



# Hypoxia Regulates the Extracellular Matrix via Mitogen-Activated Protein Kinases Pathway in Cells Retrieved from the Human Intervertebral Disc

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**Purpose:** The present study aimed to identify the physiological characteristics of cells by investigating the change in gene expression and protein levels during extracellular matrix (ECM) synthesis in the intervertebral disc (IVD) under hypoxic conditions.

**Materials and Methods:** To test the effect of oxygen on cell growth and ECM synthesis of chondrocyte-like cells, the cells from IVD were separated and cultured in two hypoxia-mimicking systems: chemical hypoxic conditions using deferoxamine (DFO), and physiological hypoxic conditions using a hypoxic chamber for 7 days. Chondrocyte like cells cultured without DFO and under the normal oxygen concentration (21% O<sub>2</sub> and 5% CO<sub>2</sub>, 37°C) served as the controls.

**Results:** Chondrocyte-like cells cultured in the presence of 6% oxygen demonstrated a 100% increase in cellular proliferation compared to the control. The cells treated with chemical hypoxic conditions demonstrated a dose-dependent increase in the mRNA expression of glucose transporter-1, GAPDH, aggrecan, and type II collagen on Day 1. When treated with 100 μM DFO, the cells showed a 50% increase in the levels of proteoglycan protein on Day 7. The cells treated with chemical hypoxic condition demonstrated increase in sulfated glycosaminoglycan (GAG) protein levels on Day 7. Moreover, the cells cultured in the presence of 6% oxygen showed a 120% increase in sulfated GAG levels on Day 7.

**Conclusion:** The oxygen concentration had an important role in the viability, proliferation, and maturation of chondrocyte-like cells in IVD. In addition, chondrocyte-like cells are sensitive to the concentration of oxygen.

**Key Words:** Intervertebral disc, low oxygen, chondrocyte-like cell, proteoglycan, extracellular matrix

## INTRODUCTION

Intervertebral disc degeneration (IDD) is a common musculoskeletal disorder that primarily affects the spine.<sup>1,2</sup> IDD is caused by proteolytic degradation of the extracellular matrix (ECM), which leads to the reorganization of the annulus fibrosus (AF),

dehydration of the nucleus pulposus (NP), and calcification of the cartilage vertebral endplates.<sup>3-6</sup> Consequently, the intervertebral disc (IVD) loses water and undergoes swelling under pressure by losing hydrophilic properties due to the loss of proteoglycan matrix in the NP region.<sup>7,8</sup>

Oxygen plays a key role in the metabolism of cells of IVD or those present in cartilage tissues.<sup>9,10</sup> As the oxygen concentration decreases from 21% to 5%, the oxygen consumption rate reduces, and lactate production increases in vivo or in vitro. This causes the inhibition of ECM synthesis and interrupts the incorporation of sulfate and proline.<sup>9,11,12</sup> During the early stages of IDD, the transport of oxygen along with nutrients into the NP and inner AF is reduced due to the low permeability of the endplate in a degenerated disc.<sup>13,14</sup> Interplay among the nutrient supply, acidic conditions, and oxygen tension in the NP might be responsible for the degenerative, regenerative, and adaptive mechanisms of aging. Glucose transporter 1 (GLUT-1) and hy-

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poxia inducible factor 1 alpha (HIF-1 $\alpha$ ), the aerobic and anaerobic metabolic regulators, regulate glucose transport, thereby controlling the properties of IVD.<sup>11</sup> The response of NP to hypoxic conditions is especially reflected in the quantitative expression of GLUT-1 and HIF-1 $\alpha$ .<sup>15</sup>

Mitogen-activated protein kinases (MAPKs) are members of a highly conserved signal transduction signaling pathway, and they are also the target molecules of anti-inflammatory therapies. MAPKs influence the gene expression through three main cascades: the extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases, and p38 mitogen-activated protein kinases.<sup>16,17</sup> The MAPK signaling pathway plays an important role in regulating the degradation or synthesis ECM, and is also involved in the degradation of proteoglycans by alternating anabolic and catabolic gene expression in IVD.<sup>18</sup> The p38 and ERK signaling pathways play a pivotal role in proteoglycan metabolism. The activation of ERK blocks apoptosis induced by mechanical stress and serum starvation under hypoxic conditions.<sup>19,20</sup> Therefore, the present study aimed to identify the physiological characteristics of the chondrocyte-like cells present in IVD by investigating the changes in gene and protein expression of matrix synthesis-related factors under hypoxic conditions.

## MATERIALS AND METHODS

### Study design

To test the effect of oxygen on cell growth and production of chondrocyte-like cells in the ECM *in vitro*, the cells were isolated from patients with degenerative disc disease (Table 1). The tissues used in this study were approved by the Institutional Review Board (4-2016-0964). We screened the tissues isolated from patients under the age of 60 years with Pfirrmann grades 3 to 5, as studies were needed for MAPK signaling to explain the changes in proteoglycan synthesis and the amount of GAG. Pfirrmann grading of the index disc level was separately performed by Dr. Lee and Dr. Yang, based on the previous literature.<sup>21</sup> The chondrocyte-like cells from IVD were separated and cultured in two hypoxia-inducing systems, chemical hypoxic conditions using deferoxamine (DFO) and physiological hypoxic conditions using a hypoxic chamber, for 7 days. Chondrocyte like cells cultured without DFO and under the normoxic concentration (21% O<sub>2</sub> and 5% CO<sub>2</sub>, 37°C) served as the controls. The mRNA levels of type II collagen, aggrecan, and GLUT-1 were analyzed using the reverse transcription-polymerase chain reaction. Furthermore, proteoglycan levels were estimated using Western blotting and by assaying the glycosaminoglycan (GAG) synthesis.

