Hypoxia promotes differentiation of pure cartilage from human induced pluripotent stem cells

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Abstract. While cartilage can be produced from induced pluripotent stem cells (iPSCs), challenges such as long culture periods and compromised tissue purity continue to prevail. The present study aimed to determine whether cartilaginous tissue could be produced from iPSCs under hypoxia and, if so, to evaluate its effects on cellular metabolism and purity of the produced tissue. Human iPSCs (hiPSCs) were cultured for cartilage differentiation in monolayers under normoxia or hypoxia (5% O_2), and chondrocyte differentiation was evaluated using reverse transcription-quantitative PCR and fluorescence-activated cell sorting. Subsequently, cartilage differentiation of hiPSCs was conducted in 3D culture under normoxia or hypoxia (5% O_2), and the formed cartilage-like tissues were evaluated on days 28 and 56 using histological analyses. Hypoxia suppressed the expression levels of the immature mesodermal markers brachyury (*T*) and forkhead box protein F1; however, it promoted the expression of the chondrogenic markers *Acan* and *CD44*. The number of sex-determining region Y-box 9-positive cells and the percentages of safranin O-positive and type 2 collagen-positive tissues increased under hypoxic conditions. Moreover, upon hypoxia-inducible factor (HIF)-1α staining, nuclei of tissues cultured under hypoxia stained more deeply compared with those of tissues cultured under normoxia. Overall, these findings indicated that hypoxia not only enhanced cartilage matrix production, but also improved tissue purity by promoting the expression of HIF-1α gene. Potentially, pure cartilage-like tissues could be produced rapidly and conveniently using this method.

Introduction

Cartilage is a type of tissue characterized by poor self-repair capacity due to the absence of blood vessels and nervous tissue. Such tissues do not heal spontaneously after sustained extensive damage due to trauma or similar events and eventually lead to the onset of osteoarthritis and impaired activities of daily living. Treatments to address cartilaginous tissue damage include bone perforation, osteochondral column transplantation, and autologous cultured cartilage transplantation. Although each of these methods has been successful to some extent, problems related to the number of procedures required and the quality of regenerated tissue prevail (1). Therefore, treatment methods that can overcome these issues are crucial for effective therapy.

In recent years, regenerative medical techniques, including transplantation of cartilaginous tissue cultured from stem cells, have been regarded as promising new therapeutic options. Among these, induced pluripotent stem cells (iPSCs) have garnered attention as a potential cell source, where clinical applications of iPSCs, such as the production of corneal and cardiac muscle sheets, continue to progress. iPSCs exhibit high self-renewal capacity and have excellent potential as a cell source owing to their ability to undergo cell division while remaining undifferentiated, even after several divisions. The formation of cartilaginous tissues from iPSCs has been reported in various studies (2-8). However, regenerative medicine utilizing iPSCs faces several obstacles, such as the associated high costs, long culture periods, risk of oncogenesis, and compromised tissue purity (9).

The application of biochemical stimulation in conjunction with recombinant proteins is a common method for inducing tissue differentiation from stem cells. However, in recent years,

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Abbreviations: iPSCs, induced pluripotent stem cells; hiPSCs, human iPSCs; FOXF1, forkhead box protein F1; SOX9, sex-determining region Y box 9; HIF-1α, hypoxia-inducible factor-1α; ES cells, embryonic stem cells; DMEM, Dulbecco's modified Eagle's medium; ITS, insulin-transferrin-selinium; FBS, fetal bovine serum; P/S, penicillin and streptomycin; RT-qPCR, reverse transcription-quantitative PCR; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorting

Key words: stem cells, articular cartilage, hypoxia, differentiation, chondrocytes

it has been revealed that mechanical signaling via physical stimulation is also important for morphogenesis/differentiation. Physiologically, chondrocytes exist in a hypoxic environment, wherein this environment is essential for their growth, differentiation, and survival (10). A previous study reported that chondrocyte differentiation was promoted under hypoxic conditions during the production of cartilaginous tissues from human embryonic stem (ES) cells (11). Therefore, it is possible that a hypoxic environment may also promote differentiation of cartilaginous tissue from iPSCs.

Based on these reports, we hypothesized that if cartilaginous tissues could be prepared from iPSCs in a hypoxic environment, it would be possible to produce such tissues faster than those cultured in a stable oxygen environment. The objective of this study was to investigate whether cartilaginous tissues can be produced from iPSCs under hypoxic conditions and to evaluate the effects of such an environment on cellular metabolism and purity of the tissue produced.

