/CCR5 interaction for recruitment of endothelial progenitor cells in mouse wound healing. The Journal of clinical investigation. 2012;122:711-21.

[2] Fang D, Seo BM, Liu Y, Sonoyama W, Yamaza T, Zhang C, et al. Transplantation of mesenchymal stem cells is an optimal approach for plastic surgery. Stem Cells. 2007;25:1021-8.

[3] Moseley TA, Zhu M, Hedrick MH. Adipose-derived stem and progenitor cells as fillers in plastic and reconstructive surgery. Plastic and reconstructive surgery. 2006;118:121S-8S.

[4] Higashiyama R, Nakao S, Shibusawa Y, Ishikawa O, Moro T, Mikami K, et al. Differential contribution of dermal resident and bone marrow-derived cells to collagen production during wound healing and fibrogenesis in mice. The Journal of investigative dermatology. 2011;131:529-36.

[5] Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med. 1994;1:71-81.

[6] Ishii G, Sangai T, Sugiyama K, Ito T, Hasebe T, Endoh Y, et al. *In vivo* characterization of bone marrow-derived fibroblasts recruited into fibrotic lesions. Stem Cells. 2005;23:699-706.

[7] Fathke C, Wilson L, Hutter J, Kapoor V, Smith A, Hocking A, et al. Contribution of bone marrow-derived cells to skin: collagen deposition and wound repair. Stem Cells. 2004;22:812-22.

[8] Deng W, Han Q, Liao L, Li C, Ge W, Zhao Z, et al. Engrafted bone marrow-derived flk-(1+) mesenchymal stem cells regenerate skin tissue. Tissue engineering. 2005;11:110-9.

[9] Sasaki M, Abe R, Fujita Y, Ando S, Inokuma D, Shimizu H. Mesenchymal stem cells are

recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. J Immunol. 2008;180:2581-7.

[10] Wu Y, Wang J, Scott PG, Tredget EE. Bone marrow-derived stem cells in wound healing: a review. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society. 2007;15.

[11] Li H, Fu X, Ouyang Y, Cai C, Wang J, Sun T. Adult bone-marrow-derived mesenchymal stem cells contribute to wound healing of skin appendages. Cell and tissue research. 2006;326:725-36.

[12] Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. Stem Cells. 2007;25:2648-59.

[13] McFarlin K, Gao X, Liu YB, Dulchavsky DS, Kwon D, Arbab AS, et al. Bone marrow-derived mesenchymal stromal cells accelerate wound healing in the rat. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society. 2006;14:471-8.

[14] Direkze NC, Forbes SJ, Brittan M, Hunt T, Jeffery R, Preston SL, et al. Multiple organ engraftment by bone-marrow-derived myofibroblasts and fibroblasts in bone-marrow-transplanted mice. Stem Cells. 2003;21:514-20.

[15] Hao L, Wang J, Zou Z, Yan G, Dong S, Deng J, et al. Transplantation of BMSCs expressing hPDGF-A/hBD2 promotes wound healing in rats with combined radiation-wound injury. Gene therapy. 2009;16:34-42.

[16] Falanga V, Iwamoto S, Chartier M, Yufit T, Butmarc J, Kouttab N, et al. Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds. Tissue engineering. 2007;13:1299-312.

[17] Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates.

Blood. 2003;101:2999-3001.

[18] Jung IH, Park JC, Kim JC, Jeon DW, Choi SH, Cho KS, et al. Novel application of human periodontal ligament stem cells and water-soluble chitin for collagen tissue regeneration: *in vitro* and *in vivo* investigations. Tissue engineering Part A. 2012;18:643-53.

[19] Song DS, Park JC, Jung IH, Choi SH, Cho KS, Kim CK, et al. Enhanced adipogenic differentiation and reduced collagen synthesis induced by human periodontal ligament stem cells might underlie the negative effect of recombinant human bone morphogenetic protein-2 on periodontal regeneration. Journal of periodontal research. 2011;46:193-203.

[20] Kim J, Park JC, Kim SH, Im GI, Kim BS, Lee JB, et al. Treatment of FGF-2 on stem cells from inflamed dental pulp tissue from human deciduous teeth. Oral diseases. 2013.

[21] Solchaga LA, Penick K, Goldberg VM, Caplan AI, Welter JF. Fibroblast growth factor-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone-marrow-derived mesenchymal stem cells. Tissue engineering Part A. 2010;16:1009-19.

[22] Marra KG, Defail AJ, Clavijo-Alvarez JA, Badylak SF, Taieb A, Schipper B, et al. FGF-2 enhances vascularization for adipose tissue engineering. Plastic and reconstructive surgery. 2008;121:1153-64.

