

# 論文内容要旨

## Differentiation of Human Bone Marrow-derived Mesenchymal Stem Cells into Early Chondrocytes in Three-dimensional Culture

(ヒト骨髄由来間葉系幹細胞の三次元培養下における軟骨細胞への分化)

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## ABBREVIATIONS

$\Delta r$	delta radius
3D	three dimensional
AD	anno domini
ATP	adenosine tri-phosphate
C	concentration
COX7B	cytochrome C oxidase subunit 7B
COX7C	cytochrome C oxidase subunit 7C
$C_m$	concentration in medium
Col II	collagen type II
Col X	collagen type X
D	diffusion coefficient
DMEM	Dulbecco's modified eagle media
GAG	glycosaminoglycan
H.E	hematoxylin and eosin
HIF-1 $\alpha$	hypoxia-inducible factor 1-alpha
IHC	immunohistochemistry
JCRB	Japanese Collection of Research Bioresources
MAPK8	mitogen-activated protein kinase 8
MEM $\alpha$	minimum essential medium alpha
MSC(s)	mesenchymal stem cell(s)
NAD+	nicotinamide adenine dinucleotide
NDUFB3	ubiquinone oxidoreductase subunit B3
NDUFS4	ubiquinone oxidoreductase subunit S4
NIHS	National Institute of Health Sciences
P.I	propidium iodide
PBS	phosphate buffered saline
PDK-1	pyruvate dehydrogenase lipoamide kinase 1
ppm	parts per million
$R, r$	radius
ROS	reactive oxygen species
SD	standard deviation
Sox9	sex determining region Y (SRY)-box 9
TBO	toluidine blue O

UQCRB	ubiquinol-cytochrome C reductase binding protein
<i>d</i>	density
<i>g</i>	gravity
hBMSC(s)	human bone marrow-derived mesenchymal stem cell(s)
<i>j</i>	flux of oxygen
ml	millimeter
mM	millimolar
n	number
nM	nanometer
<i>q</i>	oxygen uptake rate per unit volume
qPCR	quantitative polymerase chain reaction
<i>q<sub>c</sub></i>	oxygen consumption rate per single cell
<i>x</i>	distance
*	asterisk
**	double asterisks
μm	micrometer

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## GENERAL INTRODUCTION

Back to the earliest record of skin grafting in 5th century AD, an Indian surgeon performed nose repair [1]. In modern skin grafting, many allograft and autograft methods have been tested by various surgeons. Even those methods could provide better treatment options or claimed success in burn wound coverage, however low success rates, the requirement of large tissues, and the limitation of donor sites still remain a big challenges to overcome [2]. Therefore, it is likely that a recently-emerging strategy of regenerative medicine with the use of stem cells is quite attractive over allograft and autograft methods.

Dr. Green Howard, one of the founding father of stem cell research and regenerative medicine, established a method by which mouse embryonic cells can be cultured and allow them to develop into cell lines [3], enabling us to study stem cell characteristics *in vitro*. Since then, advances in biomaterials and scaffold research increased the number of tissues being engineered. Those tissues include cartilage, bone, fat, and other organ-specific tissues. In the early 1970s, a method using chondrocytes emerged as a new methodology for cartilage regeneration. This brought us the possibility of repairing cartilage by transplanting chondrocytes into a nude mouse *in*

*vivo*. Cao *et al.* [4] succeeded to generate a human ear-like tissue in nude mice. In the 1980s, researchers attempted to design scaffolds for cell delivery and nutrition supply, and to mimic micro-environments in the cartilage. In the 1990s, stem cell engineering emerged as promising technology for stem cell-based therapy and tissue engineering. Later, in the 2000s, tissue engineering using stem cells was widely examined for clinical purposes. Since then cartilage regeneration with stem cells have been tried and eventually proposed as one of promising methods for cartilage regeneration. Among human stem cells, human bone marrow-derived mesenchymal stem cells (hBMSCs) are the most widely used in research for cartilage regeneration due to their high level of Col II production. Three dimensional (3D) chondrogenic pellets have been studied by combining with or without scaffolds made of protein-based polymers, carbohydrate-based polymers, synthetic polymers, with a growth factor stimulation system, both *in vitro* and *in vivo* [5].

However, cartilage regeneration still remains a big challenge due to the low capacity of cartilage self-renewal, difficulty for regenerated cartilages to fully function, or poor integration of newly-formed cartilage with host tissues. In addition, many trials including autologous chondrocyte transplantation, collagen-covering, or matrix-assistance have provided promising strategies, while many obstacles still remain

[6]. Recently, advancement of stem cell biology has provided other options for cartilage regeneration. The current approaches are to ensure optimal and stable chondrogenic phenotype and extracellular matrix by preventing chondrocyte degradation and avoiding the hypertrophic conversion of hBMSCs [7]. According to the current dogma [8], chondrocytes and osteoblasts can be derived from a common osteochondro-progenitors.

This leads us to hypothesis that fabrication of 3D chondrogenic pellets from hBMSCs may be useful in order to maintain chondrogenic phenotype, prevent chondrocyte degradation, and avoid hypertrophic chondrocyte conversion. From this aspect, we consider a culture method that mimics natural developmental processes, as a suitable strategy for the next generation of cartilage tissue regeneration. In order to fulfill these purposes, we need to gain deeper insights into major causes that lead to chondrocyte degradation, differentiation, and conversion. As major causes of these dynamic changes, special attention was paid here to medium conditions, oxygen consumption as well as gradient, and the effect of glucose concentration, all of which may strongly influence chondrogenesis in pellets and prevent hypertrophic chondrocyte conversion.





# Chapter 1

## Effect of medium conditions on chondrogenesis in pellets

### 1. INTRODUCTION

Human bone marrow-derived mesenchymal stem cells (hBMSCs) are considered for a long time to be a promising candidate for cartilage regeneration and frequently cultured in a three-dimensional (3D) pellet. Recently, it was shown that 3D culture of cells in chondrogenic lineages, such as synovial chondrocytes, in the form of pellets improved their ability of integration and chondrogenic differentiation when transplanted into the body [9, 10]. It is obvious from these previous studies that 3D pellet culture is advantageous for the construction of chondrogenic grafts for cartilage repair.

However, it may be considered that microenvironment within a 3D pellet is quite anisotropic with regard to a distance from the pellet surface, because nutrients and oxygen are always supplied from the surface to the core of the pellet, while continuously consumed by pellet forming cells, gradually decreasing their concentrations. Under the steady-state conditions, it seems that the core regions of a 3D pellet are under hypoxia conditions, which have great impacts on the survival and

differentiation of hBMSCs within the pellet [11].

In this chapter, the effect of medium conditions on the chondrogenesis of hBMSCs in such an anisotropic pellet was examined with a special attention to chondrogenic differentiation and cell necrosis.

## 2. EXPERIMENTAL

### 2.1 Cell and medium

Immortalized hBMSCs (UE6E7T-3) were obtained from NIH (JCRB) cell bank, Japan, and cultured in a tissue-culture polystyrene dish under growth medium prepared with Dulbecco's modified eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 10% fetal bovine serum (FBS; Hyclone, Life Scientific, South Logan, UT, USA), 50 µg/ml ascorbate 2-phosphate (Wako Pure Chemical Industries, Osaka, Japan), and 1 ng/ml recombinant human FGF-2 (Gibco, Carlsbad, CA, USA). For the chondrogenic differentiation of hBMSCs, cells were cultured under chondrogenic medium prepared with minimum essential medium eagle alpha modifications (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml of streptomycin, 10% FBS, 50 µg/ml ascorbate 2-phosphate, 10 ng/ml TGF-β3 (Peprotech, Rocky Hill, NJ, USA), 30 mM D(+)-glucose

(Sigma-Aldrich), 1% ITS-Plus (Corning, Bedford, MA, USA), 0.1 nM dexamethasone (Sigma-Aldrich), 100 µg/ml pyruvate (Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich).

## 2.2 Pellet culture

After hBMSCs reached confluence, the cells were harvested by trypsinization and suspended in growth or chondrogenic medium. Then a suspension containing  $2.5 \times 10^6$  cells was centrifuged at 500 g for 5 min in a 15 ml conical tube to obtain a pellet. Then pellets were cultured under growth or chondrogenic medium, and medium was changed every 2-3 days. At 3rd day after pellet formation, pellets were transferred to a 24-well non-treated culture plate and cultured for 7, 10, 14 days under growth or chondrogenic medium.

## 2.3 Gene expression analysis

hBMSCs were cultured in monolayers under growth or chondrogenic medium, and total RNA was extracted from these cells. To assess chondrogenic differentiation, the expression of early chondrogenic marker genes including sex determining region Y (SRY)-box 9 (Sox9) and Col II was analyzed by reverse transcriptase-quantitative

polymerase chain reaction (qPCR). For assessing the oxygen consumption, mitochondrial activity assay was performed by qPCR, detecting the expression of mitochondrial redox carrier genes.

## 2.4 Histology

Pellets were fixed overnight with 4% paraformaldehyde in phosphate buffered saline (PBS). Then they were dehydrated with 70%, 80%, 90%, and 100% ethanol for 5 min each. Subsequently they were immersed in xylene for 1 hr and then embedded in paraffin wax for 2 hr at 65 °C to obtain a paraffin block. A pellet in the block was sliced with a thickness of 5 µm. Sections were stained with hematoxylin and eosin (H.E) by the standard method. The diameter of a pellet was determined on the microscopic image of the H.E stained sections.

To detect necrotic cells, sections were immersed in 0.05 µg/ml propidium iodide (P.I) solution in PBS for 5 min and then rinsed 2-3 times with PBS. The abundance of necrotic cells was assessed by image analysis using ImageJ software (ver 1.4.6, NIH, Bethesda, MD, USA).

## 2.5 Statistical analysis

Statistical significance for the differences in gene expression, cell necrosis, and pellet diameters was tested by ANOVA.

## 3. RESULTS

### 3.1 Chondrogenic differentiation and oxygen consumption by cells in monolayer

hBMSCs were cultured in monolayer under growth or chondrogenic medium. As shown in Figure 1, cells cultured in chondrogenic medium expressed early chondrogenic markers, Sox9 and Col II, more significantly than those in growth medium. This result indicates that, as expected, chondrogenic differentiation of hBMSCs is promoted by culturing them in chondrogenic medium, compared to growth medium.

Figure 2 shows the result of the mitochondrial activity assay. It can be seen that the expression of all mitochondrial redox carrier genes studied here was much less in cells cultured under chondrogenic condition than those under growth condition, suggesting that oxygen consumption by hBMSCs decreases with their differentiation into chondrocytes.

