Stem Cell Research 28 (2018) 87–95

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Enhancing proliferation and optimizing the culture condition for human bone marrow stromal cells using hypoxia and fibroblast growth factor-2

Jung-Seok Lee [a], Seul Ki Kim [a], Byung-Joo Jung [b], Seong-Bok Choi [b], Eun-Young Choi [a], Chang-Sung Kim [a,c,*]

[a] Department of Periodontology, Research Institute for Periodontal Regeneration, College of Dentistry, Yonsei University, Seoul, Republic of Korea
[b] Department of Neurosurgery, Naeun Hospital, Seoul, Republic of Korea
[c] Department of Applied Life Science, BK21 PLUS Project, College of Dentistry, Yonsei University, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 16 February 2017
Received in revised form 28 December 2017
Accepted 6 January 2018
Available online 9 January 2018

Keywords:
Bone marrow stromal cells
Hypoxia
Fibroblast growth factor
Tissue regeneration
Microenvironment interactions

ABSTRACT

This study aimed to determine the cellular characteristics and behaviors of human bone marrow stromal cells (hBMSCs) expanded in media in a hypoxic or normoxic condition and with or without fibroblast growth factor-2 (FGF-2) treatment. hBMSCs isolated from the vertebral body and expanded in these four groups were evaluated for cellular proliferation/migration, colony-forming units, cell-surface characterization, *in vitro* differentiation, *in vivo* transplantation, and gene expression. Culturing hBMSCs using a particular environmental factor (hypoxia) and with the addition of FGF-2 increased the cellular proliferation rate while enhancing the regenerative potential, modulated the multipotency-related processes (enhanced chondrogenesis-related processes/osteogenesis, but reduced adipogenesis), and increased cellular migration and collagen formation. The gene expression levels in the experimental samples showed activation of the hypoxia-inducible factor-1 pathway and glycolysis in the hypoxic condition, with this not being affected by the addition of FGF-2. The concurrent application of hypoxia and FGF-2 could provide a favorable condition for culturing hBMSCs to be used in clinical applications associated with bone tissue engineering, due to the enhancement of cellular proliferation and regenerative potential.

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1. Introduction

While the biologic responses elicited by environmental stimuli vary according to the target cells and the underlying biologic processes, many researchers in the field of cell biology have been focusing on the innate potential of various cell types (Park et al., 2011; Seo et al., 2004; Takahashi and Yamanaka, 2006). There has been particular interest in development and regeneration at the stem-cell level, stem cells of specific tissues, and reprogrammed pluripotent stem cells. Cells are controlled by their microenvironment, which includes the external stimuli, extracellular matrix, and signals from biologic processes. The environmental condition around cells can regulate and control their characteristics and behaviors (J. S. Lee et al., 2015; Rezza et al., 2014; Simon and Keith, 2008). The hypoxic condition is one of the important factors influencing the physiologic biology of stem cells and the pathologic biology of cancer cells. The environmental condition can affect cell growth depending on the cell type: while a lack of oxygen availability due to the enormously rapid proliferation of cancer cells would increase the risk of metastasis or mortality (Gilkes et al., 2014; Vaupel et al., 2004), this condition would maintain the potentiality of multidirectional differentiation and self-renewal of stem-cell niches (Maltepe and Simon, 1998; Simon and Keith, 2008). The hypoxic condition is also known to be a favorable physiologic microenvironment for the development of embryos and for the regulation of adult stromal cells. Since Morriss and New (1979) first reported the importance of the oxygen level in the *ex utero* development of the mouse embryo, some researchers have investigated how the hypoxic condition maintains and regulates the multipotency of cells during culturing (Fehrer et al., 2007; Fotia et al., 2015).

Previous studies found that an oxygen level of 20% induced genetic injury, cell senescence, and finally the loss of cell viability (Busuttil et al., 2003; Chen et al., 1995; Parrinello et al., 2003), whereas reducing the oxygen concentration to the physiologic level (2–9%) could improve the genetic stability against cell aging and alter the direction of cell differentiation in stem-cell biology (Fehrer et al., 2007; J. S. Lee et al., 2015). Low oxygen availability can modify basic cell metabolism and also enhance various pathways related to hypoxia-inducible factors (HIFs), which can be removed *via* degradation in the presence of excess oxygen. HIF-1 is especially well known to influence the transportation of glucose by cells, angiogenesis, growth-factor signaling, cellular migration, and the growth and survival of cells (Giaccia et al., 2004;

* Corresponding author at: Department of Applied Life Science, BK21 PLUS Project, College of Dentistry, Yonsei University, 50-1, Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea.
E-mail address: dentall@yuhs.ac (C.-S. Kim).

https://doi.org/10.1016/j.scr.2018.01.010
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Schofield and Ratcliffe, 2004). Many previous studies have emphasized the importance of this protein and the oxygen level in stem-cell biology for maintaining the self-renewal potential in stem-cell niches, the homing/migration of stem cells in wound-healing and inflammatory processes, and the specific direction of cell differentiation (Simon and Keith, 2008). Therefore, creating a hypoxic condition when culturing bone marrow stromal cells has been a focus of research into possible therapeutic interventions (J. S. Lee et al., 2015).

Clinical applications of stem cell grafts require tremendous numbers of target cells to be produced in *ex vivo* expansion without losing their regenerative potential. Achieving this requires an understanding of the underlying mechanisms by mimicking the physiologic biology of stem cells from quiescent stages in niches to proliferation/differentiation in the wound-healing process. In the early phase wound healing, HIFs enhance fibroblast growth factor-2 (FGF-2) and finally increase cellular proliferation. In an *in vitro* cell culture, the simultaneous use of a hypoxic condition and FGF-2 treatment would be expected to exert synergistic effects on the characteristics and behaviors of bone marrow stromal cells, even though FGF-2 can also act as a morphogen on the cells and hinder hypoxia-induced cellular responses. Therefore, the present study aimed to determine the characteristics and behaviors of cells expanded in media in a hypoxic or normoxic condition and with or without FGF-2 treatment.

2. Methods

2.1. Isolation and culture of human bone marrow stromal cells from human vertebral bodies

This study involved five volunteer patients (one male, four females; age range 41–58 years, median age, 46 years) who provided informed consent to participate based on guidelines approved by the Institutional Review Board, College of Dentistry, Yonsei University, Seoul, Korea (approval no. 2-2012-0048). Mononuclear cells were obtained by centrifugation with a density-gradient solution (Ficoll-Paque Plus, GE Healthcare Bio-Sciences, Uppsala, Sweden) from bone marrow aspirates that had been harvested directly from the vertebral body using a specific type of percutaneous puncture needle (VP Needle, OKMedinet Korea, Seoul, Korea) during vertebral surgery (Naeun Hospital, Anyang, Korea). The cells were seeded at a nonclonal density of 1000 cells/μl with media containing alpha-minimum essential medium (α-MEM; Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Gibco), 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 2 mM GlutaMAX-1 100× (Invitrogen, Carlsbad, CA, USA), and 20 μg/ml gentamicin (Gibco).