Materials and methods

Chondrogenic differentiation of human iPSCs (hiPSCs) in a monolayer culture. The established hiPSC line Toe (cell number JCRB1338) was maintained in feeder-free medium that included StemFit AK-02N (Reprocell Inc.) in 6 cm dishes coated with laminin-511 (Nippi, Inc.). The cultured hiPSCs were then transferred and maintained in StemFit AK02N in 6-well plates coated with laminin-511. The iPS cells were confirmed by fluorescent immunostaining to maintain their undifferentiated potential as appropriate (Supplemental data; Fig. S1). The hiPSCs formed high-density cell colonies consisting of 1-2x10^5 cells 10-15 days after starting maintenance under feeder-free culture conditions. Subsequently, chondrogenic differentiation of the iPSCs was initiated. First, the hiPSCs were differentiated into mesendodermal cells by culturing in Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma-Aldrich; Merck KGaA) with 10 ng/ml Wnt3A (R&D Systems), 10 ng/ml activin A (R&D Systems), 1% insulin-transferrin-selenium (ITS) (Thermo Fisher Scientific, Inc.), 1% fetal bovine serum (FBS), and 1% penicillin-streptomycin (P/S) for 3 days. On day 4, the medium was changed to chondrogenic medium [DMEM with 50 mg/ml ascorbic acid (Nacalai Tesque), 10 ng/ml BMP2 (PeproTech), 10 ng/ml TGF-β1 (PeproTech), 10 ng/ml GDF5 (PeproTech), 1% ITS, 1% FBS, 2 mM l-glutamine (Thermo Fisher Scientific, Inc.), 1x10^{-4} M non-essential amino acids (Nacalai Tesque), 1 mM sodium pyruvate (Thermo Fisher Scientific), and P/S]. bFGF (1 ng/ml; Wako Pure Chemical Industries Ltd.) was added to the medium from days 3 to 14. Two groups of cells were assessed, namely hiPSCs cultured under normoxic (21% O_2, 5% CO_2, and 74% N_2) and those under hypoxic conditions (5% O_2, 5% CO_2, and 90% N_2) in a multigas incubator (Model 9200; Wakenyaku Co., Ltd.) (Fig. 1A).

Total RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from cells using ISOGEN (Nippon Gene Co., Ltd.). The extracted RNA was reverse-transcribed using PrimeScript™ RT Master Mix (Takara Bio, Inc.) according to the manufacturer's instructions. Quantitative real-time PCR was performed using Step One Plus™ Real-Time PCR System (Applied Biosystems) with a primer probe. Each 20 μl reaction mixture contained 1 μl of cDNA (100 ng), 9 μl TaqMan™ Fast Advanced Master Mix (Applied Biosystems), and 0.33 μl of target gene primers (Table I) and probes from the Universal Probe Library (Roche). The cycle conditions were as follows: denaturation at 95˚C for 15 sec and annealing and extension at 60˚C for 1 min for 40 cycles. Relative changes in gene expression were calculated using the comparative Ct method and normalized to the expression of the internal control gene, 18S ribosomal RNA gene. The results are shown as the average of three samples in which each sample was assayed in duplicate.

Flow cytometry. Cells were detached and digested with trypsin to form a single cell suspension. For labeling of intracellular antigens, cells were fixed in 4% paraformaldehyde for 30 min at 4˚C and further permeabilized by incubating with 1% (w/v) bovine serum albumin (BSA) and 0.2% (v/v) Triton X-100 in phosphate buffered saline (PBS) for 15 min at room temperature. Cells were then incubated with 10 μl/ml primary Alexa Fluor® 488-conjugated rabbit monoclonal anti-sex-determining region Y box 9 (SOX9) antibody (EPR14335, ab196450; Abcam) diluted in PBS containing 1% BSA for 30 min at room temperature with light shielding. Cell labeling was analyzed using a FACSCalibur system (Becton-Dickinson and Company) with CellQuest software (Becton-Dickinson and Company).