[23] Kitamura M, Akamatsu M, Machigashira M, Hara Y, Sakagami R, Hirofuji T, et al. FGF-2 stimulates periodontal regeneration: results of a multi-center randomized clinical trial. Journal of dental research. 2011;90:35-40.

[24] Hayashida K, Akita S. Quality of pediatric second-degree burn wound scars following the application of basic fibroblast growth factor: results of a randomized, controlled pilot study. Ostomy/wound management. 2012;58:32-6.

[25] Akita S, Akino K, Imaizumi T, Hirano A. Basic fibroblast growth factor accelerates and improves second-degree burn wound healing. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society.

2008;16:635-41.

[26] Yonemitsu Y, Matsumoto T, Itoh H, Okazaki J, Uchiyama M, Yoshida K, et al. DVC1-0101 to treat peripheral arterial disease: a Phase I/IIa open-label dose-escalation clinical trial. Molecular therapy : the journal of the American Society of Gene Therapy. 2013;21:707-14.

[27] Solchaga LA, Penick K, Porter JD, Goldberg VM, Caplan AI, Welter JF. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. J Cell Physiol. 2005;203:398-409.

[28] Quarto N, Longaker MT. FGF-2 inhibits osteogenesis in mouse adipose tissue-derived stromal cells and sustains their proliferative and osteogenic potential state. Tissue engineering. 2006;12:1405-18.

[29] Park JC, Kim JM, Jung IH, Kim JC, Choi SH, Cho KS, et al. Isolation and characterization of human periodontal ligament (PDL) stem cells (PDLSCs) from the inflamed PDL tissue: *in vitro* and *in vivo* evaluations. Journal of clinical periodontology. 2011;38:721-31.

[30] Li B, Qu C, Chen C, Liu Y, Akiyama K, Yang R, et al. Basic fibroblast growth factor inhibits osteogenic differentiation of stem cells from human exfoliated deciduous teeth through ERK signaling. Oral diseases. 2012;18:285-92.

[31] Fang D, Seo B-M, Liu Y, Sonoyama W, Yamaza T, Zhang C, et al. Transplantation of Mesenchymal Stem Cells Is an Optimal Approach for Plastic Surgery. Stem Cells. 2007;25:1021-8.

[32] Kim YT, Park JC, Choi SH, Cho KS, Im GI, Kim BS, et al. The dynamic healing profile of human periodontal ligament stem cells: histological and immunohistochemical analysis using an ectopic transplantation model. Journal of periodontal research. 2012;47:514-24.

[33] Park JC, Kim JC, Kim YT, Choi SH, Cho KS, Im GI, et al. Acquisition of human alveolar bone-derived stromal cells using minimally irrigated implant osteotomy: *in vitro* and *in vivo* evaluations. Journal of clinical periodontology. 2012;39:495-505.

[34] Varas L, Ohlsson LB, Honeth G, Olsson A, Bengtsson T, Wiberg C, et al. Alpha10 integrin expression is up-regulated on fibroblast growth factor-2-treated mesenchymal stem cells with improved chondrogenic differentiation potential. Stem cells and development. 2007;16:965-78.

[35] Auletta JJ, Zale EA, Welter JF, Solchaga LA. Fibroblast Growth Factor-2 Enhances Expansion of Human Bone Marrow-Derived Mesenchymal Stromal Cells without Diminishing Their Immunosuppressive Potential. Stem Cells International. 2011;2011.

[36] Sun Y, Hu J, Zhou L, Pollard SM, Smith A. Interplay between FGF2 and BMP controls the self-renewal, dormancy and differentiation of rat neural stem cells. Journal of cell science. 2011.

[37] Gerstenfeld LC, Riva A, Hodgens K, Eyre DR, Landis WJ. Post-translational control of collagen fibrillogenesis in mineralizing cultures of chick osteoblasts. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 1993;8:1031-43.

[38] Maki JM, Rasanen J, Tikkanen H, Sormunen R, Makikallio K, Kivirikko KI, et al. Inactivation of the lysyl oxidase gene Lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice. Circulation. 2002;106:2503-9.

[39] Hornstra IK, Birge S, Starcher B, Bailey AJ, Mecham RP, Shapiro SD. Lysyl Oxidase Is Required for Vascular and Diaphragmatic Development in Mice. Journal of Biological Chemistry. 2003;278:14387-93.