2.2. Inducing a hypoxic condition and applying FGF-2 treatment

Isolated cells were cultured in the following four conditions according to the oxygen level and FGF-2 treatment: normoxia/FGF-2(−), normoxia/FGF-2(+), hypoxia/FGF-2(−), and hypoxia/FGF-2(+). Hypoxia was induced by placing human bone marrow stromal cells (hBMSCs) in a chamber containing 1% O_2 at 37 °C (note that in normoxia, the chamber contained 21% O_2). The cells for the two experimental groups with FGF-2 treatment were cultured in the presence of 5 ng/ml FGF-2 (Genoss, Seoul, Korea). The culture media was replaced every 2 days, which included the addition of fresh FGF-2, and cells in both hypoxia groups were removed from the hypoxic chamber for feeding but then immediately replaced in the chamber thereafter. All of the cultures were incubated at 37°C in 5% CO_2. Colony formation in each culture was observed with the aid of a light microscope (CKX41, Olympus Optical, Tokyo, Japan). Adherent hBMSC-like cells were obtained after 2 weeks of culture. Cells at passage 2 (P2) to P8 were used in the further analyses.

2.3. Comparison of hBMSC characteristics between groups

2.3.1. Assay of colony-forming efficiency

The colony-forming efficiency was evaluated based on the formation of secondary colonies in which cells at P6 were plated at 1×10^2 cells/dish onto 60-mm culture dishes (Nunc, Roskilde, Denmark) with culture media. They were fixed 10–14 days later with 10% neutral buffered formalin and then stained with crystal violet (Sigma-Aldrich). The number and size of colonies were calculated with the aid of a light microscope in order to compare the colony-forming efficiencies.

2.3.2. Surface marker expression using fluorescence-activated cell sorting

Cell-surface-marker characterization was applied to cells at P4 to further characterize the isolated hBMSCs using flow cytometry analysis [fluorescence-activated cell sorting (FACS)], the procedure for which is described in detail elsewhere (Shim et al., 2016). Harvested cells were transferred to a 15-ml tube (50015, SPL, Pocheon, Gyeonggido, Korea) and washed with phosphate-buffered saline (PBS; 10010-023, Gibco). Primary antibodies were conjugated with fluorescent protein raised against CD105, CD29, CD45, CD34, and CD14 (A07414, IM0791U, A07783, IM1870, and IM0645U, respectively; Beckman Coulter, Brea, California, USA) and CD44, CD73, and HLA-DR (555479, 550257, and 555611, respectively; BD Biosciences, Franklin Lakes, NJ, USA) for 1 h at room temperature. Two types of surface markers were selected for distinguishing adherent progenitor cells from mature cells (CD29, CD44, CD73, and CD105) and from hematopoietic/immunogenic cells (CD14, CD34, CD45, and HLA-DR). The cells were then washed with PBS three times and observed with a flow cytometer (CYTOMICS FC 500, Beckman Coulter).

2.3.3. In vitro differentiation assay: osteogenic, adipogenic, and chondrogenic induction

Cells at P5 were seeded onto six-well plates at 1×10^5 cells/well and cultured until they reached subconfluence. The protocols for *in vitro* differentiation were used based on a previous study (J. S. Lee et al., 2015). Briefly, the culture medium for osteogenic differentiation comprised α-MEM (Gibco) containing 10% FBS (Gibco), 20 μg/ml gentamicin (Gibco), 2 mM GlutaMAX-1 100× (Invitrogen), 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 0.01 μM dexamethasone (Sigma-Aldrich), and 1.8 mM potassium phosphate monobasic (Sigma-Aldrich), and was refreshed every 3–4 days. After 2–3 weeks of induction, mineralized nodule formation was confirmed as a marker of osteogenic differentiation by staining with alizarin red. The culture medium for adipogenic differentiation comprised α-MEM containing 10% FBS, 20 μg/ml gentamicin, 2 mM GlutaMAX-1 100×, 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), and 10 μg/ml insulin (Sigma-Aldrich), and was refreshed every 3–4 days. After 3 weeks of induction, the cells were stained with Oil Red O stain in order to visualize lipid droplets. Chondrogenic differentiation was induced by seeding cells at P5 into 15-ml polypropylene tubes at 4×10^5 cells/tube to prevent cell adhesion, followed by centrifugation at 150*g* for 5 min to form a pellet. The culture medium for chondrogenic differentiation comprised α-MEM (Gibco), 20 μg/ml gentamicin (Gibco), 1% insulin-transferrin-selenium (BD Biosciences), 0.1 μM dexamethasone (Sigma-Aldrich), 172 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 40 μg/ml proline (Sigma-Aldrich), and 10 ng/ml transforming growth factor-β3 (R&D Systems, Minneapolis, MN, USA), and was refreshed every 3–4 days. After 3 weeks of induction with the chondrogenic differentiation media, the pellets were fixed using 4% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. The sections were stained with toluidine blue to assess chondrogenic differentiation.

The formation of mineralized tissue was quantitatively evaluated by analyzing hydroxyapatite deposition using the Osteoimage Mineralized Assay kit (Lonza, Walkersville, MD, USA), a fluorescence plate reader

(Varioskan® Flash, Thermo Scientific, Waltham, MA, USA), and Skan-It software (version 2.4.3). In addition, a colorimetric assay for quantifying alizarin red staining was performed, wherein stained cells were incubated with a solution of 20% methanol and 10% acetic acid under shaking conditions for 30 min. Supernatants containing dissolved calcium from the cultured cells were then quantified by measuring the absorbance at 450 nm using an ELISA microplate spectrophotometer (K-BTK-EpochTak, BioTek, Winooski, VM, USA). The histomorphometric analysis involved measuring the total areas of lipid vacuole formation and chondrogenesis using an automated image-analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA).

2.3.4. In vivo regenerative potential of hBMSCs using an ectopic transplantation model

In vivo transplantation was performed to evaluate the regenerative capacity of hBMSCs. Hydroxyapatite/tricalcium phosphate (HA/TCP)-based scaffolds were used as the cell carrier. For single transplantations, 6×10^6 cells at P6 were loaded with 80 mg of HA/TCP ceramic powder (MBCP, Biomatlante, Vigneux, France) and then transplanted into subcutaneous pockets formed on the dorsal surface of immunocompromised mice (BALB/cSlc-nu, Japan SLC, Hamamatsu, Japan; 8 samples in 4 mice for each group). 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 the Medical College, Yonsei University.