### 3.2 Chondrogenic differentiation of cells in a pellet

Spherical pellets were made from cells cultured in growth or chondrogenic medium and subsequently cultured in growth or chondrogenic medium for 7, 10 and 14 days. The diameter of a pellet was measured on H.E stained sections prepared near the equatorial plane. The results are shown in Figures 3 and 4 for growth and chondrogenic conditions, respectively. The diameter of these pellets were determined and shown in Figure 5. It is seen that the diameter of a pellet cultured in both growth and chondrogenic medium steadily increased from day 7 to day 14. However, the diameter of pellets cultured in growth medium was significantly smaller than that of pellets in chondrogenic medium at day 10 and 14, even though cells had been initially seeded at the same density.

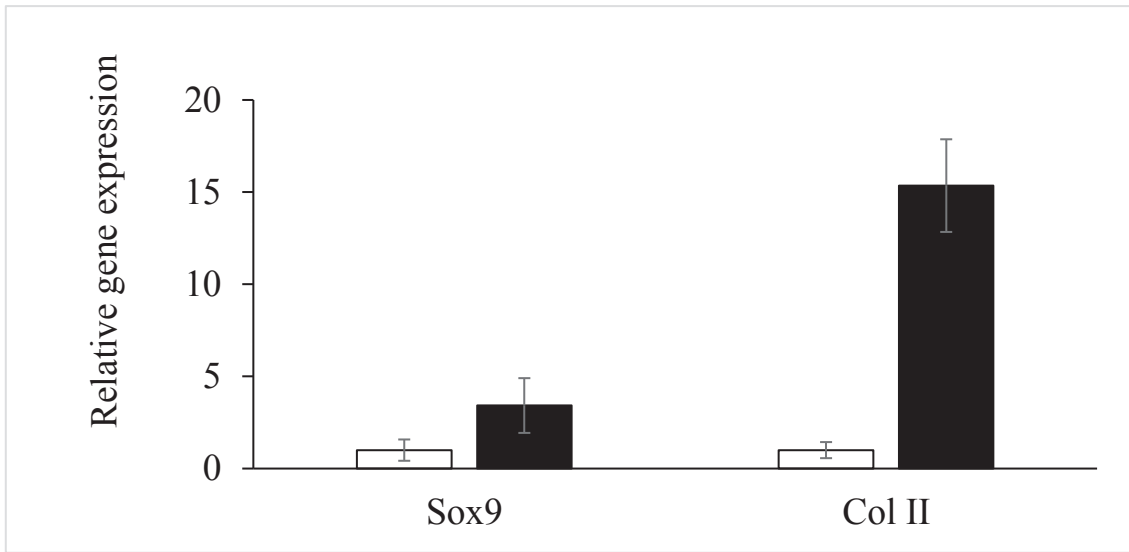


Figure 1. Expression of early chondrogenic marker genes, Sox9 and Col II, analyzed by qPCR. Cells were cultured in monolayer under (open bar) growth or (close bar) chondrogenic medium. Data are expressed as mean  $\pm$  SD (n = 3).

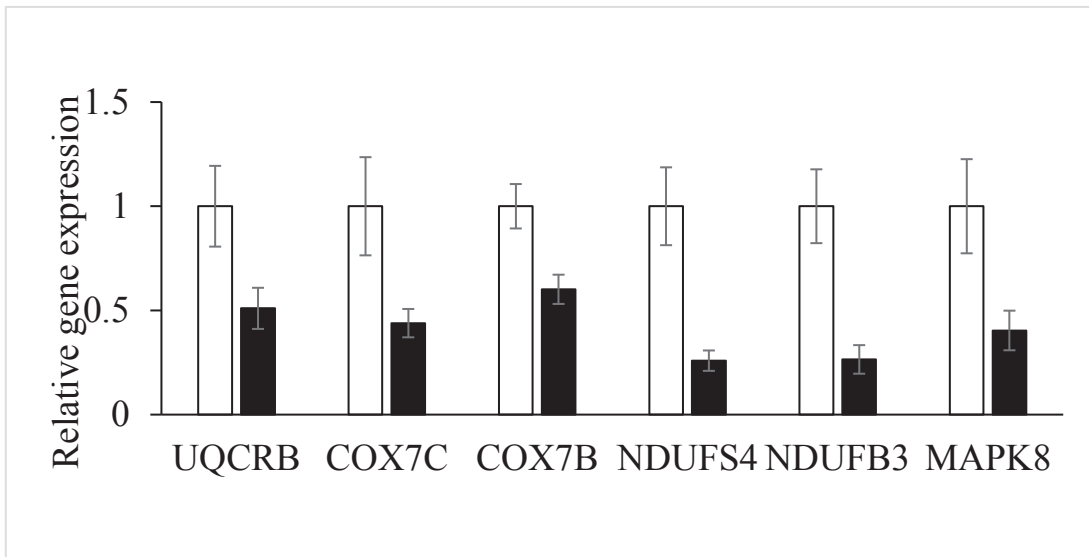


Figure 2. Mitochondrial activity assessed by qPCR for genes involved in the formation of mitochondrial redox carriers. Cells were cultured in monolayer under (open bar) growth or (close bar) chondrogenic medium. Data are expressed as mean  $\pm$  SD (n = 3).



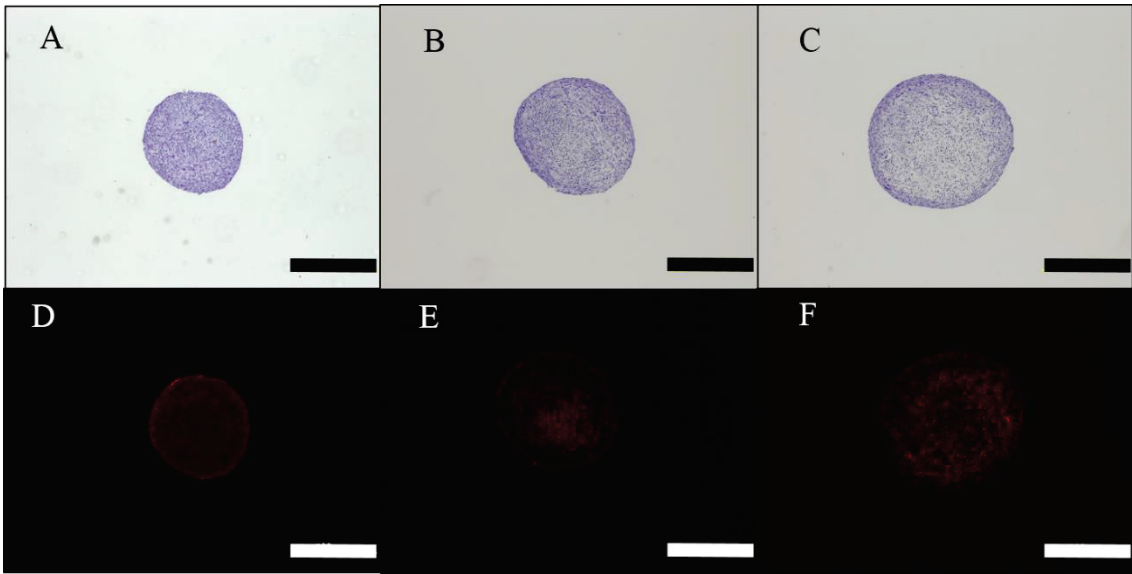


Figure 3. Histological analysis of pellets prepared from cells cultured in growth medium for (A, D) 7, (B, E) 10, and (C, F) 14 days. The cross-sections of the pellets were stained with (A–C) H.E or (D–F) P.I. Scale bar: 500  $\mu$ m.

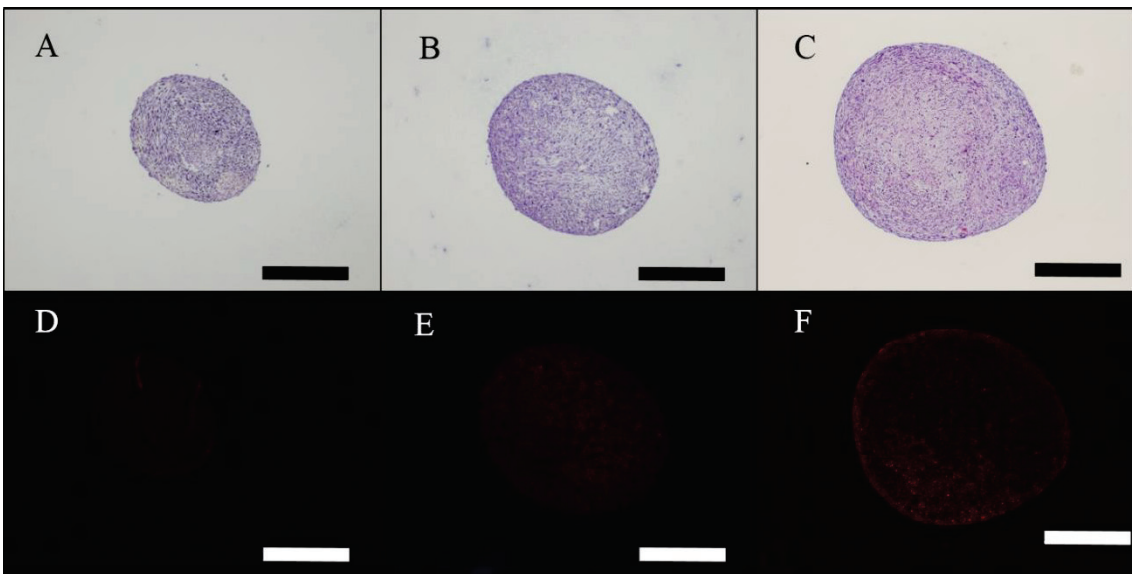


Figure 4. Histological analysis of pellets cultured in chondrogenic medium for (A, D) 7, (B, E) 10, and (C, F) 14 days. The cross-section of the pellets was stained with (A–C) H.E or (D–F) P.I. Scale bar: 500  $\mu$ m.