### Isolation and expansion of human chondrocyte-like cells

IVD tissue was obtained from 30 patients (age range: 21 to 61

**Table 1.** Patient Demographics

Patient	Sex	Age	Degree of degeneration	Experiment
#1	F	56	Grade 4, L3/4	Proliferation
#2	M	58	Grade 4, L4/5	WB
#3	F	24	Grade 4, L4/5	PCR, proliferation
#4	M	30	Grade 4, L4/5	PCR
#5	F	61	Grade 5, L4/5	WB,GAG
#6	F	54	Grade 5, L4/5/S1	WB, proliferation
#7	F	54	Grade 4, L4/5	Proliferation
#8	F	61	Grade 5, L4/5/S1	Proliferation
#9	F	56	Grade 3, L5/S1	Proliferation
#10	M	38	Grade 4, L5/S1	GAG
#11	F	60	Grade 3, L5/S1	WB
#12	M	36	Grade 5, L4/5	WB
#13	F	53	Grade 4, L3/4/5	GAG,WB
#14	F	63	Grade 4, L5/S1	PCR, WB, ICC
#15	M	55	Grade 4, L5/S1	GAG, ICC
#16	M	34	Grade 5, L5/S1	WB
#17	M	28	Grade 5, L5/S1	WB
#18	M	37	Grade 5, L4/5	WB
#19	M	36	Grade 3, L4/5	WB
#20	F	53	Grade 4, L4/5/S1	WB
#21	F	19	Grade 4, L5/S1	WB
#22	M	21	Grade 5, 4L4/5	WB
#23	F	56	Grade 4, L3/4/5	WB
#24	M	42	Grade 5, L5/S1	WB
#25	M	35	Grade 4, L4/5	WB
#26	F	26	Grade 4, L1/2	WB
#27	F	39	Grade 3, L5/S1	WB
#28	M	24	Grade 5, L5/S1	WB
#29	M	26	Grade 5, L5/S1	PCR
#30	M	26	Grade 5, L5/S1	PCR

WB, Western blot; PCR, polymer chain reaction; GAG, glycosaminoglycan; ICC, immuno cytochemistry.

years) during surgery for degenerative disc disease, such as IVD degeneration and/or herniation and IVD degeneration with spinal stenosis (Table 1). Only IVDs classified as Pfirrmann grades 3–5 were utilized. The IVD tissues were minced using a scalpel, and then digested for 2 h at 37°C under gentle agitation in Dulbecco's modified Eagle medium and nutrient mixture F-12 (Ham) (1:1) (DMEM/F12, Gibco-BRL®, Grand Island, NY, USA) containing collagenase type II (2.5 mg/mL, Sigma, St. Louis, MO, USA). The cells were then filtered through a sterile nylon mesh filter (pore size: 100  $\mu$ m, Falcon, Bedford, MA, USA) and seeded in T25 plates (Nunc™, Roskilde, Denmark) at a density of approximately 1 $\times$ 10<sup>5</sup> cells/mL. Primary cultures were maintained for 2 to 3 weeks in DMEM/F12 supplemented with 10% fetal bovine serum (FBS, Gibco-BRL®), 1% v/v penicillin, streptomycin, and nystatin (Gibco-BRL®) in a humidified 37°C incubator containing 5% CO<sub>2</sub>. The culture medium was changed three times a week.

### Chondrocyte-like cell culture in hypoxic condition

The chondrocyte-like cells from IVD were treated with 1, 10, and 100  $\mu$ M DFO (Sigma) for 7 days. Cells cultured in the absence of DFO served as the controls. Additionally, the chondrocyte-like cells were cultured in chambers containing 1% and 6% oxygen at 37°C, and in an atmosphere containing 5% CO<sub>2</sub> (Thermo<sup>®</sup>, Marietta, OH, USA) to mimic physiological hypoxic conditions for 7 days. Chondrocyte like cells cultured in normoxic conditions served as the controls. The culture medium was changed twice a week.

### Cell proliferation assay

After maintaining the chondrocyte-like cells in culture for 1, 3, 5, and 7 days, their proliferation was assessed using the EZ-Cytox Cell viability assay kit (Daeillab Service Co., LTD, Seoul, Korea). Assays were performed in triplicate. Culture medium (200  $\mu$ L) and 30  $\mu$ L of assay reagent was added to each well and incubated at 37°C for 1 h. The supernatant was transferred to a 96-well plate, and the absorbance from each well was measured at 450 nm on an enzyme-linked immunosorbent assay reader (VERSA Max, Molecular Devices, Sunnyvale, CA, USA).

### RNA extraction and reverse-transcription polymerase chain reaction analysis

The total RNA was isolated from chondrocyte-like cells cultured in hypoxic condition for 1 and 7 days using the QIAGEN RNeasy<sup>®</sup> mini kit (QIAGEN<sup>®</sup>, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was prepared using the Maxime RT premix kit (QIAGEN<sup>®</sup>). The transcript levels of HIF-1 $\alpha$ , HIF-1 $\beta$ , GLUT-1, GAPDH, aggrecan, and type II collagen were assessed (Table 2). Their relative expression levels were normalized to those of beta actin. The data were analyzed using the Image J analyzer software ver. 1.45 (National Institutes of Health, Bethesda, MD, USA).