The mice were euthanized after 8 weeks of recovery, and the implants were harvested and fixed in 10% neutral buffered formalin for 3 days. The specimens were decalcified using 5% EDTA (pH 7.2) and then embedded in paraffin. The tissue was dissected to obtain the two most-central 6-μm-thick sections, which were stained with Masson's trichrome. In ten randomly selected high-magnification (×100) images (two from the central region and two from each of the four corners on the cross-sectional image of the grafted area), three types of tissue could be found along with the residual biomaterial within the transplants: mineralized tissue, fibrous connective tissue, and bone marrow-like tissue (cell-rich zone). Two specific areas of bone formation (mineralized tissue area) and bone marrow-like tissue (cell-rich zone around or between mineralized tissues) were measured (Fang et al., 2007; Miura et al., 2006) using image-processing computer software (Photoshop CS5, Adobe, San Jose, CA, USA).

2.4. Cell proliferation assay

The proliferation rate of hBMSCs was assessed using the MTT assay and calculating the doubling time (DT). In each passage, cell growth was monitored by counting the number of cells using a hemocytometer and trypan blue (for excluding necrotic cells), and DT was calculated as $(t2–t1) \times \log(2)/\log(q2/q1)$, where $q1$ and $q2$ are the quantities that had grown at times $t1$ and $t2$, respectively. For performing the MTT assay at a specific time point, 4×10^4 cells at P5 were plated onto 6-well plates and cultured with the medium for each of the 4 groups for 5 days. After incubation with 10% MTT solution (5 mg/ml; Amresco, Solon, OH, USA) and each culture medium for 4 h, the supernatant was discarded, and the absorbance was measured at 570 nm using a microplate spectrophotometer (EPOCH, BIOTEK, Winooski, VT, USA).

2.5. Cell migration assay

hBMSCs (1.5×10^6) were seeded onto 60-mm-diameter culture dishes (BD Falcon, BD Sciences) and cultured to confluence. The cells were incubated with 5 μg/ml mitomycin C (Acros Organics, Waltham, MA, USA) to limit the effects of cellular proliferation. The monolayer cells on the dishes were scratched to make a cell-free zone, and floating cells were removed by rinsing with PBS. The distance reached by cells that migrated over the following 72 h was measured with the aid of a light microscope.

2.6. Collagen-related gene expression

Gene expression related to the formation or maturation of collagen was quantified using COL I–III for the formation of collagen and the LOX family for the cross-linking of collagen fibrils.

2.7. Quantitative real-time polymerase chain reaction

The quantitative real-time polymerase chain reaction (PCR) was performed to further confirm the other *in vitro* and *in vivo* results. Cells were harvested using Trizol (GeneAll, Seoul, Korea), and total cell RNA was prepared. Isolated total RNA was used as a template for the synthesis of cDNA with oligo dT primer (High-Capacity RNA-to-cDNA Kit, Applied Biosystems, Foster City, CA, USA). The subsequent PCR amplification was performed using a standard protocol with an SYBR real-time PCR kit (Power SYBR Green PCR Master Mix, Life Technologies, Warrington, UK), the Step One Real-Time PCR system and associated software (Applied Biosystems), and specific primers of Apo1 (NM_000043.4), p21 (NM_000389.4), BMP-2 (NM_001200.2), LPL (NM_000237.2), SOX9 (NM_000346.3), Aggrecan (NM_001135.3), VCAN (NM_004385.4), COL I–III (NM_000088, NM_033150.2, and NM_000090), LOX (NM_002317), and LOX 1–3 (NM_005576, NM_002318, and NM_032603.2). After an initial polymerase activation step at 95°C for 10 min, the samples underwent 40 amplification cycles, each comprising denaturation at 95°C for 15 s, and annealing and extension at 60°C for 60 s. The real-time PCR data were analyzed using the comparative CT method. The relative expression levels of mRNA were quantified by comparison with an internal standard (B2M; NM_004048.2) that is known to be a stable housekeeping gene regardless of the oxygen level. Each PCR was performed in triplicate with the same total RNA.

2.8. Statistical analysis

Two-way analysis of variance was used to evaluate the effects of the two factors (two oxygen levels and two conditions related to the use of FGF-2) in all analyses except for microarray gene expression profiling. Differences were considered statistically significant when $p < 0.05$.

2.9. Microarray gene expression profiling

To evaluate cellular activity in each group based on gene-expression profiling from eight types of cells in four groups of two individual samples, a bead-based microarray analysis (Human HT12 v4, Illumina, San Diego, CA, USA) was performed in accordance with a previously described protocol (J. S. Lee et al., 2015). Briefly, total RNA extraction and purification were achieved using Trizol (Invitrogen) and the RNeasy Mini kit (Qiagen, Hilden, Germany), and DNase digestion was performed to eliminate DNA contamination. RNAs were amplified using an amplification kit (Ambion, Waltham, MA, USA) in accordance to the manufacturer's instructions to yield biotinylated cRNA. After purification, the cRNA was quantified using a spectrophotometer (ND-1000, NanoDrop, Wilmington, DE, USA). Labeled cRNA samples were hybridized onto a bead-array chip according to the manufacturer's instructions, and the signals were detected/scanned by Amersham FluoroLink streptavidin-Cy3 (GE Healthcare, Buckinghamshire, UK) and an Illumina Bead Array Reader confocal scanner.

Data from the bead-based microarrays were processed using the Lumi package with background correction, normalization (quantile method), and gene annotation. Analyses were performed using the BRB-Array Tools software developed by Dr. Richard Simon and the BRB-Array Tools Development Team, and SAM (significance analysis of microarrays) was applied. A previously reported direct transcriptional target gene set of HIFs (Schofield and Ratcliffe, 2004) and the gene-set-related glycolysis/gluconeogenesis from the KEGG (Kyoto Encyclopedia of Genes and Genomes) database were used in these analyses.