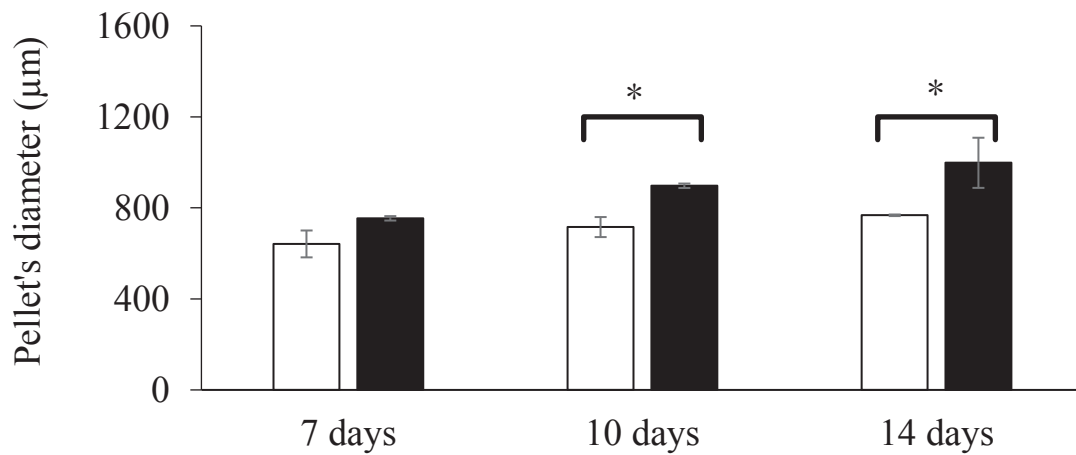


Figure 5. The diameter of pellets cultured for 7, 10 and 14 days. Pellets were prepared from (open bar) growth medium and (closed bar) chondrogenic medium. Data are expressed as mean  $\pm$  SD (n = 3). An asterisk (\*) represents significant difference by ANOVA ( $p \leq 0.05$ ).

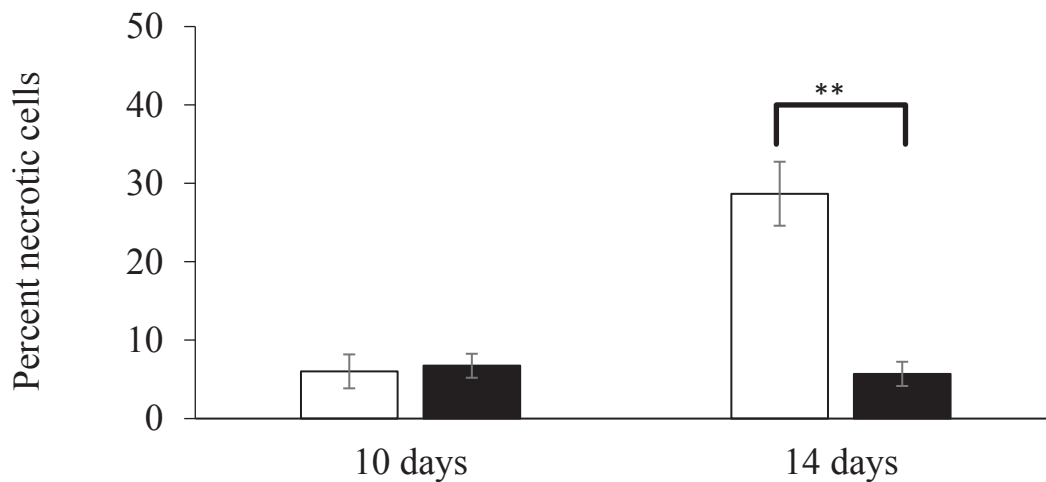


Figure 6. Percent necrotic cells in pellets obtained from (open bar) undifferentiated and (closed bar) differentiated cells. Data are expressed as mean  $\pm$  SD (n = 3). A double asterisk (\*\*) represents significant difference by ANOVA ( $p \leq 0.01$ ).

### 3.3 Cell necrosis in a pellet

As shown in Figures 3 and 6, a larger number of cells were stained with P.I in

pellets cultured under growth conditions than in those under chondrogenic condition. These results indicate the abundance of necrotic cells within a pellet cultured in growth medium compared to chondrogenic medium.

#### 4. DISCUSSION

A difference in medium conditions is considered. When hBMSCs were cultured under growth medium, differentiation is unexpectedly to occur. In other words, hBMSCs remain in the undifferentiating status. Instead, hBMSCs undergo differentiation into chondrocytes under chondrogenic medium. This is proofed by the results of expression analysis for early chondrogenic marker genes, Sox9 and Col II [12]. On the other hand, mitochondrial assay suggested that differentiating chondrocytes consume less oxygen than that of growing hBMSCs. This finding may closely related to the abundance of necrotic cells in the pellets under growth medium. As demonstrated here, pellets obtained from differentiating cells showed greater survival rate than that of growing hBMSCs.

Accordingly, it may be concluded that chondrogenic culture both before and after pellet preparation provides better condition for the survival and differentiation of hBMSCs in the 3D pellets.

## Chapter 2

### Effect of hypoxia conditions on chondrogenesis in pellets

#### 1. INTRODUCTION

In the previous chapter, it was demonstrated that oxygen consumption rate is closely related to the differentiation and survival of hBMSCs in a 3D pellet. Because oxygen is always supplied from the surface to the core of a pellet, while continuously consumed by cells within the pellet and gradually decreasing its concentration, it may be expected that there is an oxygen gradient in a pellet. This means that microenvironment within a 3D pellet is quite anisotropic with regard to a distance from the pellet surface. Under the steady-state conditions, it seems that oxygen gradients established along the radial direction have great impacts on the survival, proliferation, and chondrogenic differentiation of hBMSCs within a pellet. Therefore, this chapter is directed to study the effect of an oxygen gradient on chondrogenic differentiation and survival of hBMSCs in a pellet. It will be demonstrated that hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ ) plays a central role in the link between hypoxia conditions and chondrocyte differentiation and survival.

## 2. THEORETICAL CONSIDERATION

Oxygen concentration profile from periphery to the center of a pellet was theoretically considered for the comparison of necrotic cell distributions in a pellet. First, we assume that a spherical pellet (Figure 7) in which cells are homogeneously distributed at a density,  $d$  [cells mL<sup>-1</sup>] and the cells consume oxygen due to their metabolic activity at a constant rate,  $q$  [mol mL<sup>-1</sup>]. Oxygen is supplied from the outer medium and diffuses from the surface to the core of a pellet at a diffusion constant,  $D$  [cm<sup>2</sup> s<sup>-1</sup>]. Because oxygen diffusion obeys the Fick's first law, therefore, the flux of oxygen,  $J$  [mol cm<sup>-2</sup> s<sup>-1</sup>] can be expressed as follows:

$$J = D \frac{\partial C}{\partial x} \quad (1)$$

where  $x$  [m] is a distance and  $C$  [mol L<sup>-1</sup>] is a concentration of oxygen.

On the other hand, mass balance at steady state is applied to the spherical shell at a distance of  $r$  ( $R \geq r \geq 0$ , where  $R$  is radius of a pellet) from the center of a pellet. Considered the spherical shell with a thickness of  $\Delta r$ , the difference in inflow (pass through the plane at  $x = r + \Delta r$ ) and outflow (pass through the plane at  $x = r$ ) oxygen fluxes is attributed to oxygen consumption within the shell (thickness =  $\Delta r$ ). This can be

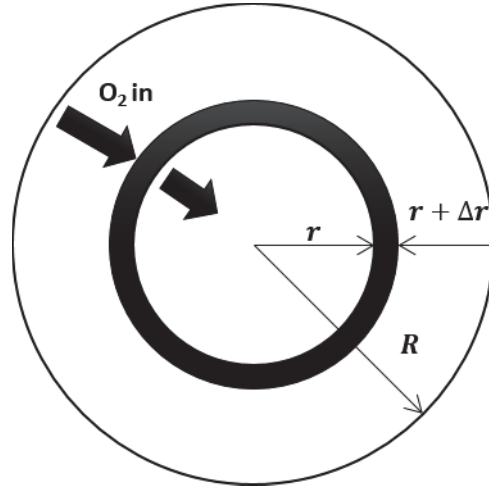


Figure 7. Illustration of a spherical shell assumed for the theoretical prediction of oxygen concentration profile in a cell pellet. A cross section of a 3D pellet with a radius of  $R$  is depicted in 2D. The shell is located at a distance of  $r$  from the center of the pellet ( $0 \leq r \leq R$ ), and the thickness of the shell is  $\Delta r$ .

expressed by equation (2):

$$[\text{Inflow oxygen}]_{r+\Delta r} - [\text{Outflow oxygen}]_r - [\text{Oxygen consumption}]_{dr} = 0 \quad (2)$$

Since oxygen flow into the pellets through a unit area of the spherical shell surface is expressed as  $(4\pi r^2 J)_{r+\Delta r}$ , while outflow of oxygen from the shell is expressed as  $(4\pi r^2 J)_r$ . Therefore, equation (2) can be substituted as follows:

$$[4\pi(r + \Delta r)^2(-D \frac{\partial c}{\partial r})]_{r+\Delta r} - [4\pi r^2(-D \frac{\partial c}{\partial r})]_r - 4\pi r^2 \Delta r q = 0 \quad (3)$$

By giving the limit as  $\Delta r \rightarrow 0$ , then we obtain equation (4):

$$\frac{1}{r^2} D \frac{\partial}{\partial r} \left( r^2 \frac{\partial C}{\partial r} \right) - q = 0 \quad (4)$$

Using a boundary condition of  $C = C_m$  at  $r = R$ , the differential equation (4) can be solved as below:

$$C = \left[ \frac{q}{6D} (r^2 - R^2) \right] + C_m \quad (5)$$

Where  $C_m$  is oxygen concentration in a medium, and  $q$  is an oxygen uptake rate per unit volume. The  $q$  value can be determined from cell density ( $d$ ) and the oxygen consumption rate per single cells ( $q_c$ ). Thus,  $q$  can be obtained by equation (6).

$$q = (d / \frac{4}{3} \pi r^3) \times q_c \quad (6)$$

Streeter and Cheema [13] reported that the rate of oxygen consumption ( $q_c$ ) by a single hBMSC in 3D culture was determined to be  $7.91 \times 10^{-18}$  mol cell<sup>-1</sup> sec<sup>-1</sup>. For the diffusion constant of oxygen ( $D$ ), the value ( $D = 1.7 \times 10^{-5}$  cm<sup>2</sup> s<sup>-1</sup>) previously

described by Ehsan for 3D vascular tissues composed of endothelial cells and fibroblasts [14] was used in this study.