Chondrogenic differentiation of hiPSCs in 3D culture. hiPSCs were transferred and maintained in StemFit AK-02N in 6-well plates coated with Matrigel GFR (Thermo Fisher Scientific, Inc.). The cells formed high-density colonies (1-2x10^5) 10-15 days after the start of maintenance. Subsequently, chondrogenic differentiation of iPSCs was induced in the same manner as for monolayer culture. Multilayered nodules were formed by day 14, and the nodules were physically separated from the bottom of the dishes to form particles. Then, the particles were transferred to suspension culture in 3.5 cm non-attachment culture dishes (Prime surface®; Sumitomo Bakelite Co., Ltd.) on day 14 and cultured in chondrogenic medium until day 42. On day 42, the medium was replaced with conventional medium (DMEM with 10% FBS and 50 units and 50 mg/ml of penicillin and streptomycin, respectively). The medium was changed every 2-7 days. Two groups of cells were assessed, namely hiPSCs cultured under normoxic and those under hypoxic conditions (5% O_2, 5% CO_2, and 90% N_2) in a multigas incubator.

Histological and immunohistochemical analyses. Pellets were fixed in 4% paraformaldehyde (Wako Pure Chemical Industries Ltd.), embedded in paraffin, and sectioned (thickness: 5 μm). The sections were stained with hematoxylin and eosin or safranin O. For immunohistochemical analysis of type 1 and type 2 collagen, paraffin-embedded sections were deparaffinized in xylene, rehydrated using a graded alcohol series, and washed with PBS. Endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in methanol for 5 min. The sections were incubated at 4˚C with mouse polyclonal anti-type 1 collagen antibody (1:150; ab6308; Abcam)

Table I. Primers used for reverse transcription-quantitative PCR.

Gene	Direction	Primer sequence (5'-3')
18S rRNA	Forward	ATGAGTCCACTTTAAATCCTTTAACGA
	Reverse	CTTTAATATACGCTATTGGAGCTGGAA
T (Brachyury)	Forward	AGACACGTTCACCTTCAGCA
	Reverse	GCTCACCAATGAGATGAYCG
FOXF1	Forward	CAGCCTCTCCACGCACTC
	Reverse	CCTTTCGGTCACACATGCT
Acan	Forward	GACGGCTTCCACCAGTGT
	Reverse	TCGAGGGTGTAGCGTGTAGA
CD44	Forward	GCAGTCAACAGTCGAAGAAGG
	Reverse	TGTCCTCCACAGCTCCATT
HIF-1α	Forward	TTTTCAAGCAGTAGGAATTGGAA
	Reverse	TTCCAAGAAAGTGATGTAGTAGCTG

HIF-1α, hypoxia-inducible factor-1α; *FOXF1,* forkhead box protein F1.