3. Results

3.1. Assay of colony-forming efficiency

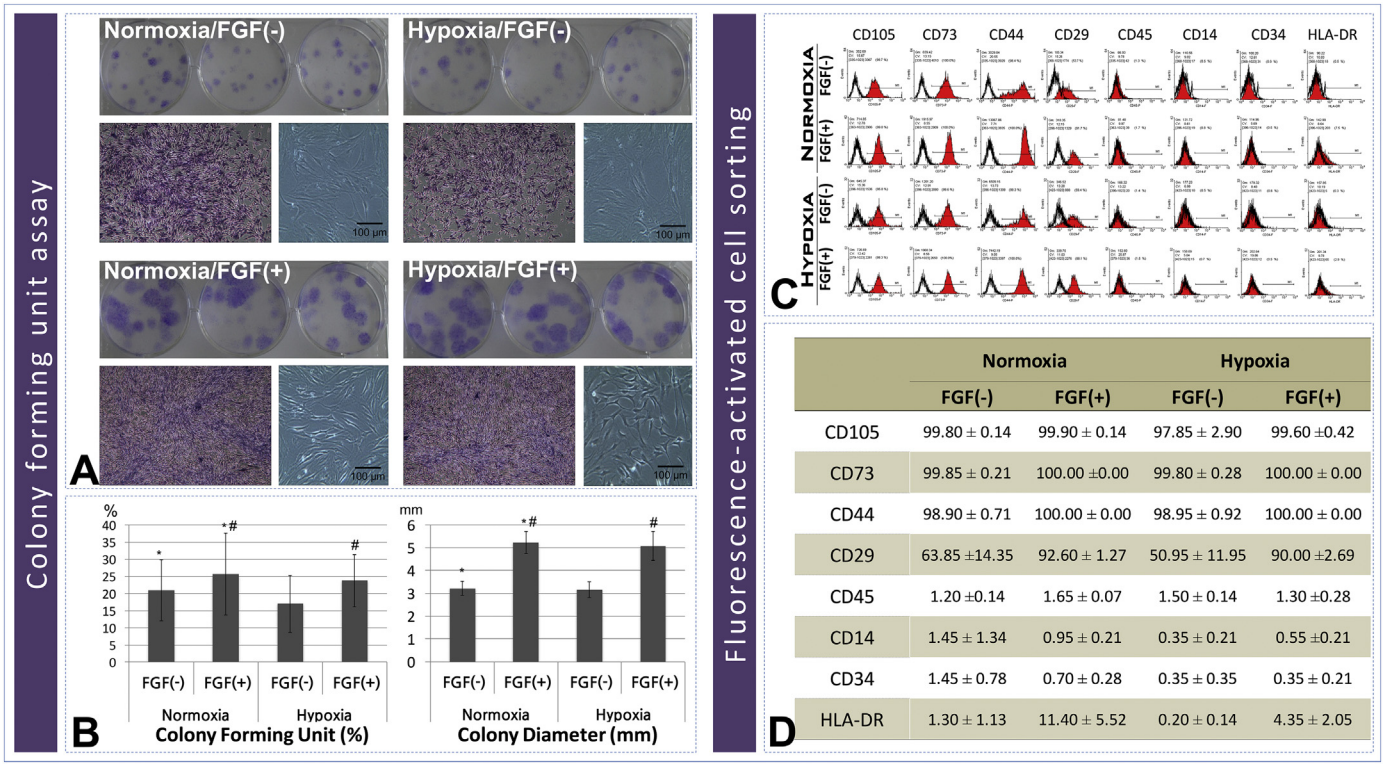
Various numbers of plastic adherent colonies of various sizes were formed from the suspended single cells depending on the oxygen concentration and FGF-2 treatment in the culture environment (Fig. 1A and B). The number of colonies was lower for cells cultured without FGF-2 treatment in the hypoxia group than in the normoxia group (17.00 ± 8.28% and 20.89 ± 8.88%, respectively; mean ± SD); however, the sizes of the colonies did not differ significantly with the oxygen concentration (3.15 ± 0.34 and 3.21 ± 0.30 mm). FGF-2 treatment significantly increased the number of colonies [25.67 ± 11.95% and 23.78 ± 7.66% for the normoxia/FGF-2(+) and hypoxia/FGF-2(+) groups, respectively] and their size (5.22 ± 0.48 and 5.07 ± 0.64 mm, correspondingly) and the density of cells within a colony, compared to both the normoxia and hypoxia groups without FGF-2 treatment. All of the specimens exhibited spindle-shaped cells in highly magnified views, and a particularly interesting finding was that cells cultured with FGF-2 treatment showed clearly distinguishable cellular margins with a pericellular halo.

3.2. Surface marker expression (FACS)

All cells cultured in the four types of environment showed positivity for CD29, CD44, CD73, and CD105 and negativity for hematopoietic/immune cell markers (CD14, CD34, CD45, and HLA-DR). FGF-2 treatment enhanced the expression levels of CD29 and HLA-DR in both oxygen levels: CD29 expression was increased from 63.85 ± 14.35% to 92.60 ± 1.27% in normoxia and from 50.95 ± 11.95% to 90.00 ± 2.69% in hypoxia; the corresponding changes for HLA-DR were from 1.30 ± 1.13% to 11.40 ± 5.52% and from 0.20 ± 0.14% to 4.35 ± 2.05%. However, the increased amount of HLA-DR was reduced under hypoxia. Except for CD29 and HLA-DR, the use of FGF-2 did not modify the cell-surface markers. These patterns were also observed for cells from another passage (Fig. 1C and D).

3.3. In vitro differentiation

The multidirectional differentiation capacity was evaluated by inducing three types of cells from hBMSCs in each condition. Alizarin-red-positive mineral depositions were significantly enhanced in cells cultured at the low oxygen level, but this was decreased by FGF-2 treatment, whereas Oil-Red-O-positive vacuoles were significantly reduced and could be minimally observed in the cells at the low oxygen level. These results were in accordance with BMP-2 gene expression increasing in the hypoxic condition, while the ALPL and Runx2 gene expression levels were significantly reduced, regardless of the presence or absence of FGF-2 treatment (Fig. 2A). However, the expression levels of all of the adipogenic genes (LPL and AP2) were in agreement with the results from the *in vitro* induction assay, and were significantly decreased in samples cultured in the hypoxic condition (Fig. 2B). Exceptionally, PPAR-γ was significantly enhanced in cells cultured in hypoxia and with FGF-2. There was significantly more glycosaminoglycan formation within the pellet in the low-oxygen condition or with FGF-2 treatment, with increased pellet size and a hyperchromic shift. Although no cartilage formation was observed when using the late passage of hBMSCs regardless of the environmental conditions, hypoxia and FGF-2 treatment enhanced the formation of chondrogenesis-related products. The expression levels of related genes (SOX9, Col2A1, and Aggrecan) were increased in cells cultured in the hypoxic condition, with the expression of



	Normoxia		Hypoxia	
	FGF(-)	FGF(+)	FGF(-)	FGF(+)
CD105	99.80 ± 0.14	99.90 ± 0.14	97.85 ± 2.90	99.60 ±0.42
CD73	99.85 ± 0.21	100.00 ±0.00	99.80 ± 0.28	100.00 ± 0.00
CD44	98.90 ± 0.71	100.00 ± 0.00	98.95 ± 0.92	100.00 ± 0.00
CD29	63.85 ±14.35	92.60 ± 1.27	50.95 ± 11.95	90.00 ±2.69
CD45	1.20 ±0.14	1.65 ± 0.07	1.50 ± 0.14	1.30 ±0.28
CD14	1.45 ± 1.34	0.95 ± 0.21	0.35 ± 0.21	0.55 ±0.21
CD34	1.45 ± 0.78	0.70 ± 0.28	0.35 ± 0.35	0.35 ± 0.21
HLA-DR	1.30 ± 1.13	11.40 ± 5.52	0.20 ± 0.14	4.35 ± 2.05