### Protein extraction and Western blot analysis

The cells were seeded at a density of  $5 \times 10^5$  cells per well and cultured in hypoxic conditions for 1 and 7 days. Then, they were lysed in a buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5 mM EDTA (pH 7.4), 1 mM Tri-HCl (pH 7.4), and

protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Mannheim, Germany). After treatment in two hypoxia-inducing systems for 3 days, nuclear and cytoplasmic proteins from cells were isolated using the NE-PER<sup>®</sup> Nuclear and cytoplasmic Extraction reagents (Thermo scientific, Rockford, IL, USA). The lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred onto 0.45- $\mu$ m pore size polyvinylidene difluoride (PVDF, Pierce, Rockford, IL, USA) membranes using an electrophoretic transfer system (Mini Trans-Blot<sup>®</sup> Cell and systems, Bio-Rad, Hercules, CA, USA). The blots were incubated with antibodies against proteoglycan (Abcam<sup>®</sup>, Milton, UK), phospho-MEK1/2, MEK1/2, phospho-ERK1/2, ERK1/2, phospho-P90RSK, and P90RSK (Cell Signaling Technology<sup>®</sup> Inc, Danvers, MA, USA). After incubation with the secondary antibodies, immunoreactive bands were visualized using a Western blot detection system (West-Zo<sup>®</sup> plus, iNtRON Biotechnology, Seongnam, Korea). The blots were stripped of bound antibodies and reprobated using antibodies against actin (Abcam<sup>®</sup>) to verify the amounts of loaded protein.

### Immunofluorescence staining

Chondrocyte-like cells were seeded in 35-mm glass bottom culture dishes (10000 cells/well), and treated with DFO (10  $\mu$ M) and cultured in the presence of 6% O<sub>2</sub> for 3 days. The cells were then fixed using 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS for 20 min, blocked with PBS containing 5% BSA, and incubated with antibodies against RSK1/RSK2/RSK3 (Cell Signaling Technology<sup>®</sup> Inc) at 4°C overnight. After washing, the cells were incubated with fluorescent-labeled secondary antibodies (anti-rabbit IgG Alexa Fluor-488) and rhodamine phalloidin (Thermo Scientific) (for F-actin staining) for 1 h at room temperature. The nuclei were counterstained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). The cells were imaged using a Zeiss LSM700 confocal microscope (Carl Zeiss, berlin, Germany).

### Sulfated GAG assay

Chondrocyte-like cells were cultured for 7 days under the indicated conditions in DMEM/F12 medium. Sulfated GAG was harvested from these cells after lysis in papain extraction buffer containing 0.1 M sodium acetate, 0.01 M sodium EDTA, and 5 mM cysteine HCl (pH 6.4). The sulfated GAG samples were concentrated using a sulfated GAG assay protocol, and measured at a wavelength of 650 nm as per the manufacturer's instructions (Blyscan<sup>™</sup>, Biocolor LTD., Carrickfergus, UK). The amount of sulfated GAG was calculated based on a standard curve generated from the absorption values of known concentrations of bovine tracheal chondroitin 4-sulfate, provided with the sulfated GAG assay kit.

### Statistical analysis

Data were compiled from three independent experiments that

**Table 2.** List of Primers Used for PCR Analyses

Gene	Upstream primer (5'-3')	Downstream primer (3'-5')
Aggrecan	GAATCTAGCAGTGAGACGTC	CTGCAGCAGTTGATTCTG AT
GLUT-1	GGTGATCGAGGAGTTCTACA	GATCAGCATCTCAAAGGACT
HIF-1 $\alpha$	GAGTTCGCATCTTGATAAGG	CATGGTCACATGGATGAGTA
HIF-1 $\beta$	GGTGCACCTGATCATAGAGT	AAA ACCAGACAAGCTAACCA
Type II collagen	CTACTGGAGTGACTGGTCCTAA	ACCATCTTTCCAGAAGGAA
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTTC
$\beta$ -actin	GGCGGACTATGACTTAGTTG	AAACAACAATGTGCAATCAA

PCR, polymer chain reaction; HIF-1 $\alpha$ , hypoxia inducible factor 1 alpha; HIF-1 $\beta$ , hypoxia inducible factor 1 beta; GLUT-1, glucose transporter 1.

were performed on separate cultures using chondrocyte-like cells isolated from 15 donors. Data are expressed as the mean± standard deviation from the results of three independent experiments. A paired t-test was performed to compare the results between two groups in the biochemical assays, and Mann-Whitney U test was used for non-parametric data analysis using SPSS 21.0 (IBM Corp., Armonk, NY, USA). A  $p$  value<0.05 was considered to be statistically significant.

## RESULTS

### Proliferation of chondrocyte-like cells

Chondrocyte-like cells treated with 1, 10, and 100  $\mu$ M DFO did not show a significant increase in cellular proliferation with respect to the control cells cultured in the absence of DFO. However, on Day 7, chondrocyte-like cells cultured in the presence of 6% oxygen demonstrated a 100% increase in cellular proliferation compared to the control cells cultured in the presence of 21% oxygen ( $p$ <0.05). However, the chondrocyte-like cells exposed to 1% oxygen showed no increase in proliferation compared to the control culture at Day 1 and Day 7 (Fig. 1).