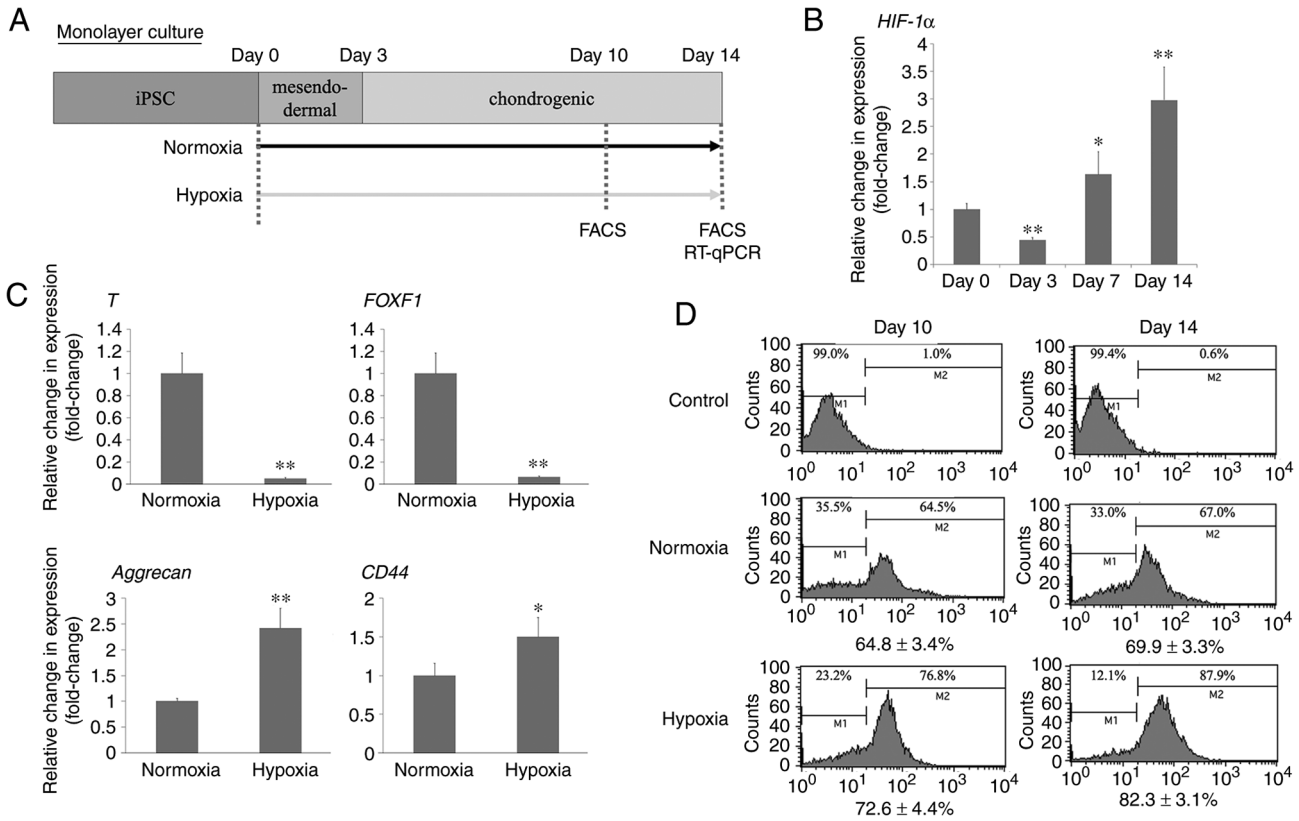

Figure 1. Hypoxia promotes chondrogenic differentiation. (A) hiPSCs cultured in monolayer under normal oxygen or 5% O_2 hypoxic conditions during cartilage differentiation for 14 days (n=3). (B) Changes in HIF-1α expression over time under hypoxic conditions by RT-qPCR analysis normalized to day 0. (C) RT-qPCR analysis of the gene expression levels of *T* and *FOXF1* (immature mesodermal markers), and *Acan* and *CD44* (chondrogenic markers) on day 14 normalized to normoxia. (D) FACS analysis of the percentage of SOX9-positive cells on days 10 and 14. *P<0.05, **P<0.01 compared with day 0 or normoxia groups. RT-qPCR, reverse transcription-quantitative PCR; hiPSC, human induced pluripotent stem cells; FOXF1, forkhead box protein F1; T, brachyury.

or anti-type 2 collagen antibody (1:50; F-57; Kyowa Pharma Chemical Co., Ltd.) overnight. After extensive washing with PBS, the sections were incubated in Histofine Simple Stain Rat MAX-PO (Nichirei Biosciences Inc.) for 30 min at room temperature following the manufacturer protocol. Immunostaining was detected by 3,3-Diaminobenzidine staining. Counterstaining was performed with Mayer's hematoxylin. Any three arbitrary locations stained red with safranin O, which is considered to indicate advanced chondrogenic differentiation, were observed at 400x. The number of cell nuclei in the field of section was counted using ImageJ software (developed by Wayne Rasband), and the mean value was calculated.

Cartilage-like tissues stained with Safranin O on day 28 or 56 were qualitatively evaluated using the Bern Score system (12).

Immunofluorescent staining. For immunohistochemistry of hypoxia-inducible factor (HIF)-1α, paraffin-embedded sections were deparaffinized in xylene, rehydrated in a graded alcohol series, and immersed in PBS, followed by application of a protein block (Dako) for 10 min. The sections were incubated at 4˚C with rabbit polyclonal anti-HIF-1α antibody (1:250; ab2185; Abcam) for 1 h. After extensive washing with PBS, the sections were incubated with Alexa Fluor® 568-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:500; A-11036; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. After extensive washing with PBS, the sections were counterstained using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (H-1200; Vector Laboratories).

Statistical analysis. All duplicate and triplicate experiments yielded almost identical results. All data in this study are expressed as the mean value ± standard deviation. We used the parametric one-way analysis of variance to test the differences between groups. The Tukey-Kramer test was used to determine the differences between groups when the results were considered significant. For all analyses, differences at $P<0.05$ were considered statistically significant.