[40] Hong HH, Pischon N, Santana RB, Palamakumbura AH, Chase HB, Gantz D, et al. A role for lysyl oxidase regulation in the control of normal collagen deposition in differentiating osteoblast cultures. J Cell Physiol. 2004;200:53-62.

[41] Kagan HM, Li W. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. Journal of cellular biochemistry. 2003;88:660-72.

[42] Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends in genetics : TIG. 2004;20:33-43.

[43] Maki JM, Sormunen R, Lippo S, Kaarteenaho-Wiik R, Soininen R, Myllyharju J. Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. Am J Pathol. 2005;167:927-36.

[44] Scarless Wound Healing. New York, NY, USA: Marcel Dekker; 2000.

[45] Ishii G, Sangai T, Ito T, Hasebe T, Endoh Y, Sasaki H, et al. *In vivo* and *in vitro* characterization of human fibroblasts recruited selectively into human cancer stroma. International journal of cancer Journal international du cancer. 2005;117:212-20.

[46] Desmouliere A, Chaponnier C, Gabbiani G. Tissue repair, contraction, and the myofibroblast. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society. 2005;13:7-12.

[47] Harty M, Neff AW, King MW, Mescher AL. Regeneration or scarring: an immunologic perspective. Developmental dynamics : an official publication of the American Association of Anatomists. 2003;226:268-79.

FIGURE LEGENDS

Figure 1. Characterization of BMSCs from fresh bone marrow of human vertebral body.

A) Isolated cells showed the ability to form CFU, which is well maintained during several passages (stained by crystal violet). B and C) Cell proliferation of hBMSCs at several passages was examined and doubling time was also calculated. D) Immunophenotype analysis of hBMSCs by flow cytometry. Cultured hBMSCs were incubated with specific monoclonal antibodies against the cell-surface marker antigens including CD105, CD73, CD90, CD44, and CD29, CD14, CD34, and CD45, followed by florescence conjugated secondary antibodies. E) hBMSCs in this study showed expression of surface antigens. E) *In vitro* multipotency of hBMSCs was examined. Osteogenic, adipogenic and chondrogenic differentiation were determined by Alizarin red, Oil red O, and Toluidine blue stains respectively. All the test (Test) group successfully induced specific differentiation responding to specific biologic stimuli while all the control (CTL) group which were cultured in normal growth media did not induce corresponding differentiations. F) Real-time PCR confirmed each differentiation at the mRNA level. All the specific mRNAs indicating each cell-lineage were expressed compared to the CTL group. Osteocalcin and RUNX2 were used for osteogenic differentiation, LPL and PPARr2 for adipogenic differentiation, and Aggrican, collagen IIA1, and SOX9 for chondrogenic differentiation. G) When hBMSCs were transplanted with HA/TCP carriers (HA),

histologic analysis using masson trichrome stain after 8 weeks revealed that they induced bone (B) and bone marrow (BM) like tissue regeneration. Positive immunohistochemical staining (red arrow head) with human specific mitochondria (hMito) indicate that cells and regenerated tissue are originated form transplanted cells (X400; scale bar = 100um).

Figure 2. Effect of FGF-2 treatment on the biologic activity of hBMSCs.

A) Under the microscopic observation, hBMSCs expanded with FGF-2 treatment showed the changed phenotype of smaller in size and less flattened morphology compared to that of hBMSCs without FGF-2 (0 ng/ml). B) MTT test at various time point showed that proliferative potency was significantly increased in hBMSCs expanded with FGF-2 in a dose-dependent manner. C) FGF-2 treatment enhanced the CFU efficiency of hBMSCs in terms of size and number of colony formed.

Figure 3. Enhancement of *in vitro* collagen tissue formation by hBMSCs expanded with FGF-2.

A) Assay for collagen production using Sircol assay kit showed that production of soluble collagen in a culture media gradually decreased while insoluble collagen deposition on the culture dishes was increased as the concentration of FGF-2 was increased. B) Hydroxyproline production as an indicator of collagen tissue showed similar pattern to both collagen types. C) Real-time PCR using collagen related genes

showed that mRNA expression of collagen type I, IV and V were decreased, while mRNA expression of collagen type II and III was significantly up-regulated. All the Lox family genes were increased with FGF-2 treatment with different level according to the type of collagen, suggesting that more mature type collagen tissue was produced and deposited when FGF-2 was added to hBMSCs.

Figure 4. Enhancement of *in vivo* collagen tissue formation by hBMSCs expanded with FGF-2.