### 3. EXPERIMENTAL

#### 3.1 Cell and medium

Immortalized hBMSCs (UE6E7T-3) were obtained from NIH (JCRB) cell bank, Japan, and cultured in a tissue-culture polystyrene dish under growth medium prepared with Dulbecco's modified eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 10% fetal bovine serum (FBS; Hyclone, Life Scientific, South Logan, UT, USA), 50 µg/ml ascorbate 2-phosphate (Wako Pure Chemical Industries, Osaka, Japan), and 1 ng/ml recombinant human FGF-2 (Gibco, Carlsbad, CA, USA). For the chondrogenic differentiation of hBMSCs, cells were cultured under chondrogenic medium prepared with minimum essential medium eagle alpha modifications (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml of streptomycin, 10% FBS, 50 µg/ml ascorbate 2-phosphate, 10 ng/ml TGF-β3 (Peprotech, Rocky Hill, NJ, USA), 30 mM D(+)-glucose (Sigma-Aldrich), 1% ITS-Plus (Corning, Bedford, MA, USA), 0.1 nM dexamethasone (Sigma-Aldrich), 100 µg/ml pyruvate (Sigma-Aldrich), and 2 mM L-glutamine



(Sigma-Aldrich).

### 3.2 Pellet culture

hBMSCs were cultured in monolayer under growth or chondrogenic medium. After hBMSCs reached confluence, the cells were harvested by trypsinization and suspended in growth or chondrogenic medium. Then a suspension containing  $2.5 \times 10^6$  cells were centrifuged at 500 g for 5 min in a 15 ml conical tube to obtain a pellet. Then pellets were cultured under growth or chondrogenic medium, and medium was changed every 2-3 days. At 3rd day, pellets were transferred to a 24-well non-treated culture plate and cultured for 7, 10, 14 days. Here pellets prepared from undifferentiated cells (cultured under growth medium at the 1st stage in a monolayer) and then cultured in chondrogenic medium are referred as "Non-pre-chondrogenic pellets", whereas pellets prepared from differentiated cells (cultured under chondrogenic medium at the 1st stage in a monolayer) then cultured in chondrogenic medium are referred as "Pre-chondrogenic pellets".

### 3.3 Histology

Pellets were fixed overnight with 4% paraformaldehyde in phosphate buffered

saline (PBS). Then they were dehydrated with 70%, 80%, 90%, and 100% ethanol for 5 min each. Subsequently they were immersed in xylene for 1 hr and then embedded in paraffin wax for 2 hr at 65 °C to obtain a paraffin block. A pellet in the block was sliced with a thickness of 5 µm.

To assess the formation of cartilage-like tissues, the sections were stained with toluidine blue O (TBO). The sections were also stained with hematoxylin and eosin (H.E). To detect necrotic cells, slices were stained using 0.05 µg/ml propidium iodide (P.I) solution in PBS for 5 min and then rinsed 2-3 times with PBS. The abundance of necrotic cells was assessed by image analysis using ImageJ software (ver 1.4.6, NIH, Bethesda, MD, USA).

Immunofluorescent staining was carried out using anti-HIF-1 $\alpha$  antibody (1:400, Novus Biologicals, Littleton, CO, USA) and fluorescently-conjugated secondary antibody. The sections were observed with a fluorescent microscope (IX73, OLYMPUS, Tokyo, Japan).

### 3.4 Oxygen concentration

The concentration of dissolved oxygen (DO) in a medium was measured using a digital polarographic DO probe with integrated temperature sensor (Hanna edge®

Multiparameter DO meter HI2040, Hanna Instruments, Woonsocket, RI, USA). The measurement was performed at close proximity to the pellet surface in a medium.

### 3.5 Statistical analysis

Statistical significance for the differences in gene expression, cell necrosis, and pellet diameters was tested by ANOVA.

## 4. RESULTS AND DISCUSSION

### 4.1 Oxygen gradient and the distribution of necrotic cells in a pellet

Oxygen gradient was theoretically determined and shown in Figure 8 for the case of a pellet with a typical diameter (400  $\mu\text{m}$ ) in a medium containing 5.5 ppm dissolved oxygen (determined using an oxygen electrode). As is seen in Figure 8, oxygen concentration gradually decreased with a distance from the surface of a pellet. In addition, the graph shows that, under the condition considered here (400  $\mu\text{m}$  in a pellet diameter), sufficient oxygen is not able to reach to the core region of a pellet, where cells may not be able to survive the hypoxia condition.

Figures 9 and 10 show the presence of necrotic cells in pellets that were prepared from undifferentiated (cultured under growth medium in monolayers;

Non-pre-chondrogenic pellets) and differentiated (cultured under chondrogenic medium in monolayers; Pre-chondrogenic pellets) cells then cultured in chondrogenic medium in a pellet for both cases. As is seen, the necrotic cells were significantly larger in pellets initially prepared with undifferentiated cells than in those with differentiated

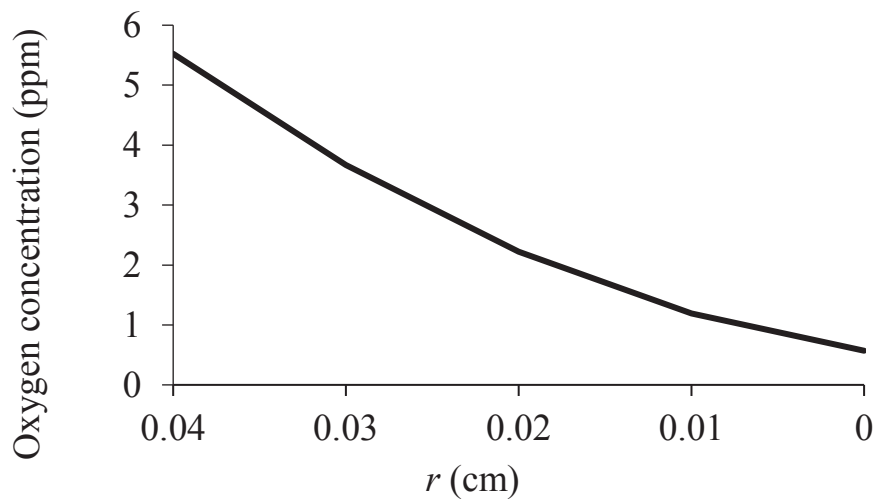


Figure 8. Theoretically predicted profile of oxygen concentration along the radial direction in a spherical pellet with a radius of 400  $\mu\text{m}$  in a medium at an oxygen concentration of 5.5 ppm.  $r$  represents distance from the core of a pellet.

cells. This result is generally in good agreement with the finding in Chapter 1: Differentiated chondrocytes require less oxygen and therefore remain viable at high survival rate. However, the result tells us nothing about the distribution of necrotic cells within a pellet.

To gain deeper insights into the distribution of necrotic cells, the density necrotic

cells stained by P.I was determined for consecutive concentric layers (thickness of each layer: 50  $\mu\text{m}$ ). The results are shown in Figures 11 and 12 for the case of growth and chondrogenic media, respectively.

It can be seen in Figure 11 (growing pellets prepared with undifferentiated hBMSCs) that, regardless the distance from a pellet surface, the percentage of necrotic cells after 7-day culture was relatively low comparing to those after 10- and 14-day culture. Although necrotic cell densities at day 7 and 10 were slightly lower than that at day 14 at any analyzed zones, the number of living cells was higher in the peripheral layers than that in the middle or core regions. Necrotic cell density gradually increases with a distance from the surface to the core of a pellet. It was observed that almost 70% of total cells died in the central region after 14 days. It should be noted that the observed distribution of necrotic cells with regard to the distance from the pellet surface is in accordance with the theoretical curve in Figure 8 for an oxygen gradient, suggesting that necrosis is closely linked to oxygen concentration and that the core regions are under hypoxia condition. A similar simple dependence was also reported by others [15].

In the case of chondrogenic pellets (Figure 12), necrotic cells distributed in a manner which is distinct from the case with growing pellets: First, the overall density of necrotic cells is relatively low compared to the case with growing pellets (Figure 11) at

any time points. It is striking that, at day 14, the density of necrotic cells increased with a distance from the surface of a pellet and reached the highest percentage at a distance of 250  $\mu\text{m}$  from the pellet surface. In the region deeper than this ( $> 300 \mu\text{m}$ ), the density of necrotic cells decreased with a distance. Because cells in chondrogenic pellets at day 14 can be expected to differentiate into chondrocytes, they are likely to require less oxygen and therefore can survive even under hypoxia condition in the core regions. The discrepancy between the observed distribution of necrotic cells (Figure 12) and the simple theoretical prediction for oxygen gradient (Figure 8) can be ascribed to the changes in oxygen requirement by cells upon their chondrogenic differentiation.

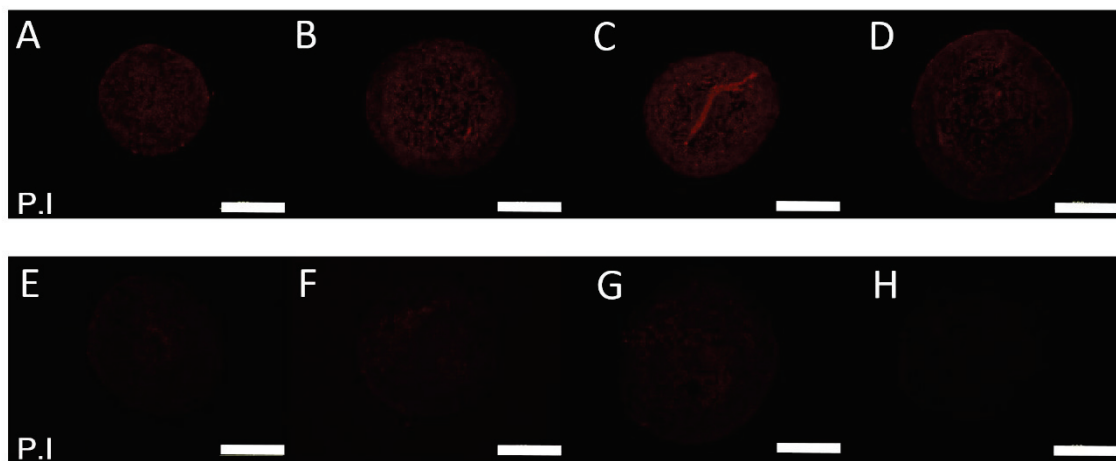


Figure 9. Propidium iodide (P.I) staining of necrotic cells in pellets cultured for 10, 14, 21, and 28 days. (A–D) Non-pre-chondrogenic pellets, (E–H) Pre-chondrogenic pellets. Scale bar: 500  $\mu\text{m}$ .