D

Fig. 1. Results from the colony-forming assay and fluorescence-activated cell sorting. (A and B) Cells cultured under a low oxygen level without FGF-2 treatment [hypoxia/FGF(−)] showed a slightly reduced number of colony-forming units compared to the normoxia/FGF(+) group. FGF-2 treatment significantly enhanced the proportion and size of colonies formed in both the normoxia and hypoxia conditions ($p < 0.001$). Smaller cells were observed with reduced cellular processes in highly-magnified views of cultured cells under hypoxia compared to normoxia, regardless of whether or not FGF-2 was applied. (C and D) All cells under the four conditions showed positivity for hBMSC-related markers (CD29, CD44, CD73, and CD105) and negativity for hematopoietic/immune cell markers (CD14, CD34, CD45, and HLA-DR). A restricted supply of oxygen reduced the proportion of HLA-DR-positive cells, despite the enhancement by FGF-2 treatment. *Statistically different from the hypoxic condition. #Statistically different from culturing without FGF-2.

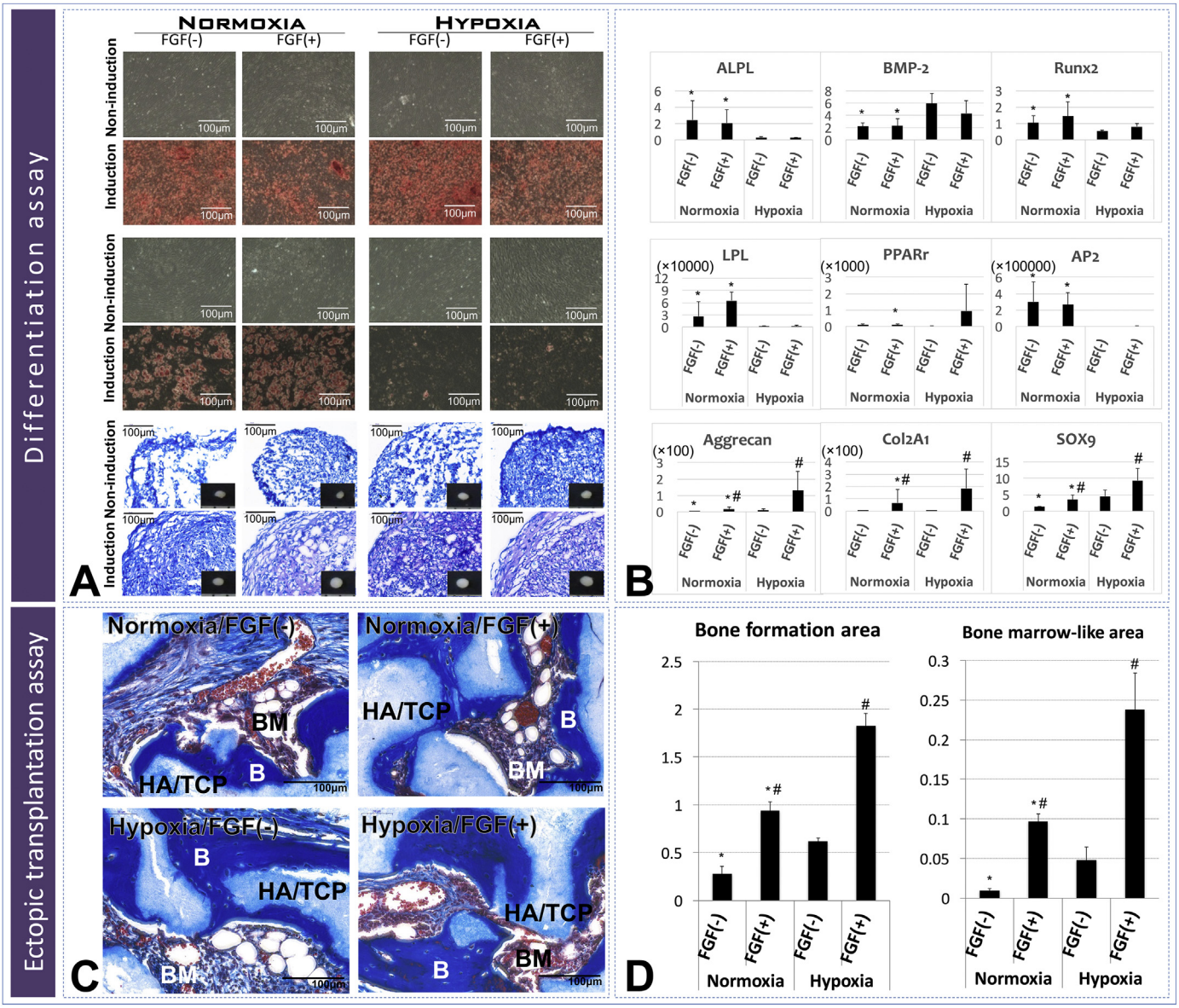


Fig. 2. Results from the differentiation and ectopic transplantation assays. (A to C) Adipogenesis was significantly reduced in cells cultured under hypoxia compared to normoxia ($p = 0.01$ for LPL and $p = 0.05$ for AP2), while osteogenesis and chondrogenesis were enhanced. Although two markers (ALPL and Runx2) for osteogenesis were slightly but not significant reduced in hypoxia groups, BMP-2 significantly increased in cells from hypoxia groups ($p = 0.01$), which might be related to the enhanced osteogenesis. The expression level of SOX9 was higher in hypoxia groups than in normoxia groups, and in FGF-2-treated groups than in untreated groups ($p = 0.01$). (D and E) In the ectopic transplantation model, the formation of mineralized tissue (bone; $p = 0.001$) and bone marrow-like zone ($p = 0.03$) around the grafted particles was increased in the hypoxic condition and with FGF-2 treatment, and also synergistically when combined. *Statistically different from the hypoxic condition. #Statistically different from culturing without FGF-2.

Col2A1 in particularly being synergistically enhanced in the simultaneous-treatment condition (Fig. 2C).