### mRNA expression

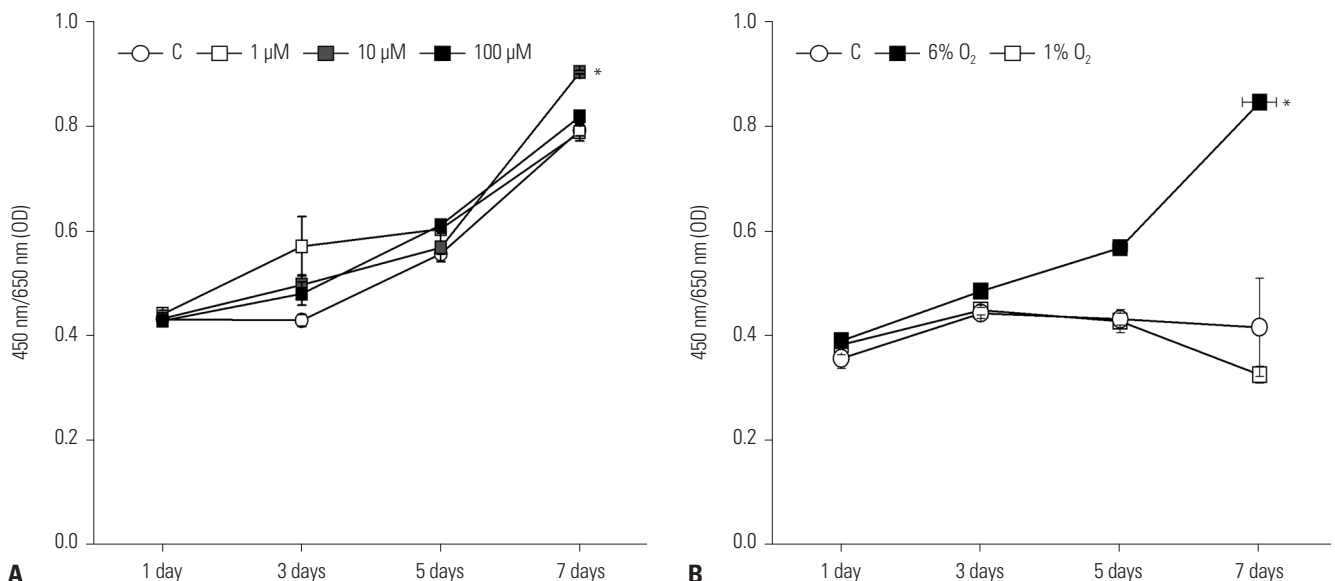
Chondrocyte-like cells treated with 1, 10, and 100  $\mu$ M DFO demonstrated a dose-dependent increase in the mRNA expression of GLUT-1, GAPDH, aggrecan, and type II collagen on Day 1. In addition, these cells showed a further increase in the mRNA expression of HIF-1 $\beta$ , GLUT-1, aggrecan, and type II collagen on Day 7 with respect to the control cells (Fig. 2). However, no changes in GAPDH and HIF-1 $\alpha$  mRNA levels were observed. Chondrocyte-like cells cultured in the presence of 1% and 6%

of oxygen, on Day 1, showed no significant changes in the mRNA expression of GLUT-1, GAPDH, aggrecan, and type II collagen when compared to the control cells. HIF-1 $\alpha$  was not expressed, and HIF-1 $\beta$  was induced in the presence of 1% oxygen on Day 1. However, the chondrocyte-like cells cultured in the presence of 1% oxygen showed more than a 70% increase in GLUT-1, aggrecan, and type II collagen mRNA levels on Day 7 when compared to the control cells (Fig. 2).

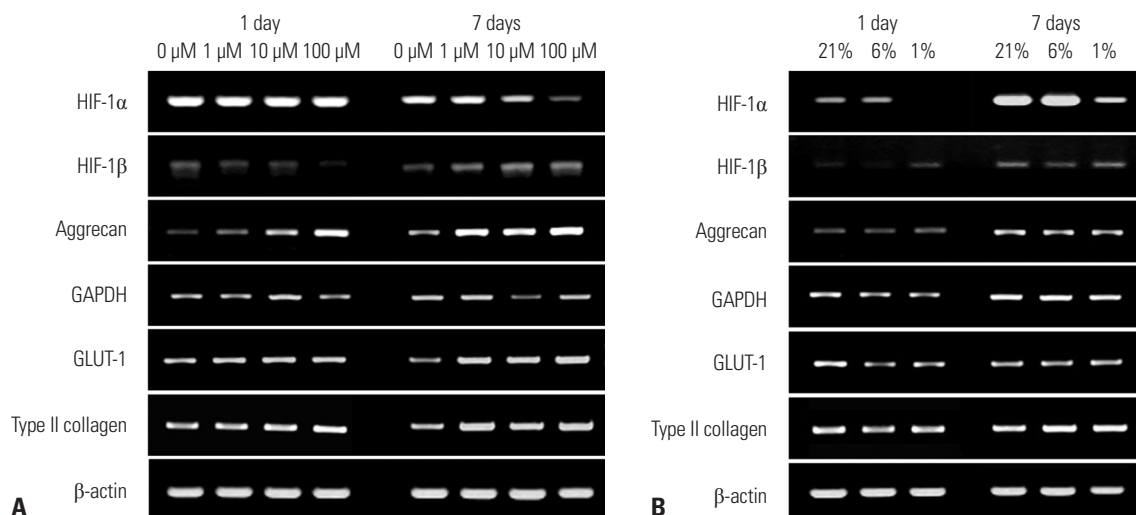
### MAPK signaling pathways

Chondrocyte-like cells treated with 1, 10, or 100  $\mu$ M DFO demonstrated increased levels of phospho-MEK1/2 and phospho-ERK proteins at 30 min and 60 min post-treatment compared to the control cells (Fig. 3A). When cultured in the presence of 1% and 6% oxygen, chondrocyte-like cells showed increased levels of phospho-MEK1/2 and phospho-ERK proteins, after incubation for 30 min and 60 min compared to the control cells (Fig. 3B). Chondrocyte-like cells treated with 10  $\mu$ M DFO and cultured in the presence of 6% oxygen demonstrated increased the levels of phospho-MEK1/2 and phospho-ERK proteins on Day 3 compared to the control cells (Fig. 3C)