A and B) Regeneration of collagen fibers at 8 weeks by hBMSCs *in vivo* was analyzed under microscopic observation. Masson trichrome staining showed that hBMSCs induced collagenous ECM (red arrowhead) regeneration with hyaluronic acid carrier. The capacity of collagen production was significantly enhanced in hBMSCs/FGF-2 group (magnification X400, scale bar = 100 um, * $P<0.05$). C) Immunohistochemistry of human specific mitochondria (hMito), hydroxyproline, and collagen types I (Col I) and III (Col III). The regenerated collagenous ECM was originated from hBMSCs transplanted and it was confirmed by immunohistochemical staining with an anti-hMito antibody. The newly formed collagen tissue showed distinct positive response to hydroxyproline, collagen type I and III. Expression of each antibody (red arrowhead) was more intensive in hBMSCs/FGF-2 group compared to hBMSCs (magnification X400, scale bar = 100 um).

FIGURES

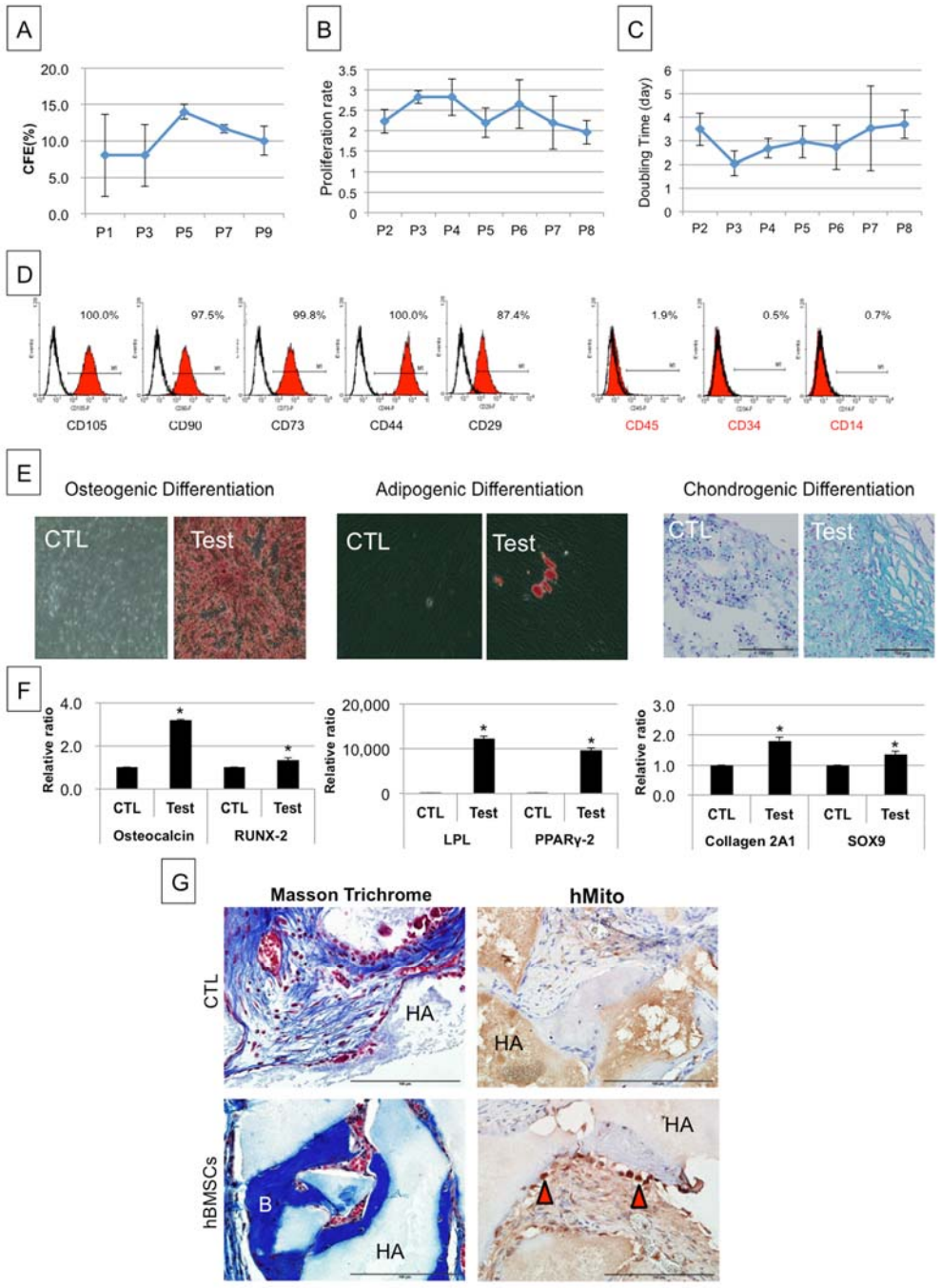

Figure 1

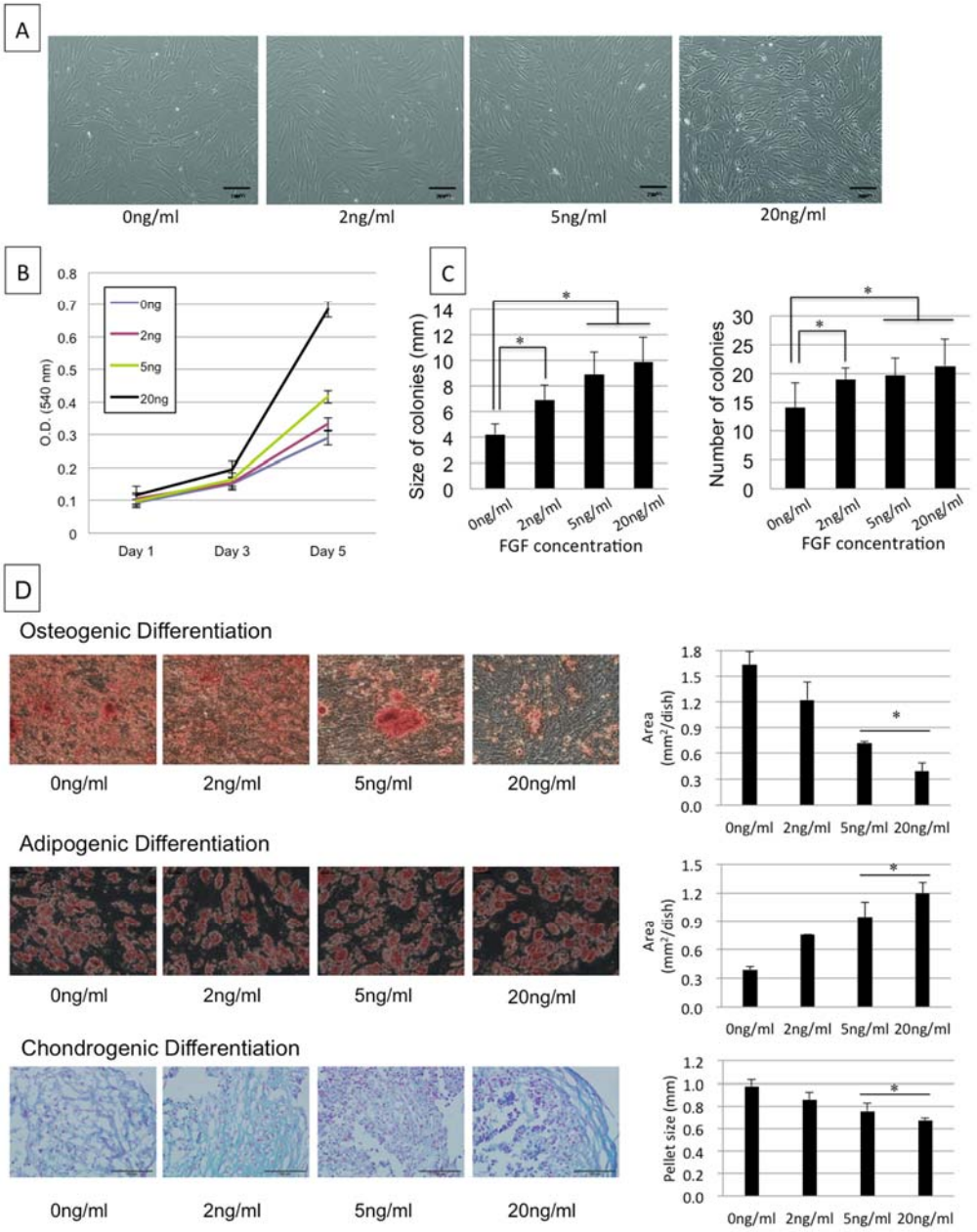
A
0ng/ml
2ng/ml
5ng/ml
20ng/ml
B
O.D. (540 nm)
0ng
2ng
5ng
20ng
Day 1
Day 3
Day 5
C
Size of colonies (mm)
Number of colonies
FGF concentration
FGF concentration
D
Osteogenic Differentiation
Adipogenic Differentiation
Chondrogenic Differentiation
Area (mm²/dish)
Area (mm²/dish)
Pellet size (mm)