3.4. In vivo transplantation

In an ectopic transplantation model, cells cultured in the four different conditions were evaluated for their *in vivo* differentiation capacities. All of the samples exhibited mineralized tissue and bone marrow formation; mineralized tissues could be observed on the surfaces of grafted carrying biomaterials, and fibrovascular marrow tissues were present in the spaces between the grafted particles (Fig. 2D). The amount of bone formation was significantly increased in two conditions (hypoxia and FGF-2 treatment), and this increased synergistically in samples from the hypoxia/FGF-2(+) group: 0.28 ± 0.08, 0.94 ± 0.09, 0.62 ± 0.03, and 1.83 ± 0 (mm^2) in the normoxia/FGF-2(−), normoxia/FGF-2(+), hypoxia/FGF-2(−), and hypoxia/FGF-2(+) groups, respectively. A particularly interesting observation was that (bone marrow-like) cell-rich zones in fibrovascular spaces appeared more frequently at sites that received cells from both hypoxia groups, in which zonal clusters of mononuclear cells were found: 0.010 ± 0.002, 0.10 ± 0.01, 0.05 ± 0.02, and 0.24 ± 0.05 (mm^2), respectively (Fig. 2E).

3.5. Cell proliferation

Cellular proliferation assays were performed by calculating DT during cell expansion and applying the MTT assay. Both assays revealed similar patterns of proliferation: FGF-2 treatment reduced DT and increased the formazan color intensity, and the low-oxygen condition affected cell proliferation in an opposite way to FGF-2 treatment (Fig. 3A). However, the gene expression levels of two types of representative senescence markers demonstrated that cell aging could be delayed in tandem with the reduction of proliferation in the low-oxygen condition, with this not being enhanced by FGF-2 treatment despite the enhancement of cell proliferation—the normalized values of Apo1 and p21 were

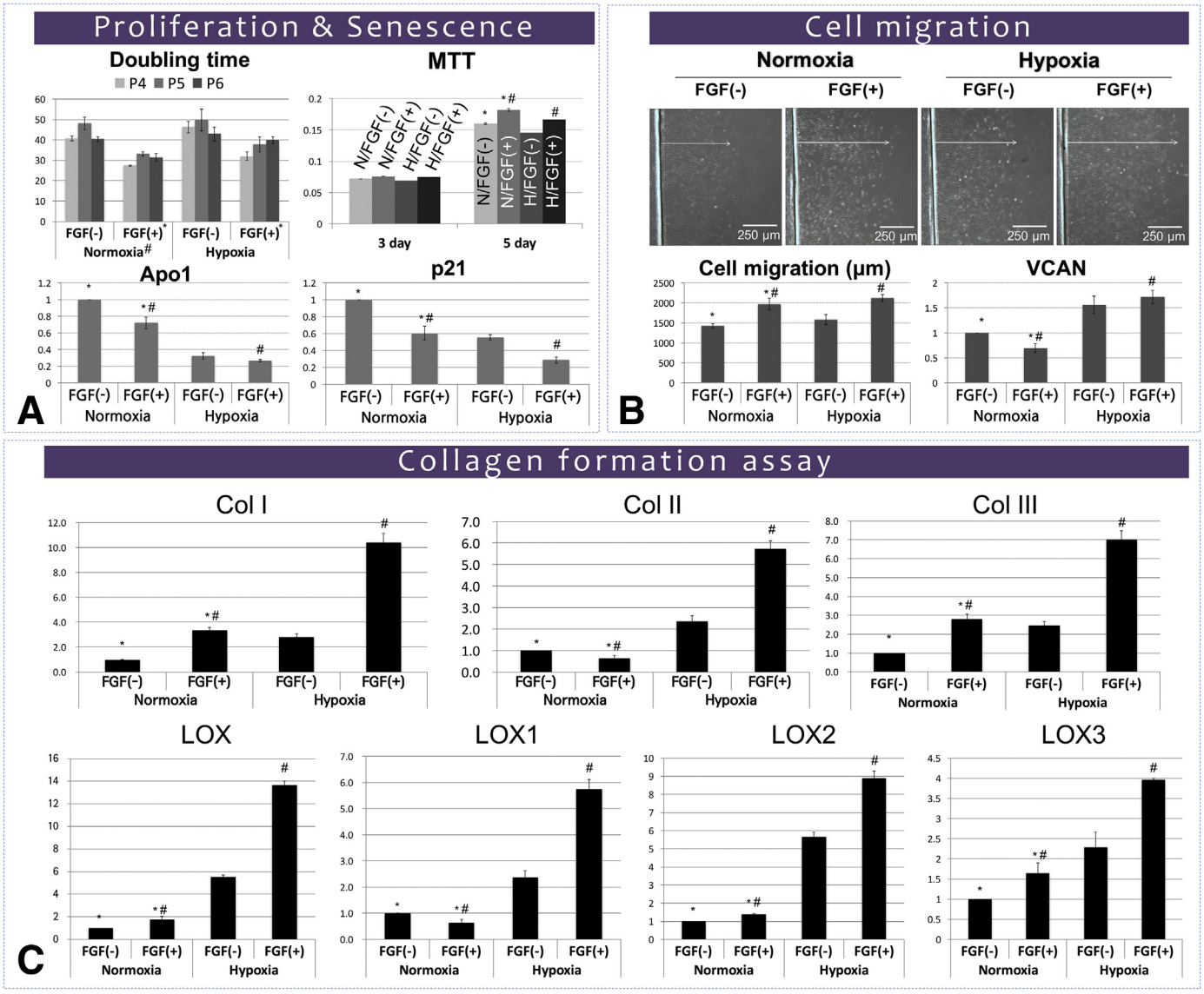


Fig. 3. Results from assays for proliferation, senescence, cell migration, and collagen formation. (A) The cellular proliferation rate was decreased in the hypoxia groups [extended DT ($p < 0.001$ for all passages) and reduced value in the MTT assay ($p = 0.001$ for 5-day samples)], while FGF-2 treatment had the opposite effect on cell proliferation. The expression levels of representative markers for senescence (Apo1 and p21; $p < 0.001$ for both genes) were decreased in cells under the hypoxic condition and with FGF-2 treatment. (B) Cellular migratory activity was also enhanced in the hypoxic condition and with FGF-2 treatment ($p = 0.001$). VCAN gene expression was increased in the hypoxic condition ($p < 0.001$), which is related to the reduced adhesion and migration of cells, and this was not affected by FGF-2 treatment. (C) The expression levels of all genes associated with collagen formation (COL I–III) and maturation (LOX family) were significantly enhanced in a hypoxic condition, and this pattern was synergistically strengthened in a hypoxic condition accompanied with FGF-2 treatment. *Statistically different from the hypoxic condition. #Statistically different from culturing without FGF-2.