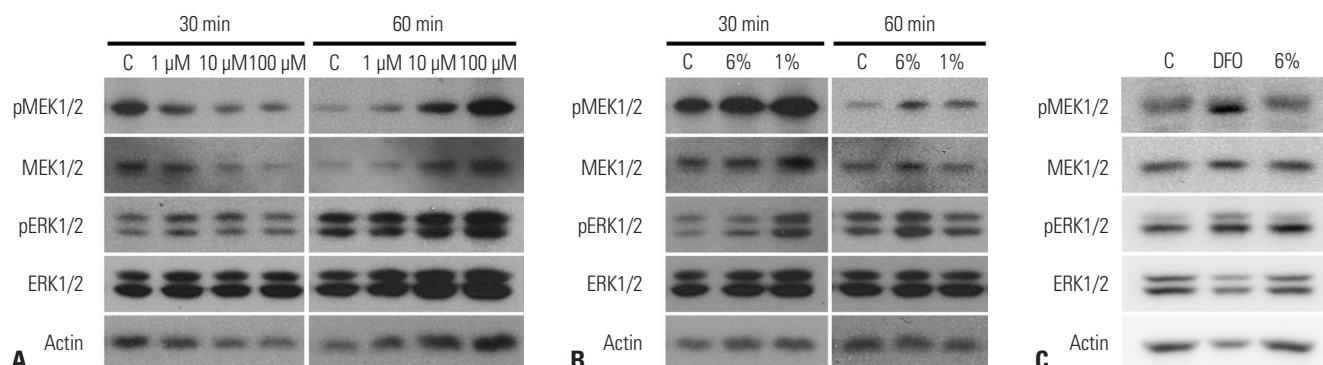
When treated with 10  $\mu$ M DFO, chondrocyte-like cells demonstrated increased levels of phospho-P90RSK protein at 30 min and 60 min post-treatment compared to the control cells (Fig. 4A), Chondrocyte-like cells exposed to 1% and 6% oxygen showed no significant change in the levels of phospho-P90RSK protein at 30 min and 60 min compared to the control cells (Fig. 4B). When treated with 10  $\mu$ M DFO and cultured in the presence of 6% oxygen, chondrocyte-like cells showed decreased levels of phospho-P90RSK protein on Day 3 compared to the control culture (Fig. 4C). RSK1/RSK2/RSK3 protein was found to be localized in the nuclei of these cells (Fig. 4D).



**Fig. 1.** Proliferation of human chondrocyte-like cells from Day 1 to Day 7 in culture. (A) Cells treated with 1, 10, and 100  $\mu$ M deferoxamine. (B) Cells cultured in the presence of 1% and 6% oxygen. C, control.



**Fig. 2.** Expression of HIF-1 $\alpha$ , HIF-1 $\beta$ , aggrecan, GAPDH, GLUT-1, and type II collagen mRNA in human chondrocyte-like cells at Day 1 and Day 7 of culture. (A) mRNA expression in cells treated with 1, 10, and 100  $\mu$ M deferoxamine. (B) mRNA expression in cells cultured in the presence of 1% and 6% oxygen. HIF-1 $\alpha$ , hypoxia inducible factor 1 alpha; HIF-1 $\beta$ , hypoxia inducible factor 1 beta; GLUT-1, glucose transporter-1; C, control.



**Fig. 3.** Expression of MAPK signaling pathway components in human chondrocyte-like cells. (A) Levels of pMEK1/2, MEK1/2, pERK1/2, and ERK1/2 proteins in cells treated with 1, 10, and 100  $\mu$ M DFO for 30 min and 60 min. (B) Expression of pMEK1/2, MEK1/2, pERK1/2, and ERK1/2 proteins in cells cultured in the presence of 1% and 6% oxygen at 30 min and 60 min of culture. (C) The levels of pMEK1/2, MEK1/2, pERK1/2, and ERK1/2 proteins in cells treated with 10  $\mu$ M DFO and cultured in the presence of 6% oxygen on Day 3 of culture. MEK, MAPK/ERK kinase; ERK, extracellular signal-regulated kinase; DFO, deferoxamine; C, control.

### Proteoglycan and type II collagen levels

When treated with 100  $\mu$ M DFO, chondrocyte-like cells showed a 50% increase in the levels of the proteoglycan protein on Day 7 compared to the control cells (Fig. 5A). However, treatment with 1 and 10  $\mu$ M DFO only resulted in a 10% increase in the proteoglycan protein levels (Fig. 5A). Chondrocyte-like cells cultured in the presence of 1% oxygen showed an 80% increase in the proteoglycan level compared to the control cells and cells cultured in the presence of 6% oxygen culture on Day 7 (Fig. 5B). Type II collagen and aggrecan proteins, with the exception of proteoglycan, showed a decrease in their levels in the chondrocyte-like cells treated with 10  $\mu$ M DFO and in the presence of 6% oxygen on Day 3 compared to the control cells (Fig. 5C).