Figure 2

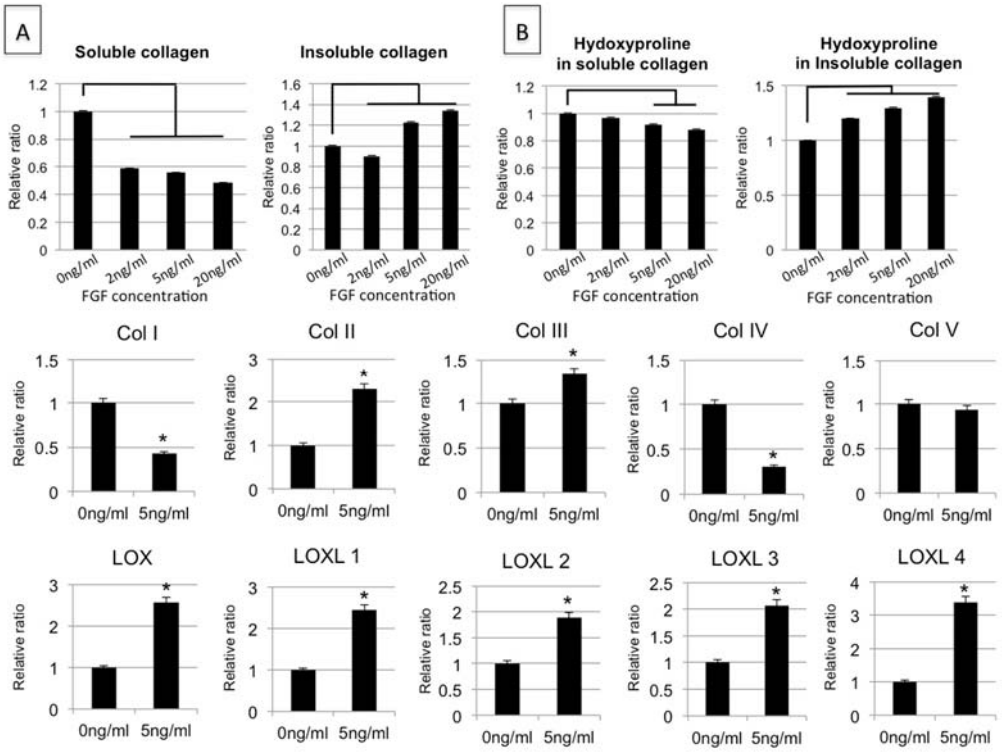
A
Soluble collagen
Insoluble collagen
B
Hydoxyproline in soluble collagen
Hydoxyproline in Insoluble collagen
Relative ratio
0ng/ml
2ng/ml
5ng/ml
20ng/ml
FGF concentration
Col I
Col II
Col III
Col IV
Col V
LOX
LOXL 1
LOXL 2
LOXL 3
LOXL 4
0ng/ml 5ng/ml

Figure 3

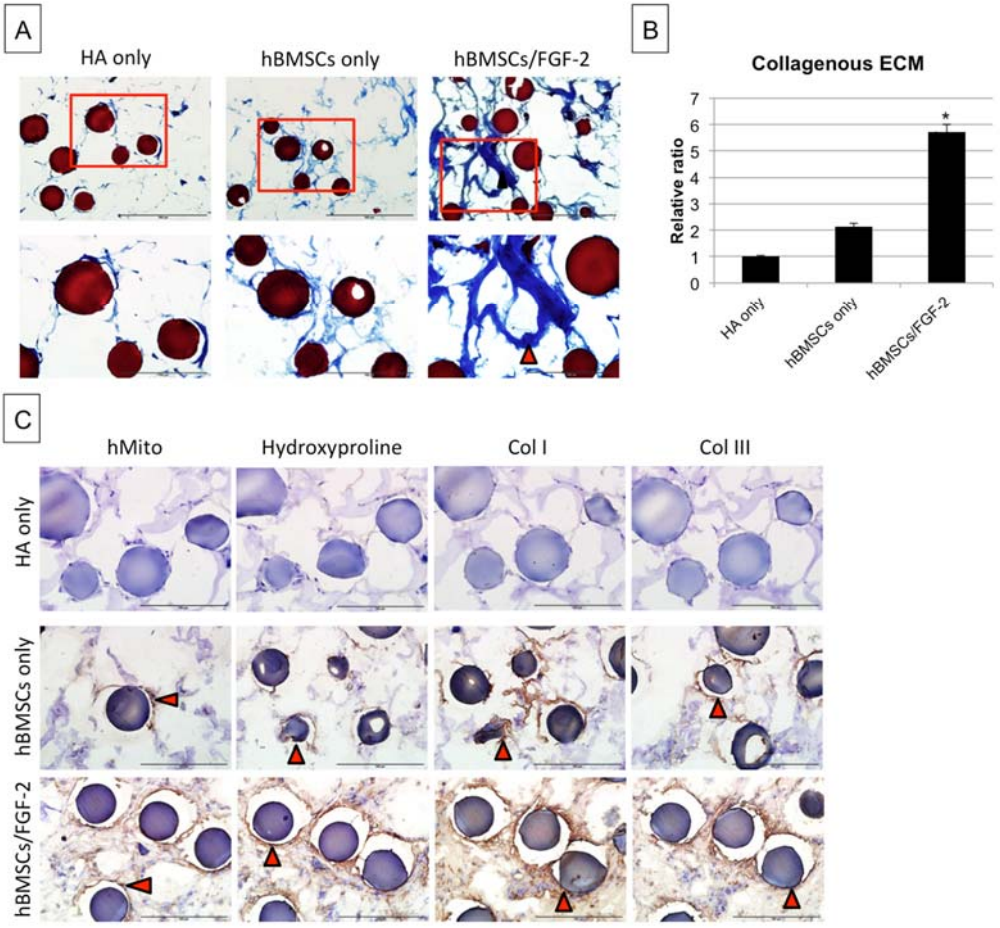
A
HA only
hBMSCs only
hBMSCs/FGF-2
B
Collagenous ECM
Relative ratio
0
1
2
3
4
5
6
7
*
HA only
hBMSCs only
hBMSCs/FGF-2
C
hMito
Hydroxyproline
Col I
Col III
HA only
hBMSCs only
hBMSCs/FGF-2