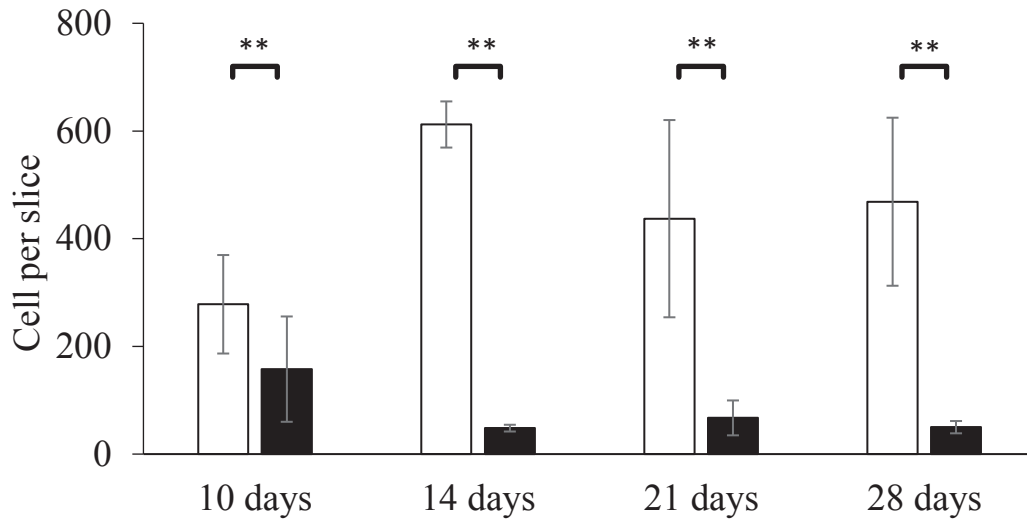


Figure 10. Quantification of necrotic cells present in pellets cultured for 10, 14, 21, and 28 days. (Open bar) Non-pre-chondrogenic pellets, (Closed bar) Pre-chondrogenic pellets. Data are expressed as mean  $\pm$  SD (n = 3). Statistical analysis was performed by ANOVA (\*\*:  $p \leq 0.01$ ).

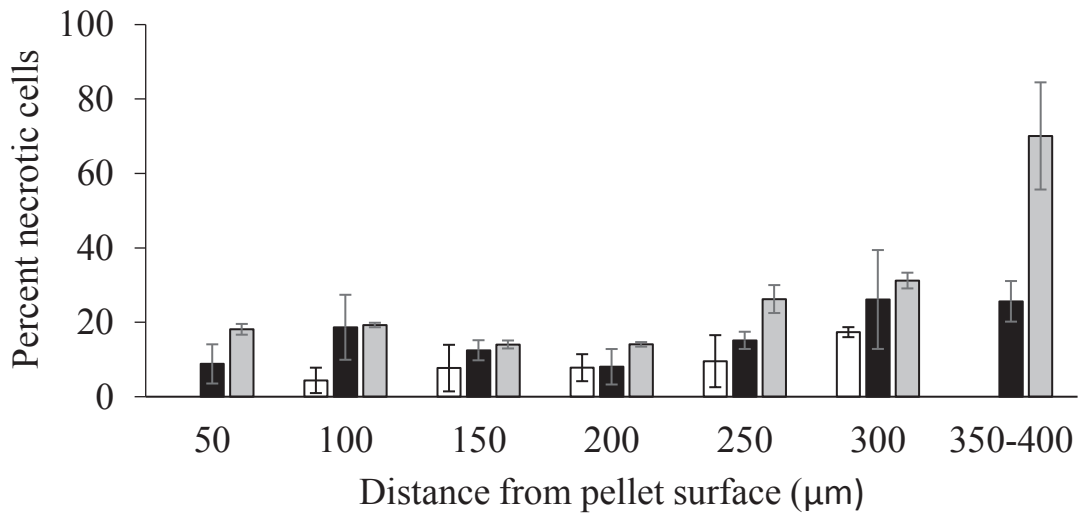


Figure 11. Distribution of necrotic cells in Non-pre-chondrogenic pellets cultured in growth medium for (open bar) 7, (closed bar) 10 and (gray bar) 14 days. Data are expressed as mean  $\pm$  SD (n = 3).

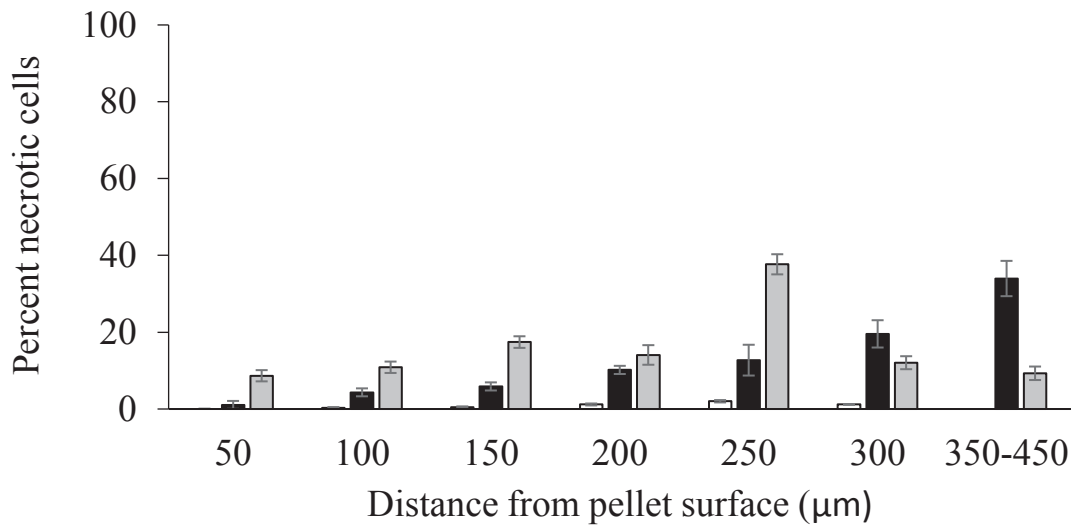


Figure 12. Distribution of necrotic cells in Pre-chondrogenic pellets cultured further in chondrogenic medium for (open bar) 7, (closed bar) 10 and (gray bar) 14 days. Data are expressed as mean  $\pm$  SD (n = 3).

#### 4.2 Anisotropy of chondrogenesis in a pellet

TBO staining was performed for Non-pre-chondrogenic and Pre-chondrogenic pellets and the results are shown in Figures 13 and 14. As seen in Figure 13, the core region exhibited dense staining especially in the case of Pre-chondrogenic pellets. This is indicative of extracellular matrix production and the differentiation of hCMSCs into chondrocyte lineage. As shown in Figure 14, the level of chondrogenic differentiation is more significant in the case of Pre-chondrogenic pellets compared to the case of Non-pre-chondrogenic ones. This observation is in good agreement with the finding that cell necrosis is unlikely to occur in the core region,



since chondrocytes require less oxygen compared to cells of much undifferentiated states.

It is interesting to note that co-culture of chondrocytes and hBMSCs has been shown to enhance expression of cartilaginous extracellular matrix [16], and co-culture of hMBSCs with chondrocytes exhibit a superior microenvironment for chondrogenesis than when they were cultured separately [17]. This may be implicated in the fact that Pre-chondrogenic method is favorable for cartilage regeneration compared to Non-pre-chondrogenic method.

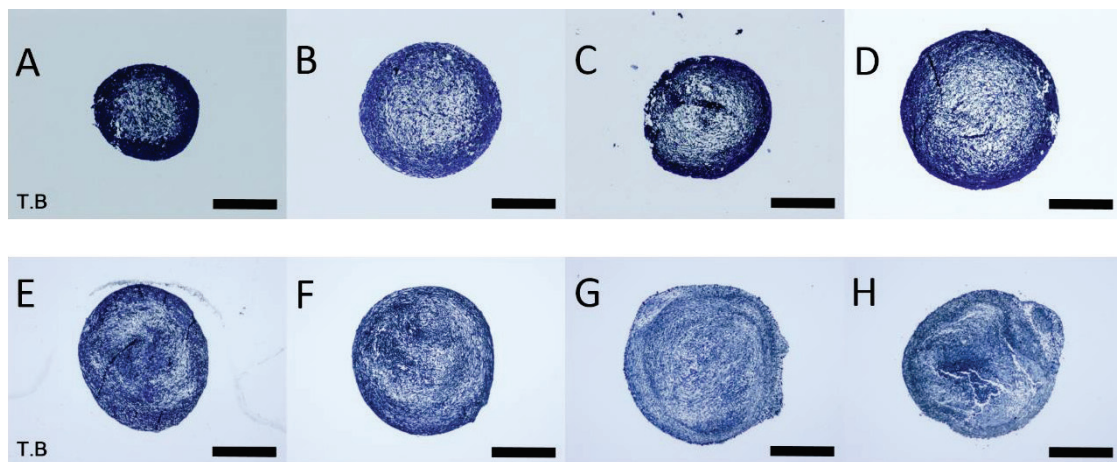


Figure 13. Cartilage-like tissues stained with TBO. Pellets were cultured for (A, E) 10, (B, F) 14, (C, G) 21, and (D, H) 28 days. (A–D) Non-pre-chondrogenic pellets and (E–H) Pre-chondrogenic pellets (E–H) were cultured in chondrogenic medium. Scale bar = 500  $\mu$ m.

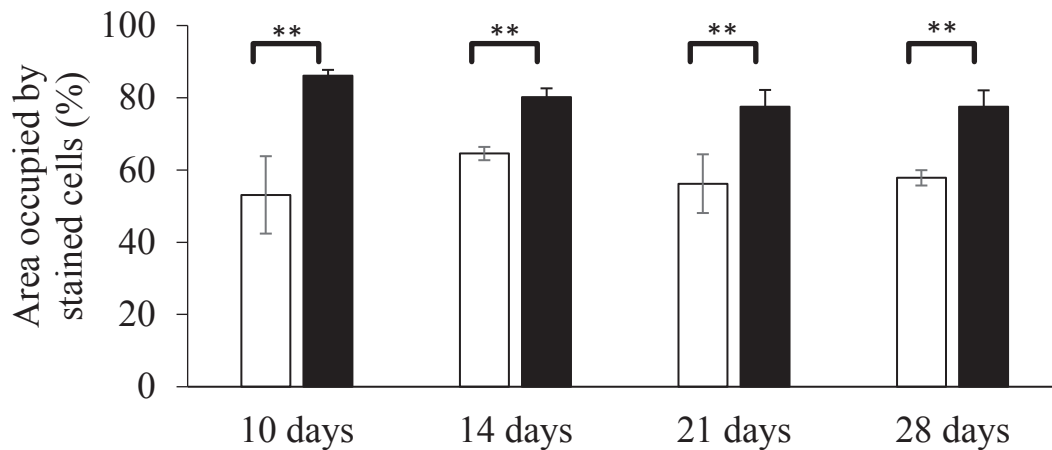


Figure 14. Quantification of TBO-stained chondrocytes. Pellets were cultured for 10, 14, 21, and 28 days. (open bar) Non-pre-chondrogenic and (closed bar) Pre-chondrogenic pellets were cultured in chondrogenic medium for up to 28 days. Data are expressed as mean  $\pm$  SD (n = 3). Statistical analysis was performed by ANOVA (\*\*:  $p \leq 0.01$ ).