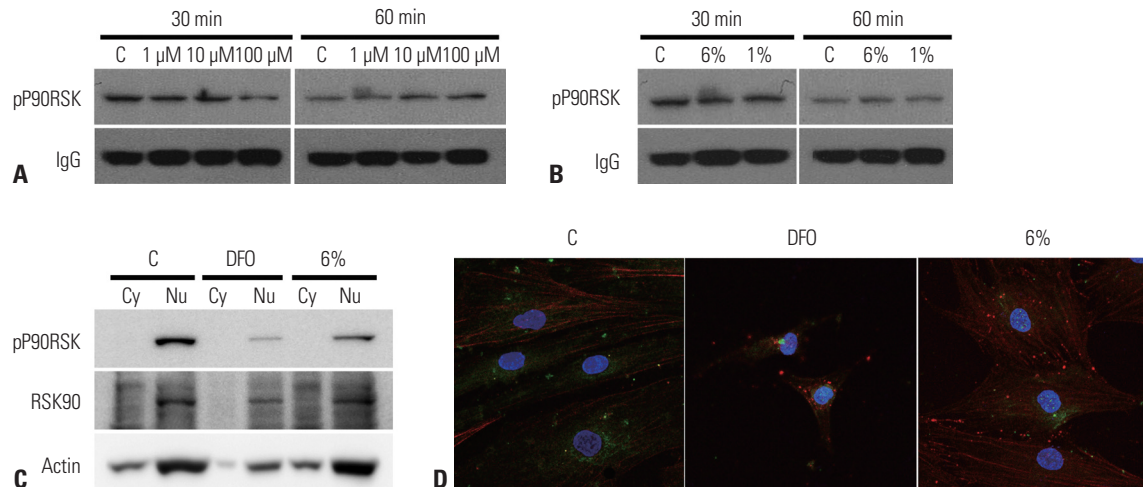
### Sulfated GAG synthesis

Chondrocyte-like cells treated with 1, 10, and 100  $\mu$ M DFO dem-

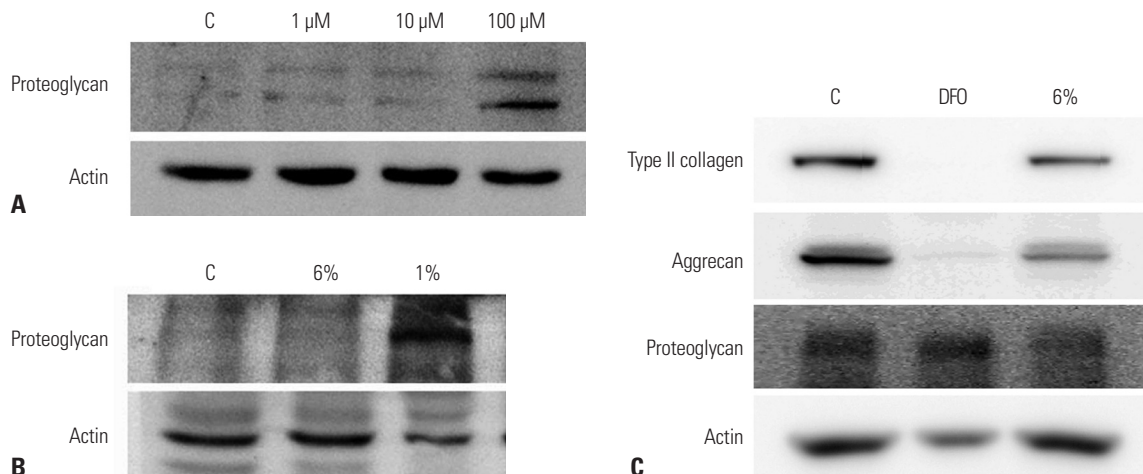
onstrated 200%, 250%, and 200% increase in sulfated GAG protein levels on Day 7 compared to the control cells ( $p < 0.05$ ) (Fig. 6A) Moreover, chondrocyte-like cells cultured in the presence of 6% oxygen showed a 120% increase in sulfated GAG levels on Day 7 compared to the control culture ( $p < 0.05$ ) (Fig. 6B), while chondrocyte like cells exposed to 1% oxygen demonstrated no difference in the sulfated GAG levels on Day 7 compared to the control cells.

## DISCUSSION

The IVD is the largest avascular tissue in the human body. The NP is located at the center of the human lumbar disc, and is approximately 6 to 8 mm away from any blood vessel. Consequently, it has a low oxygen supply.<sup>22-24</sup> The oxygen consumption rate is dependent on the oxygen tension in the NP and



**Fig. 4.** pP90RSK protein expression in human chondrocyte-like cells. (A) Expression of pP90RSK in cells treated with 1, 10, and 100 μM DFO for 30 min and 60 min using immunoprecipitation protocol. (B) The levels of pP90RSK in cells cultured in the presence of 1% and 6% oxygen at 30 min and 60 min of culture using immunoprecipitation protocol. (C) The expression of RSK1/RSK2/RSK3 proteins in the cytosol and nucleus of cells treated with 10 μM DFO and cultured in the presence of 6% oxygen after 3 days of culture. (D) RSK1/RSK2/RSK3 expression in the cells treated with 10 μM DFO and cultured in presence of 6% oxygen after 3 days of culture. Red, actin; blue, nucleus; green; RSK1/RSK2/RSK3; RSK, ribosomal protein S6 kinase; DFO, deferoxamine; C, control.