1.00 ± 0.00 and 1.00 ± 0.00, 0.72 ± 0.07 and 0.61 ± 0.08, 0.32 ± 0.04 and 0.56 ± 0.03, and 0.27 ± 0.02 and 0.29 ± 0.04 in the normoxia/FGF-2(−), normoxia/FGF-2(+), hypoxia/FGF-2(−), and hypoxia/FGF-2(+) groups, respectively (Fig. 3A).

3.6. Cell migration

Cells migrated at various speeds depending on their culture conditions. Both experimental conditions (FGF-2 treatment and a low oxygen level) significantly enhanced the migration distance from the preexisting cell culture margin, especially for FGF-2 treatment: 1425.21 ± 58.26, 1969.12 ± 146.94, 1578.95 ± 126.06, and 2124.23 ± 90.36 μm in the normoxia/FGF-2(−), normoxia/FGF-2(+), hypoxia/FGF-2(−), and hypoxia/FGF-2(+) groups, respectively. The expression of the VCAN gene, which is related to the anti-adhesion and migration of cells, was increased in the hypoxic condition, and this was not affected by FGF-2 treatment (Fig. 3B).

3.7. Collagen-related gene expression

The expression levels of all genes associated with collagen formation (COL I–III) and maturation (LOX family) were significantly enhanced by a low oxygen level, and this pattern was synergistically strengthened in a hypoxic condition accompanied with FGF-2 treatment. However, COL II and LOX 1 were exceptionally decreased in samples cultured in a normoxic condition with FGF-2 treatment (Fig. 3C).

3.8. Microarray gene expression profiling

For evaluating the effects of FGF-2 treatment on the hypoxia-induced cellular responses at the gene expression level, microarray

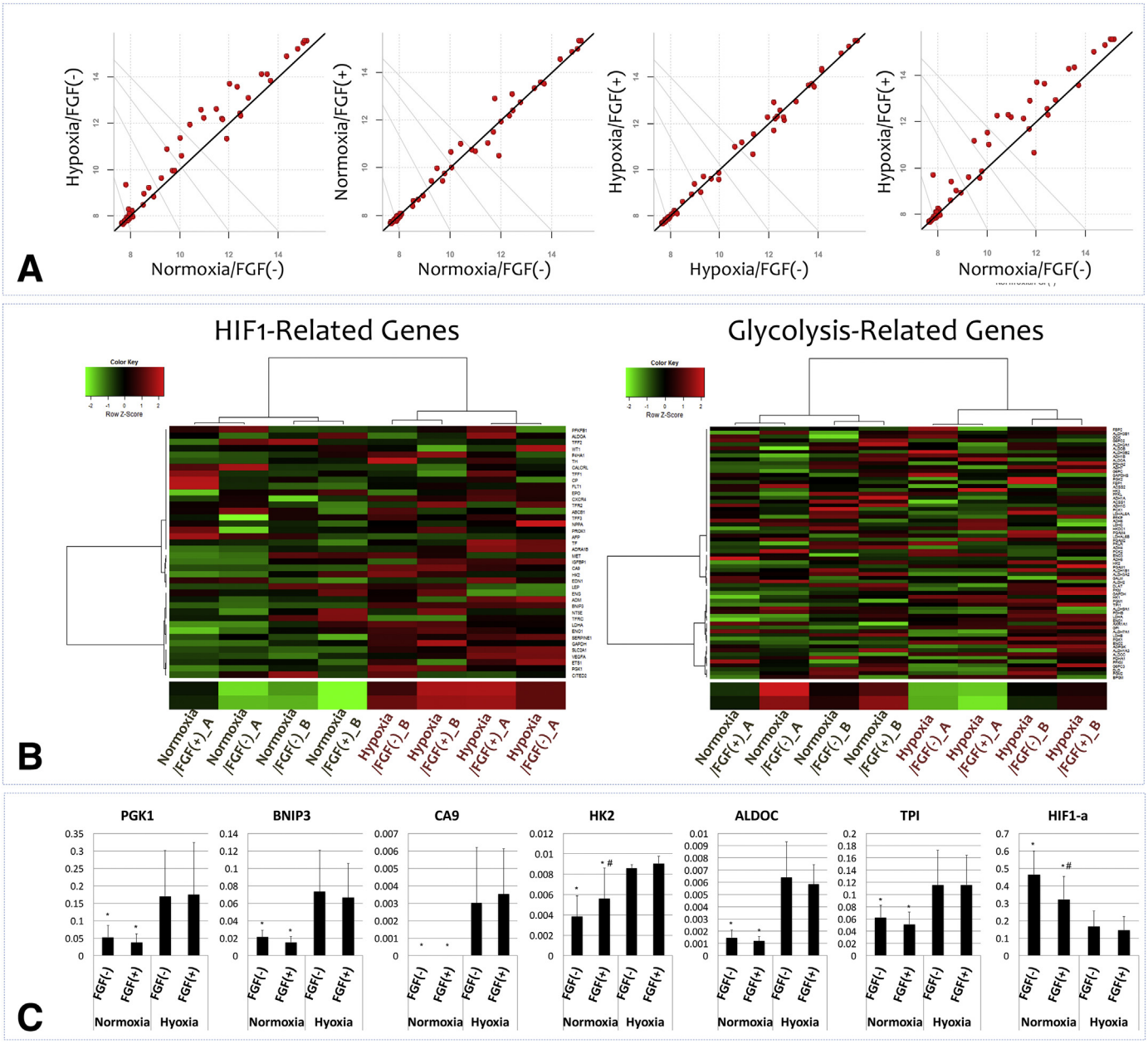


Fig. 4. Analytic results from microarray gene expression profiling. (A) Scatter plots of the expression levels of HIF-related genes between different groups showed fluctuations in the gene expression patterns between the hypoxic and normoxic conditions, but only minor effects of FGF-2 treatment. (B) In clustering analyses based on gene sets related to the HIF pathway and glycolysis (Supplementary Table 1), all samples clustered according to the two oxygen levels, independently of FGF-2 treatment. (C) The quantitative PCR revealed the same pattern of expression levels of representative HIF-related genes (PGK1, BNIP3, CA9, HK2, ALDOC, TPI, and HIF-1a) as in the microarray data. *Statistically different from the hypoxic condition. #Statistically different from culturing without FGF-2.

analysis was performed using the gene sets of HIF direct targets and glycolysis, which are known to be representative genes related to hypoxia-induced cellular responses. Scatter plots of the expression levels of HIF-related genes between different groups revealed fluctuations in the gene expression patterns between the hypoxic and normoxic conditions, regardless of the FGF-2 treatment. In both normoxic and hypoxic conditions, the data points in scatter plots were concentrated around the line indicating a directly proportional relationship between groups with and without FGF-2 treatment (Fig. 4A). In clustering analyses based on these two gene sets, all samples were clustered according to the two oxygen levels, regardless of FGF-2 treatment (Fig. 4B and Supplementary Table 1). The quantitative PCR revealed the same pattern of expression levels of representative HIF-related genes (PGK1, BNIP3, CA9, HK2, ALDOC, TPI, and HIF-1a) as in the microarray data (Fig. 4C).