#### 4.3 HIF-1 $\alpha$ expression in a pellet

To gain deeper insights into the effect of hypoxia conditions, pellets were prepared separately from Non-pre-chondrogenic and Pre-chondrogenic cells and then cultured under chondrogenic medium up to 28 days. As is seen in Figures 15 and 16, HIF-1 $\alpha$  expression is much more notable with Pre-chondrogenic pellets than Non-pre-chondrogenic pellets. This result suggests that chondrogenic medium provides an inductive environment for cells to express HIF-1 $\alpha$ .

#### 4.4 Involvement of HIF-1 $\alpha$ expression in cell differentiation and survival

As predicted by theoretical consideration, it appears that necrotic cells distribute

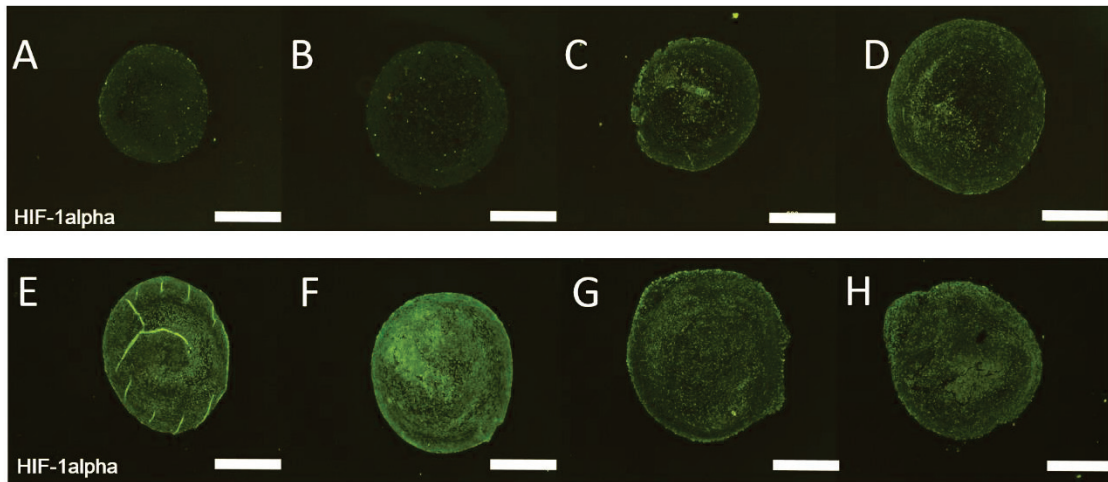


Figure 15. Immunofluorescent staining of HIF-1 $\alpha$  expressed in pellets. (A–D) Non-pre-chondrogenic and (E–H) Pre-chondrogenic pellets were cultured in chondrogenic medium for (A, E) 10, (B, F) 14, (C, G) 21, and (D, H) 28 days. Scale bar: 500  $\mu$ m.

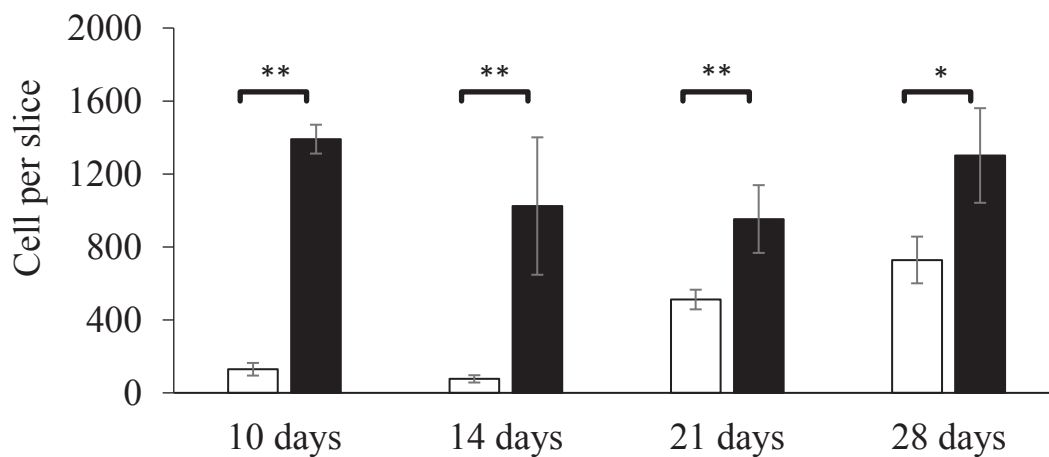


Figure 16. Quantification of HIF-1 $\alpha$  immunofluorescence. (open bar) Non-pre-chondrogenic and (closed bar) Pre-chondrogenic pellets were cultured in chondrogenic medium and then immunologically stained using antibody to HIF-1 $\alpha$ . Data are expressed as mean  $\pm$  SD (n = 3). Statistical analyzed was performed by ANOVA (\*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ ).

unevenly within a pellet prepared from Non-pre-chondrogenic cells and are more

abundant in the core regions of the pellet. These results can be simply explained from the theoretical consideration for oxygen gradient established within a pellet. However, when pellet culture was initiated with more differentiated chondrocytes, namely Pre-chondrogenic cells, the density of necrotic cells are relatively low in the core regions. This is likely because of reduced requirement of oxygen by differentiated chondrocytes. These results suggest that hypoxia condition established especially in the core regions of a pellet has rather a positive effect on the enrichment of chondrocytes.

It was reported that chondrogenesis of hBMSCs was enhanced by the hypoxic condition at approximately 1-5% of oxygen and that GAG production was further upregulated in this state than at normal oxygen tension [18]. In addition, it was reported that hypoxic conditions were advantageous to maintain chondrogenic environments [19] and to improve growth and genetic stability by activating glycolysis [20].

As one of the most important molecules, HIF-1 $\alpha$  is known to promote chondrocyte differentiation and also suppress mitochondrial activity. Therefore, a special attention was paid here to this molecule. This protein is known as a transcription factor that binds to Sox9 promoter to induce chondrogenesis [21] In addition, loss of HIF-1 $\alpha$  leads to cell necrosis in HIF-1 $\alpha$  knockout mice [22], while the expression of several proteins forming mitochondrial redox carriers is impaired by HIF-1 $\alpha$ . The later

effect gives rise to a decrease in intracellularly-generated reactive oxygen species (ROS), a by-product of ATP synthesis, which may cause cell necrosis. In addition, increase in ROS has been characterized by an increased mitochondrial function [23]. Furthermore, the expression of HIF-1 $\alpha$  serves to transform cells so that cells are able to use available glucose in anaerobic metabolism in order to keep the maximum rate of glycolytic ATP production through the upregulation of PDK-1 [24, 25] and lactate dehydrogenase A [26]. Through this pathway, HIF-1 $\alpha$  expression can promote effectively the recycle of glycolytic cofactor NAD<sup>+</sup>. As a result, HIF-1 $\alpha$  expression can prevent cell necrosis [27]

It was observed in the present study that pellets prepared with Pre-chondrogenic cells yielded more cartilage-like extracellular matrices, as shown by TBO staining, than Non-pre-chondrogenic pellets. In addition, the level of HIF-1 $\alpha$  gene expression was higher in pellets prepared with Pre-chondrogenic cells than those with Non-pre-chondrogenic cells. Both results can be well explained from the diverse functions of HIF-1 $\alpha$  as described above. In addition, it was reported that chondrocytes should be cultured under hypoxia condition which contains 1-5% of oxygen for bio-safety and preventing endochondral ossification [28]. Taking together into consideration, the Pre-chondrogenic method is suitable for chondrogenic differentiation

in 3D pellet culture for cartilage regeneration in term of promoting chondrogenic differentiation, and reducing cell necrosis.



## Chapter 3

### Effect of glucose on chondrogenesis in pellets

#### 1. INTRODUCTION

In 3D pellets, hBMSCs undergo over time differentiation into hypertrophic chondrocytes associated with cell apoptosis, which raises a significant concern in cartilage regeneration. Besides apoptosis, conversion of early chondrocytes into hypertrophic chondrocytes promotes vascular invasion, endochondral ossification, and bone formation. This represents that, with current culture conditions, it is not easy to avoid to induce differentiation of hBMSCs into long growth plate bone *in vitro*. Previous study has suggested that thyroid hormones, TGF- $\beta$ , and dexamethasone both of which have potential to inhibit hypertrophic chondrocyte conversion [29]. Glucose has been added to a high concentration to chondrogenic media with the purpose to stimulate chondrogenesis and reduce cell necrosis [30, 31]

Therefore, in this chapter, different glucose concentrations were tested for their potential to prevent hypertrophic chondrocyte conversion.



## 2. EXPERIMENTAL

### 2.1 Cell and medium

Immortalized hBMSCs (UE6E7T-3) were obtained from NIHS (JCRB) cell bank, Japan, and cultured in a tissue-culture polystyrene dish under growth medium prepared with Dulbecco's modified eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 10% fetal bovine serum (FBS; Hyclone, Life Scientific, South Logan, UT, USA), 50 µg/ml ascorbate 2-phosphate (Wako Pure Chemical Industries, Osaka, Japan), and 1 ng/ml recombinant human FGF-2 (Gibco, Carlsbad, CA, USA).

For the chondrogenic differentiation of hBMSCs, cells were cultured in both monolayer and pellet culture using chondrogenic medium prepared with minimum essential medium eagle alpha modifications (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml of streptomycin, 10% FBS, 50 µg/ml ascorbate 2-phosphate, 10 ng/ml TGF-β3 (Peprotech, Rocky Hill, NJ, USA), 6 or 30 mM D(+)-glucose (Sigma-Aldrich), 1% ITS-Plus (Corning, Bedford, MA, USA), 0.1 nM dexamethasone (Sigma-Aldrich), 100 µg/ml pyruvate (Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich). Pellet culture was performed in the same manner as in Chapters 1 and 2.