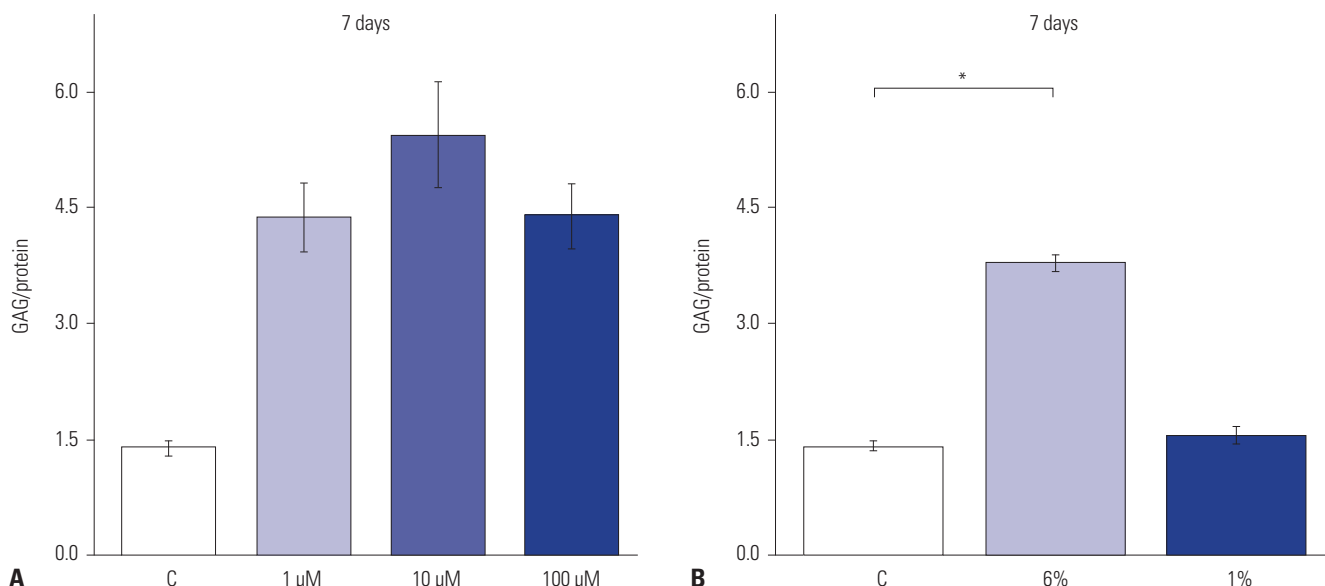


**Fig. 5.** Expression of proteoglycan proteins in human chondrocyte-like cells. (A) Proteoglycan expression in cells treated with 1, 10, and 100 μM DFO for 7 days. (B) Proteoglycan expression in cells cultured in the presence of 1% and 6% oxygen. (C) Type II collagen, aggrecan, and proteoglycan expression in cells treated with DFO and cultured in presence of 6% oxygen after 3 days of culture. DFO, deferoxamine; C, control.

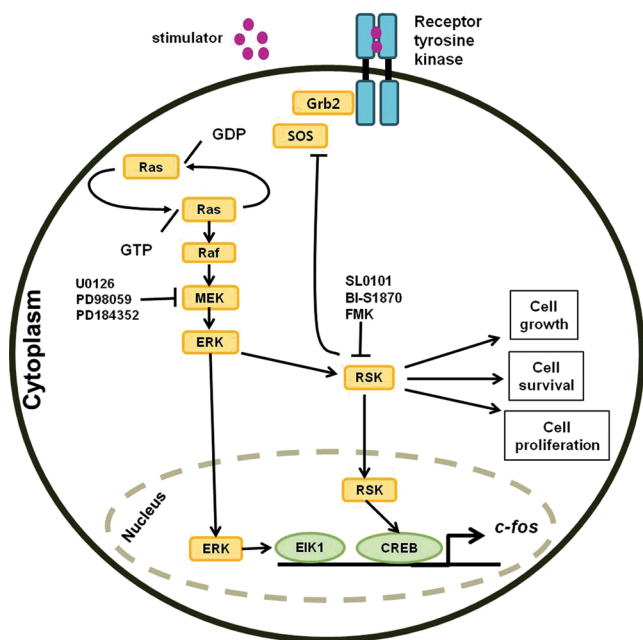
outer AF tissue. In vitro, the oxygen consumption rate is regulated by the oxygen content and pH of the culture medium.<sup>9,10</sup> There are steep gradients in O<sub>2</sub> concentration over the avascular disc, with pO<sub>2</sub> falling to as low as 1% within the center of a large disc, and it is possible for the NP cells to be adjusted to the metabolism under low O<sub>2</sub> concentrations.<sup>25</sup> The steep gradient of oxygen concentration between the NP and outer AF promotes cell differentiation.<sup>11,12</sup> The pO<sub>2</sub> levels in large discs can range from 1% to 5%, and there has been proof that the disc cell metabolism can shift with O<sub>2</sub> concentration in vivo.<sup>14</sup> Human synovium-derived stem cells or mesenchymal stem cells differentiate into chondrocyte-like cells in a low oxygen environment.<sup>26-29</sup> Furthermore, low oxygen conditions are important for the re differentiation of cells in the NP region.<sup>15,25,30</sup>

Our results showed that 1% oxygen had no effect on cell proliferation. However, culturing of cells in the presence of 6% oxygen induced a 200% increase in cell proliferation. Treatment with 10 μM DFO also enhanced the cell proliferation compared to treatment with other doses and the untreated control (Fig. 1).