Figure 4

Table 1. Primer sequences

Gene (GenBank no.)	**Primer sequence** (forward and reverse)	**Size** (bp)
Collagen type Ⅰ	GAT CTG CGT CTG CGA CAA C (forward)	68
(NM_000088)	GGC AGT TCT TGG TCT CGT CA (reverse)	
Collagen type Ⅱ	GAA CAT CAC CTA CCA CTG CAA G(forward)	500
(NM_001844)	GCA GAG TCC TAG AGT GAC TGA G(reverse)	
Collagen type Ⅲ	GCC AAA TAT GTG TCT GTG ACT CA(forward)	145
(NM_000090)	GGG CGA GTA GGA GCA GTT G(reverse)	
Collagen type Ⅳ	ATG TCA ATG GCA CCC ATC AC(forward)	382
(NM_001845)	CTT CAA GGT GGA CGG CGT AG(reverse)	
Collagen type Ⅴ	ACG CGG CGA TCT TCC AAA G(forward)	387
(NM_000093)	GGA AAT GCA GAC GCA GGG TA(reverse)	
LOX	CGG CGG AGG AAA ACTGTC T(forward)	128
(NM_002317)	TCG GCT GGG TAA GAA ATC TGA(reverse)	
LOXL 1	CCA CRA CGA CCT ACT GGA TGC(forward)	97
(NM_005576)	GTT GCC GAA GTC ACA GGT G(reverse)	
LOXL 2	GGG TGG AGG TGT ACT ATG ATG G(forward)	137
(NM_002318)	CTT GCC GTA GGA GGA GCT G(reverse)	
LOXL 3	CGA GCA GAG TGT GAC TGA ATG(forward)	147
(NM_032603.2)	CTC CTC CAC TTG CAG GTG AT(reverse)	
LOXL 4	CTG GGC ACC ACT AAG CTC C(forward)	119
(NM_032211.6)	CTC CTG GAT AGC AAA GTT GTC AT(reverse)	
β-actin	CAT GTA CGT TGC TAT CCA GGC(forward)	250
(NM_001101)	CTC CTT AAT GTC AACG CAC GAT(reverse)	
LPL	TGG ACT GGC TGT CAC GGG CT(forward)	167
(NM_000237.2)	GCC AGC AGC ATG GGC TCC AA(reverse)	
PPARγ2	ACA GCA AAC CCC TAT TCC ATG CTG T(forward)	159
(NM_015869.4)	TCC CAA AGT TGG TGG GCC AGA A(reverse)	
Runx-2	CAC TGG CGC TGC AAC AAG A(forward)	127
(NM_004348)	CAT TCC GGA GCT CAG CAG AAT AA(reverse)	
Osteocalcin	CAA AGG TGC AGC CTT TGT GTC(forward)	150
(X53698)	TCA CAG TCC GGA TTG AGC TCA(reverse)	
Aggrecan	AGG GCG AGT GGA ATG ATG TT(forward)	68
(NM_001135.3)	GGT GGC TGT GCC CTT TTT AC(reverse)	
collagen 2A1	CTG CAA AAT AAA ATC TCG GTG TTC T(forward)	101
033150.2)	GGG CAT TTG ACT CAC ACC AGT(reverse)	
SOX9	CTT TGG TTT GTG TTC GTG TTT TG(forward)	101
(NM_000346.3)	AGA GAA AGA AAA AGG GAA AGG TAA GTT T(reverse)	
GAPDH	GTG GTG GAC CTG ACC TGC (forward)	208
(NM_002046.3)	TGA GCT TGA VAA AGT GGT C(reverse)	