4. Discussion

A low oxygen level reduces the proliferation rate of cells (Itahana et al., 2003; Kondoh et al., 2005; Parrinello et al., 2003) despite it also suppressing cell senescence (Chen et al., 1995; A. C. Lee et al., 1999). The present study focused on two issues related to *ex vivo* hBMSC cultures: (1) maintaining cellular characteristics in a hypoxic condition and (2) enhancing cellular proliferation using FGF-2. In senescence characteristics of laboratory cell cultures changed with the culture passage, and our

preliminary experimental data also showed that the number of colonies decreased significantly for cells after P5 on average (Supplementary Fig. 1). The expression of a specific progenitor cell marker (CD29) was significantly reduced in late-passage cells in the present study, and so all of the experiments used stromal cells at P6 when evaluating the effects of hypoxia and FGF-2 on the late passage of cells.

A restricted supply of oxygen alters cellular metabolism to the oxygen-independent pathway, glycolysis, and this could modulate the cellular life span (Kondoh et al., 2005) and stabilize the multipotency of human bone marrow stromal cells (Estrada et al., 2012). These mechanisms are consistent with the present study finding that eight samples could be clustered into two other groups (hypoxia and normoxia) by microarray analysis based on the glycolysis-related gene set. Along with the changes in gene expression, the *in vitro* cellular responses differed in cells cultured in the hypoxic condition, with the reduction of cellular proliferation and the expression of senescence markers in hypoxia. These findings are in agreement with previous studies showing continuous increases in population doubling *via* activation of the glycolytic enzyme (Kondoh et al., 2005) and the reduction of telomere shortening in bone marrow stromal cells (Estrada et al., 2012). In the present study, the hypoxic condition also modified the differentiation patterns of hBMSCs. A low oxygen level limits the utilization of fatty acid, whose metabolism includes the use of oxygen in mitochondrial respiration (Simon and Keith, 2008; Yun et al., 2002).

For results for osteogenic differentiation indicated that the patterns of gene expression were inconsistent with the mineral deposition (increased BMP-2 but reduced ALP and Runx2), which was consistent with the results from our previous study (J. S. Lee et al., 2015). The limitations of the *in vitro* cell culture system mean that these observations should be interpreted in combination with previous results (including from *in vivo* transplantation) that showed similar patterns of enhanced new bone formation under a hypoxic condition. Although a lower oxygen level may enhance osteogenic differentiation, the present results suggest that FGF-2 has only minor effects during this process. Considering these cellular responses, the additional application of FGF-2 might not interrupt the shifted cellular conditions when culturing in hypoxia (J. S. Lee et al., 2015; Rajpurohit et al., 1996; Schipani et al., 2001). The present microarray data showed that samples clustered according to the oxygen level only (*i.e.*, independent of FGF-2 treatment), which might indicate that FGF-2 treatment does not significantly affect the two critical pathways for hypoxia-induced cellular responses. Scatter plots for an HIF-1-related gene set also showed significant alterations of expression in the hypoxic condition, while HIF-1-related genes were expressed similarly in groups with and without FGF-2 treatment in both the normoxic and hypoxic conditions.

The homing of mesenchymal stromal cell is the critical first step in the process of regeneration. Progenitor cells would be detached and migrate to the wound or remodeling site in the response to a chemotactic stimulus, and greater migratory activity of marrow stromal cells can enhance the process of tissue regeneration. In the present study, hBMSCs cultured in the environment of hypoxia with or without FGF-2 showed significant increases in cellular migration, even though the hypoxic condition reduced cellular proliferation. This is in accordance with the enhanced regenerative potential of differentiation and colony-forming efficiency in late-passage cells. Functions from HIF-related pathways were controlled by degradation of HIF-1 in the presence of sufficient oxygen, rather than by controlling its production. Previous studies found that reducing HIF-1 degradation by restricting the supply of oxygen affected various pathways related to the biologic characteristics of cancer cells and stem cells (Gilkes et al., 2014; Simon and Keith, 2008), including the HIF-1 pathway and glycolysis. The changes in the underlying mechanisms will result in cellular phenotypes differing significantly in terms of their proliferation, migration, and differentiation.

One of the concerns when using FGF-2 in a culturing technique for bone marrow stromal cells is the possibility of limiting the multipotency of cells, with increasing cellular proliferation. In the present result, the surface markers for bone marrow stromal cells were not changed in any of the experimental groups, whereas HLA-DR increased with FGF-2 treatment. A previous study similarly showed increases in HLA-DR of mesenchymal stromal cells by FGF-2 treatment, and suggested that the numbers of human mesenchymal stromal cells could be controlled by the engagement of immunologic processes from increased HLA-DR expression (Bocelli-Tyndall et al., 2010). However, hypoxia can reduce the expression of HLA-DR induced by FGF-2 and also recover the increase in CD29 expression, and both environmental conditions may synergistically and complementarily affect hBMSCs so as to enhance the regenerative potential of hBMSC and increase their proliferation rate while maintaining the cellular purity in populations of mesenchymal stromal cells.

Culturing hBMSCs using a particular environmental condition (hypoxia) with the addition of FGF-2 increased the cellular proliferation rate while enhancing the regenerative potential for bone tissue engineering, modulating multipotency-related processes (enhanced chondrogenesis-related processes, reduced adipogenesis, and with a compensatory increase in the reduced osteogenesis induced by hypoxia only), and increased cellular migratory/collagen formation activity. However, hypoxia did not reverse the senescence of hBMSCs, such as a loss of chondrogenesis. The gene expression levels in the experimental samples showed activation of the HIF-1 pathway and glycolysis in the hypoxic condition, but the addition of FGF-2 did not affect the hypoxia-induced biologic activity.

We propose the concurrent application of the hypoxic condition and FGF-2 as a putative methodology for culturing hBMSCs for use in bone tissue engineering. However, this conclusion is based on experiments of pooled hBMSCs rather than each individual colony, and so future studies need to investigate the clinical application of grafting colonies of hBMSCs cultured in hypoxia and FGF-2.

Role of sponsor

The funding organization played no role in the design of the study, the choice of enrolled patients, the review and interpretation of data, or the preparation or approval of the manuscript.

Conflict-of-interest disclosure

The authors declare that they have no competing financial interests.

Acknowledgement

This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science, ICT & Future Planning (Grant No. 2012M3A9B2052521), and by an NRF grant funded by the Korea government (MSIP) (No. 2015R1A2A1A15053961).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.01.010.

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