## 2.2 Immunostaining

Pellets were prepared from differentiated cells (hBMSCs cultured in chondrogenic medium in monolayers; Pre-chondrogenic condition) or undifferentiated cells (hBMSCs cultured in growth medium in monolayers; Non-pre-chondrogenic condition) and then cultured under chondrogenic medium. Thin sections of 5  $\mu\text{m}$  in thickness were prepared in a manner similar to that in Chapter 2 and immunologically stained using primary antibody against hypertrophic chondrocyte marker, collagen type X (Col X; 1:1000, Abcam, Cambridge, UK) and reacted with secondary antibody (Alexa Fluor 594, goat anti-mouse IgM.). The abundance of stained cells was assessed by image analysis using ImageJ software (ver 1.4.6, NIH, Bethesda, MD, USA).

## 2.3 Apoptosis

Cell apoptosis was analyzed by the TUNEL assay using an apoptosis assay kit (Deadend™ Fluorometric TUNEL System, Promega, Madison, WI, USA). The abundance of necrotic cells was assessed by image analysis using the ImageJ software.

## 2.4 Statistical analysis

Statistical significance for the differences in Col X expression and apoptosis was

tested by ANOVA.

### 3. RESULTS

Figures 17 and 18 show the expression of Col X and the abundance of apoptotic cells in Non-pre-chondrogenic or Pre-chondrogenic pellets cultured in chondrogenic medium. Col X is known to be specifically expressed in hypertrophic chondrocytes, whereas cell apoptosis is also an indicative of the conversion process. As seen in Figures 17 and 18, pellets cultured in a medium with 30 mM glucose exhibited higher Col X expression with more notable apoptosis especially in more differentiated Pre-chondrogenic pellets at day 28, suggesting that hypertrophic chondrocyte conversion really takes place during the maturation of chondrocytes in pellets.

In order to examine the effect of glucose concentration on hypertrophic chondrocyte conversion, Pre-chondrogenic pellets were cultured in chondrogenic medium containing 6 or 30 mM glucose. As shown in Figures 19 to 22, Col X expression and cell apoptosis, thus hypertrophic chondrocyte conversion is much notable in the medium at higher glucose concentration (30 mM), compared to the lower case (6 mM). These results indicate that lower concentration of glucose level is better for avoiding hypertrophic chondrocyte conversion, thus maintaining the early chondrocyte phenotype.

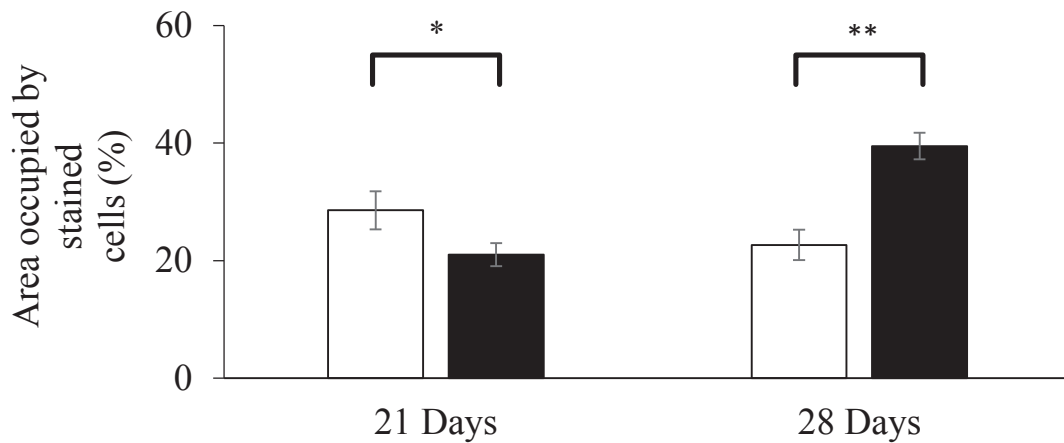


Figure 17. Quantification of cells immunologically stained by antibody to collagen type X. (open bar) Non-pre-chondrogenic and (closed bar) Pre-chondrogenic pellets were cultured for 21 or 28 days in chondrogenic medium. Data are expressed as mean  $\pm$  SD (n = 3). Statistical analysis was performed by ANOVA (\*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ ).

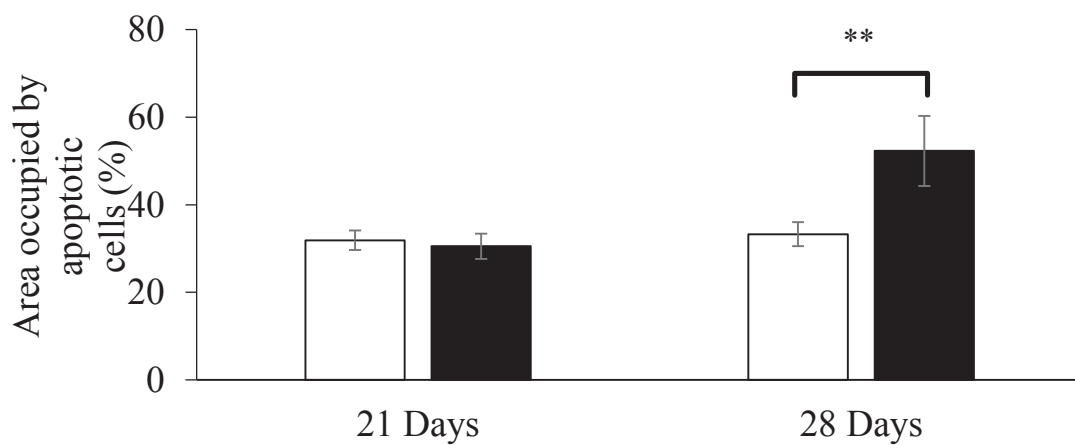


Figure 18. Quantification of apoptotic cells. (open bar) Non-pre-chondrogenic and (closed bar) Pre-chondrogenic pellets were cultured for 21 or 28 days in chondrogenic medium. Data are expressed as mean  $\pm$  SD (n = 3). Statistical analysis was performed by ANOVA (\*\*:  $p \leq 0.01$ ).

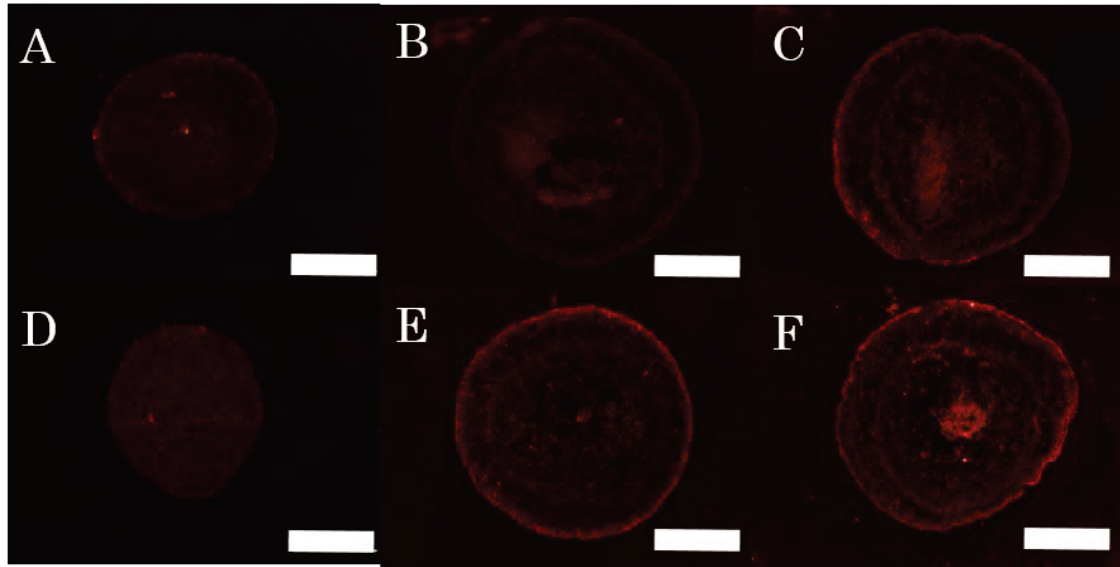


Figure 19. Expression of collagen type X in Pre-chondrogenic pellets cultured for (A, D) 7, (B, C) 21, and (C, F) 28 days in chondrogenic medium containing (A–C) 6 or (D–F) 30 mM glucose. Scale bar: 500  $\mu$ m.

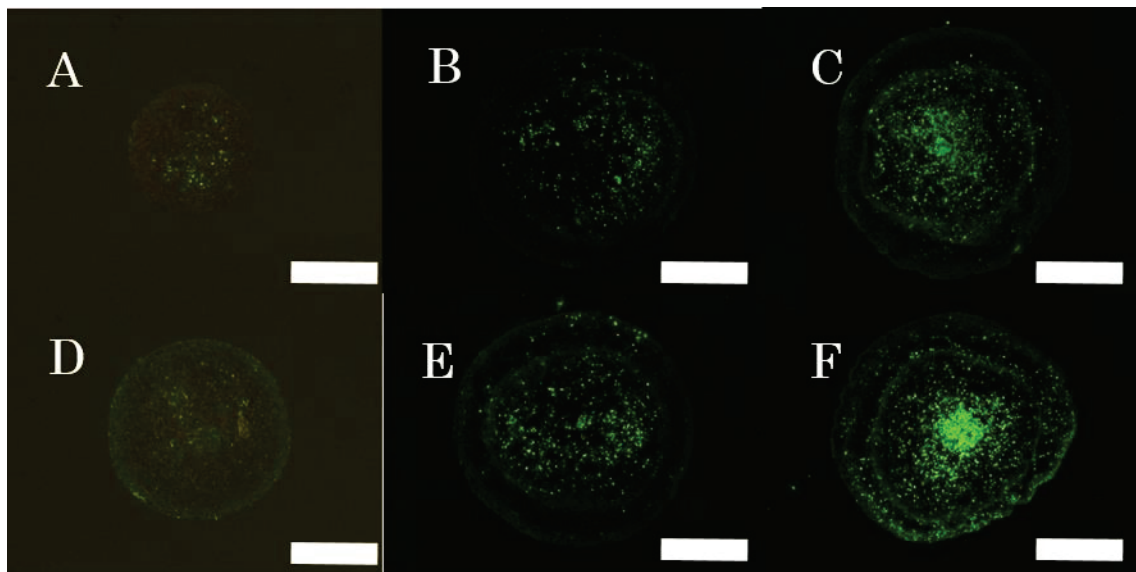


Figure 20. Apoptotic cells in Pre-chondrogenic pellets cultured (A, D) 7, (B, C) 21, and (C, F) 28 days in chondrogenic medium containing (A–C) 6 or (D–F) 30 mM glucose. Scale bar: 500  $\mu$ m.