ABSTRACT (IN KOREAN)

사람 골수 줄기세포 유도 콜라겐 조직 재생에 미치는 2형 섬유아세포 성장인자의 역할

<지도교수 김 창 성>

연세대학교 대학원 치의학과

박 동 수

목적 : 골수 줄기세포의 콜라겐 조직 재생은 창상 치유의 필수적 요소로서 다양한 생물학적 자극요소에의해 변화될 수 있다. 본 연구의 목적은 사람 골수 줄기세포로 유도되는 콜라겐 조직 재생에 미치는 2형 섬유아세포성장인자의 효과를 다양한 연구모델을 이용하여 분석하는 것이다.

재료 및 방법 : 본 연구에서 사용된 사람 골수줄기세포는 척추 수술시 얻어지는 척추골 골수로부터 분리하였다. 분리된 세포의 줄기세포특성을 확인하기위하여 다양한 실험 모델을 이용하여 특성 분석을 시행하였다. 사람 골수 줄기세포의 특성 변화에 미치는 2형 섬유아세포성장인자의 처치 효과를 다양한 농도(0, 2, 5, 20 ng/ml)를 이용하여 심도있게 분석하였다. 2형 섬유아세포성장인자가 사람

골수 줄기세포의 콜라겐 조직 재생에 미치는 효과를 분석하기위하여 실험실적 연구 모델을 이용하여 가용성/비가용성 콜라겐과 상응하는 하이드록시프로린 합성을 분석하였고 실시간 중합효소 연쇄반응분석법을 이용하여 종류별 콜라겐과 록스 유전자군의 발현을 측정하였다. 생체내 콜라겐 조직 재생을 분석하기위하여 콜라겐 재생 분석을 위한 생체내 분석 모델을 이용하여 사람 골수줄기세포를 이식하였다. 이식 후 8주에 이식물을 채취하고 조직학적 그리고 면역조직화학적 분석을 시행하였다.

결과 : 본 연구에서 사용된 사람 골수 줄기세포는 간엽줄기세포의 특성을 잘 나타내었다. 골수 줄기세포의 특성에 미치는 2형 섬유아세포성장인자의 효과는 콜로니 형성능, 증식, 그리고 실험실적 분화 측면에서 이전에 보고된 연구 결과들과 일치하는 결과를 나타내었다. 비가용성 콜라겐의 합성능은 2형 섬유아세포성장인자의 처치에의해 향상되었지만 가용성 콜라겐은 감소되었는데 2형 섬유아세포성장인자의 농도 의존성을 나타내었다. 콜라겐 형성과 상응하는 하이드록시프로린 형성도 비슷한 양상의 결과를 나타내었다. 종류별 콜라겐의 유전자 발현은 콜라겐 종류마다 다른 변화를 보였다. 2형 섬유아세포성장인자는 I, IV과 V형 콜라겐 유전자 발현은 감소시키지만 II와 III형 콜라겐 유전자의 발현은 증가시켰다. 모든 록스 유전자군의 발현은 2형 섬유아세포성장인자에 의해 향상되었다. 이식 후 8주에 시행된 조직학적 그리고 면역조직화학적 분석 결과에서 사람 골수 줄기세포는 2형 섬유아세포성장인자에 의해 유의하게

증가된 양의 콜라겐 형성을 유도하였고 보다 성숙된 구조의 콜라겐이 형성됨을 확인하였다. ($p< 0.05$)

결론: 2형 섬유아세포성장인자는 사람 골수줄기세포의 줄기세포학적 성격과 콜라겐 형성능을 향상시키므로 콜라겐 형성이 매개되는 사람골수줄기세포의 창상치유에 효과적으로 적용할 수 있을 것이다.

핵심되는 말 : Fibroblast Growth Factor-2, human bone marrow stem cell, collagen tissue regeneration, wound healing, colony forming unit, proliferation, differentiation