HIF is a member of the basic helix-loop-helix-PER-ARANT-SIM family, comprising an β-subunit that is degraded under normoxic conditions and a β-subunit.<sup>31</sup> HIF-1α is mainly expressed under hypoxic condition or in poorly vascularized sites, and is constitutively expressed in IVD cells.<sup>32-34</sup> It induces aggrecan and type II collagen expression in human articular chondrocytes.<sup>35</sup> HIF-1α expression has been shown to decrease with an increase in DFO concentration over 7 days of culture. It is



**Fig. 6.** Sulfated GAG levels in human chondrocyte-like cells on Day 7 of culture. (A) GAG content in cells cultured with 1, 10, and 100 μM deferoxamine. (B) GAG content in cells cultured in presence of 1% and 6% oxygen. C, control; GAG, glycosaminoglycan.



**Fig. 7.** Activation of the ribosomal protein S6 kinase (RSK) family of protein kinases in the Ras/MAPK pathway. MAPK, mitogen activated protein kinase.

believed that the cells are stabilized by partially adapting to low oxygen pressure. HIF-1α is sensitive to the oxygen concentration, and can be degraded under normoxic conditions.<sup>9,36,37</sup> In our study, HIF-1β expression decreased under chemical hypoxic condition on Day 7, while increasing under physiological hypoxic conditions. However, HIF-1β expression increased under both chemical and physiologic hypoxic conditions on Day 7. These results suggest that stabilized HIF-1α expression induces the expression of HIF-1β (Fig. 2). The

GLUT-1 gene expression, related to glucose metabolism, was found to increase by more than 200% under chemical hypoxic conditions. In addition, treatment with DFO induced the expression of aggrecan and type II collagen mRNAs by 200%. Moreover, the mRNA expression of type II collagen in 1% and 6% oxygen increased by 30% when compared to the control cells (Fig. 2). These results suggest that hypoxia stimulated the expression of HIF-1α, HIF-1β and GLUT-1, and that increased GLUT-1 levels were necessary for the synthesis of glycoprotein and GAG.

RSK (90 kDa ribosomal S6 kinase) consists of four isoforms (RSK1,<sup>38</sup> RSK2, RSK3,<sup>39</sup> and RSK4<sup>38</sup>) which have non-identical phosphotransferase domains and two functional domains in the same polypeptide. All RSK isoforms have four essential phosphorylation sites (Ser221, Ser363, Ser380, and Thr573 in human RSK1) in the cytoplasmic region and act via mitogenic stimulation, such as growth factors. RSK isoforms and their substrates are involved in transcriptional and translational regulation, cell growth, motility, proliferation and survival.<sup>40</sup> In our study, the levels of phospho-MEK1/2 and phospho-ERK1/2 increased under hypoxic conditions (Fig. 3). Moreover, we demonstrated the presence of phosphorylated p90RSK in the nucleus (Fig. 4C). It is believed that the phosphorylated P90RSK translocates to the nucleus via the MAPK signaling pathway. The phosphorylated P90RSK and ERK might stimulate transcription factors in the nucleus and affect cell growth, survival, and proliferation (Fig. 7). Hypoxic conditions might stimulate proliferation and survival, and also maintain the phenotype of chondrocyte-like cells. The oxygen concentration might regulate transcription factors in the nucleus and control the characteristics of the ECM components through complex molecular rearrangements in the cells (Fig. 6).

In this study, a low oxygen concentration induced an increase in proteoglycan and GAG protein levels (Figs. 5 and 6) The oxygen concentration affected the proliferation of chondrocyte-like cells isolated from IVD and increased the mRNA expression levels of GLUT-1, aggrecan, and type II collagen. Furthermore, the protein levels of proteoglycan and GAG were increased by both chemical and physiological hypoxic conditions.

The findings from this study shed light on the mechanism of IVD degeneration and its adaptation during aging. First, with aging, disruptions in the oxygen and nutrient supply prompt adaptation of IVD cells, leading to their reduced cellular proliferation (conditions mimicking culturing in presence of 1% oxygen) and promoting differentiation into a discogenic phenotype (i.e., increase in type II collagen, aggrecan, and sulfated GAG production). Second, low oxygen tension activates the MAPK pathway, resulting in cellular proliferation and matrix synthesis. Third, low oxygen tension affects glucose metabolism by upregulating the GLUT-1 expression. Furthermore, IVD cell culture with low oxygen tension leads to an increase in cellular proliferation and the synthesis of discogenic matrix, which could be a useful tool for ex vivo cell therapy in degenerative disc disease. To consider the natural course of disc degeneration with endplate sclerosis and related deprivation of oxygen and nutrients, the results in the present study can also be applied in the clinical setting. The whole process of aging and adaptation of chondrocyte-like cells of IVD in hypoxic condition would be helpful to understand the degeneration of IVD as well as the related clinical presentation of back pain and radicular pain in degenerative disc diseases, such as the disc herniation and stenosis. This study was limited in that the cells were not separated into NP and AF regions; therefore, it remains unclear through which mechanism the oxygen concentration affected ECM synthesis. The findings of our study suggest the need for further studies to investigate the communication among chondrocyte-like cells in IVD. A future study co-culturing mesenchymal stem cells and IVD cells at various oxygen tensions is needed to optimize the discogenic differentiation of mesenchymal stem cells for such ex vivo cell therapy.

In conclusion, oxygen concentration plays an important role in the viability, proliferation, and maturation of chondrocyte-like cells in IVD. Chondrocyte-like cells were found to be sensitive to oxygen concentration. The cells were suitable for growth in low oxygen for their regular metabolism. We understand the physiological activity of cells in IVD, and believe our findings will contribute to understanding the physiological environment of IVD.

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