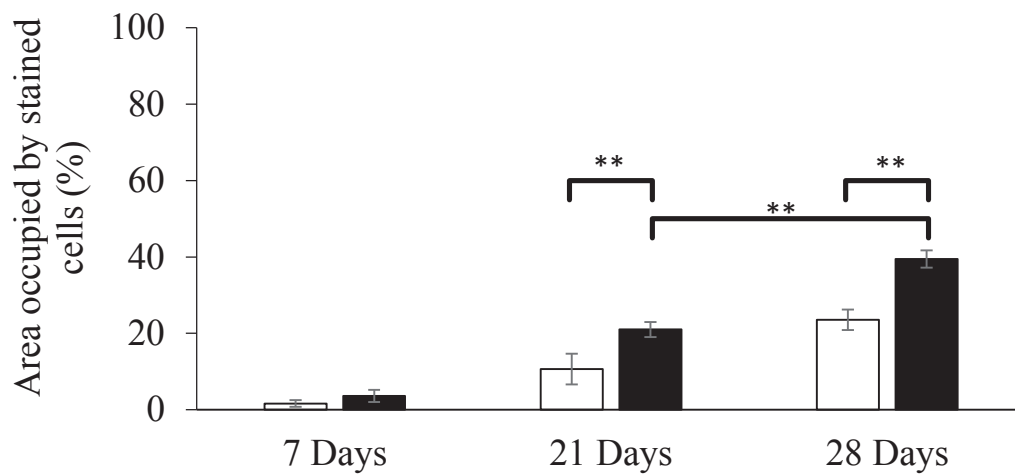


Figure 21. Quantification of cells immunologically stained by antibody to collagen type X. Pre-chondrogenic pellets cultured for 7, 21, and 28 days in chondrogenic medium containing (open bar) 6 or (closed bar) 30 mM glucose. Data are expressed as mean  $\pm$  SD (n = 3). Statistical analysis was performed by ANOVA (\*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ ).

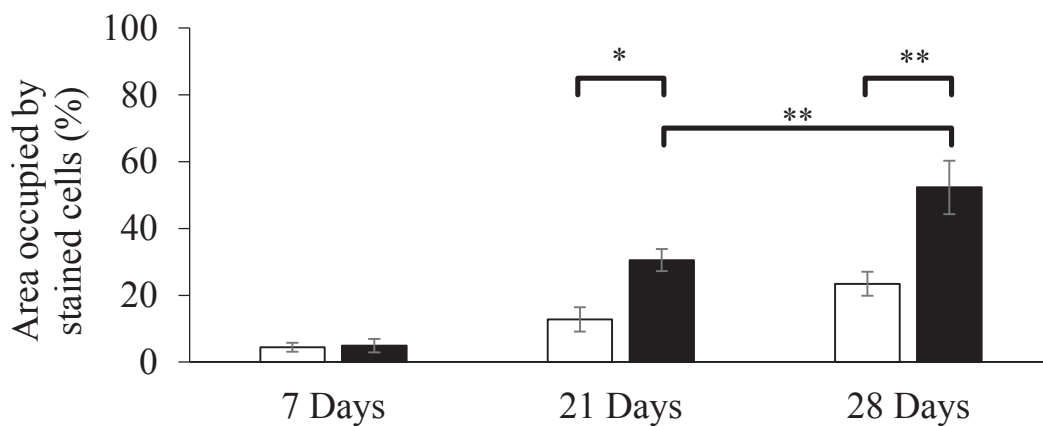


Figure 22. Quantification of apoptotic cells. Pre-chondrogenic pellets cultured for 7, 21, and 28 days in chondrogenic medium containing (open bar) 6 or (closed bar) 30 mM glucose. Data are expressed as mean  $\pm$  SD (n = 3). Statistical analysis was performed by ANOVA (\*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ ).

#### 4. DISCUSSION

It was found that high concentration of glucose in medium was found to have a strong effect on chondrogenic differentiation of hBMSCs. High glucose concentration was adopted to induce chondrogenesis of chick MSCs in pellets [32]. In addition, mineral deposition of hBMSCs during osteogenesis in high-glucose medium is greater than that of hBMSCs in low-glucose medium [33]. Therefore, we hypothesis here that low glucose concentration in chondrogenic medium may be able to retard hypertrophic chondrocyte conversion.

The chondrogenic differentiation of MSCs can be regulated by glucose concentration in medium [34]. Furthermore, hBMSCs, a heterogeneous population, undergo hypertrophic chondrocyte conversion [35, 36, 37] in both culture methods here, Non-pre-chondrogenic and Pre-chondrogenic methods with a medium at high glucose concentration. This undesirable effect was also discussed by others [38] as the default mode of chondrocytes, and an unnatural pathway of chondrogenic differentiation of MSCs pellets underwent hypertrophic chondrocyte conversion [39]. In addition, it has been demonstrated that chondrocytes and osteocytes were derived from a common osteochondroprogenitors [8]. Thus it is likely that hBMSC pellet cultured in chondrogenic medium will ultimately differentiate into hypertrophic chondrocytes and

later will contribute to full osteocytes lineage.

During chondrogenesis, progenitor cells ultimately differentiate into early and subsequently late chondrocytes, a so-called hypertrophic chondrocytes, and finally contribute to full osteogenic lineage. Therefore, hypertrophic chondrocyte conversion should be suppressed as much as possible for cartilage regeneration. In this chapter, it was found that culturing pre-chondrogenic pellets in a medium at low glucose concentration facilitated to significantly decrease hypertrophic chondrocyte conversion in the pellets.





## SUMMARY

Human bone marrow-derived mesenchymal stem cells (hBMSCs) are considered to be promising as cell sources for cartilage regeneration. However, differentiation of hBMSCs into chondrocytes can ultimately lead to hypertrophic chondrocytes which later become osteoblasts and contribute to the full osteogenic lineage. Therefore, suppressing hypertrophic chondrocyte differentiation must be carefully taken into consideration for cartilage regeneration. This study aimed at studying the effect of conditions, such as differentiation stages and nutrient concentrations, in three-dimensional (3D) pellet culture of hBMSCs on the promotion of chondrogenic differentiation and also the prevention of hypertrophic chondrocyte conversion or, in other words, the stabilization of early chondrocytes differentiated in the pellets.

hBMSCs (UE6E7T-3, JCRB Cell Bank) were cultured in growth or chondrogenic medium. After cells reached confluence,  $2.5 \times 10^6$  cells were centrifuged at 500 g for 5 min in a 15 ml conical tube to obtain a pellet. Then the pellet was cultured under growth or chondrogenic medium, and the medium was changed every 2–3 days. Pellets were sliced with a thickness of 5  $\mu$ m and stained with hematoxylin–eosin (H.E) and toluidine blue O (TBO). Moreover, the sections were immunologically stained to

detect HIF-1 $\alpha$  (a marker for hypoxia), Col X (a marker for hypertrophic chondrocytes), and necrotic and apoptotic cells were analyzed by propidium iodide (P.I) staining and TUNEL assay, respectively. Quantitative PCR was performed to analyze the expression of chondrogenic marker genes (Sox9 and Col II) and the activity of mitochondria. ImageJ software was used to quantify the stained cells. Statistical analysis was performed by ANOVA.

First, the difference in medium conditions was considered: hBMSCs were cultured under growth or chondrogenic medium in monolayer. It was seen that cells under the chondrogenic medium exhibited a lower level of mitochondrial activity than those under the growth medium. This result suggests that chondrocytes differentiated under the chondrogenic medium consume less oxygen than growing hBMSCs. In 3D culture, pellets in chondrogenic medium contained a smaller number of necrotic cells than those in growth medium. This may be due to the fact that, as mentioned above, differentiated chondrocytes require less oxygen and therefore that these cells could survive even in the core regions of a pellet where microenvironment might be under hypoxia conditions.

To gain deeper insights into the effect of hypoxia conditions on the differentiation of chondrocytes, pellets were prepared separately from undifferentiated

and pre-differentiated cells, and then cultured under chondrogenic medium for 10 – 28 days. It appears that necrotic cells distribute unevenly within a pellet prepared from undifferentiated cells and are more abundant in the core regions of a pellet. This result can be simply explained from the theoretical consideration for the oxygen gradient established within a pellet. However, when pellet culture was initiated with more differentiated chondrocytes, the density of necrotic cells are relatively low in the core regions, probably because of reduced requirement of oxygen by differentiated chondrocytes. This result suggests that hypoxia condition, found especially in the core regions of a pellet, has rather a positive effect on the enrichment of chondrocytes.

As one of the most important molecules that promote chondrocyte differentiation and also suppress mitochondrial activity, a special attention was paid here to hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ). This protein is known as a transcription factor that binds to Sox9 promotor to induce chondrogenesis, while the expression of several proteins forming mitochondrial redox carriers is impaired by HIF-1 $\alpha$ . The later effect gives rise to a decrease in intracellularly-generated reactive oxygen species, a by-product of ATP synthesis, which may cause cell necrosis. It was observed in the present study that pellets prepared with pre-chondrogenic differentiation produced cartilage-like extracellular matrices, as shown by TBO staining, more abundantly than

those with undifferentiated cells. In addition, the level of HIF-1 $\alpha$  gene expression was higher in pellets prepared with pre-differentiated cells than those with undifferentiated cells. The both results can be well explained from the diverse functions of HIF-1 $\alpha$  as described above.

During chondrogenesis, progenitor cells ultimately differentiate into early and subsequently late chondrocytes, a so-called hypertrophic chondrocytes, and finally contribute to full osteogenic lineage. Therefore, hypertrophic chondrocyte conversion should be suppressed as much as possible for cartilage regeneration. In this study, it was found that culturing pre-differentiated pellets in a medium at low glucose concentration facilitated to significantly decrease hypertrophic chondrocyte conversion in the pellets.

In conclusion, chondrocyte consume less oxygen than that of hBMSCs. Since chondrocyte and hBMSCs consume oxygen at different rate, oxygen gradient and oxygen consumption influence cell necrosis and chondrogenic differentiation in the pellets. HIF-1 $\alpha$  seems to play a key role in these effects. Hypertrophic chondrocyte conversion can be suppressed in a medium at low glucose concentration. Accordingly, pre-chondrogenic differentiation with low glucose concentration may provide optimal conditions for the formation of early chondrocytes and thus for cartilage regeneration.

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