Results

Effects of hypoxic stimulation on the purity of differentiated cartilage. To confirm the effects of hypoxic stimulation on cell viability, we measured the number of cells in the tissue specimen on day 14. Although hypoxic stimulation at 5% O_2 did not adversely affect cell viability during the differentiation of cartilaginous tissue from iPSCs, cell viability decreased by approximately 80% in the 2% O_2 hypoxic environment (data not shown). Therefore, a hypoxic environment with 5% O_2 was used for the subsequent analyses. We also evaluated the gene expression change in immature mesodermal markers (*T*) and chondrogenic markers (*sox9*). The expression of mesoderm markers increased during culture in mesoderm medium by day 3. Thereafter, the expression of mesoderm markers decreased, and instead, the expression of chondrogenic markers increased after changing the mesodermal medium to a chondrogenic medium, consistent with the results of a previous study (8) (Fig. S2). We observed changes in HIF-1α expression over time under hypoxic conditions. HIF-1α expression increased as chondrogenic differentiation progressed from day 3 to day 14 (Fig. 1C). However, on day 3, HIF-1α expression was decreased rather than increased.

The effect of a hypoxic environment on cartilage differentiation was investigated using real-time RT-PCR performed during plate culture. Culturing was carried out in accordance with the differentiation protocol described in Fig. 1A, and gene expression was assessed in the group cultured under normoxic conditions and in the group cultured in a 5% O_2 hypoxic environment on day 14 after the start of culture, the point at which cartilage differentiation had advanced to some extent, and gene expression was assessed. The expression of *T* (*Brachyury*) and *FOXF1*, markers of undifferentiated mesodermal tissue, was markedly reduced in cultures grown under hypoxic conditions compared to those grown under normoxic conditions. Additionally, the expression of *Acan*, a marker indicating cartilage matrix production, and *CD44*, a surface marker of chondrocytes, was significantly increased in the hypoxic group compared to that in the normoxic group (Fig. 1B).

To confirm the expression of SOX9, a master regulator related to chondrocyte differentiation, fluorescence-activated cell sorting (FACS) targeting SOX9 was performed on days 10 and 14 in both the groups (cultured under steady oxygen conditions and in a 5% O_2 hypoxic environment). We observed that the proportion of SOX9-positive cells not only increased by day 10 under hypoxic culture conditions, but this number increased even more by day 14 (Fig. 1D).

Effect of hypoxic stimulation on substrate production during cartilage differentiation. Similarly, we also performed 3D culture using Matrigel according to the differentiation protocol and established a suspension culture from day 14. The groups cultured under normoxic and hypoxic conditions were examined histologically on days 28 and 56 after the start of differentiation culture. Histological examination performed on day 28 revealed that only a portion of the cells grown under normoxic conditions were stained with safranin O, while the tissue grown under hypoxic conditions exhibited uniform staining with safranin O and for type 2 collagen (Fig. 2). Neither tissue exhibited positive staining for type 1 collagen. In addition, there was no remarkable difference between the normoxic and hypoxic groups in terms of the maximum X-axial diameters of the cell masses, as measured on day 28. By day 56, favorable staining was obtained with safranin O and for type 2 collagen in both the normoxia and hypoxia groups, and tissues similar to normal cartilaginous tissue were produced. The cell phenotype showed chondrocyte-like cells surrounded by pericellular matrix. On the high magnification Safranin O staining images, on day 28, the normoxia group had more cell numbers and less cartilage matrix. On the other hand, in the hypoxia group, the number of cells at the margins was high, and matrix production was poor, but chondrocyte-like cells with cartilaginous lumen were scattered in the center. On day 56, the number of cells decreased in both normoxia and hypoxia groups, and the tissue developed a rich matrix. On day 28, the Bern score, a qualitative score about cartilage generated *in vitro*, was higher in the hypoxia group than in the normoxia group. On the other hand, the normoxia and hypoxia groups had similarly high scores on day 56 (Fig. 2D-G).

Investigation of the promotive effects of HIF-1α on cartilage differentiation under hypoxia. Next, we investigated the mechanisms that promote cartilage differentiation in a hypoxic environment. The expression of HIF-1α is promoted under hypoxic conditions and accelerates cartilage differentiation. We also performed 3D culture using Matrigel according to the differentiation protocol up to day 28, followed by immunostaining for HIF-1α. The expression of HIF-1α was observed under both normoxic and hypoxic conditions (Fig. 3). However, nuclei of tissues cultured under hypoxic conditions were more deeply stained than those of the tissues cultured under normoxic conditions.

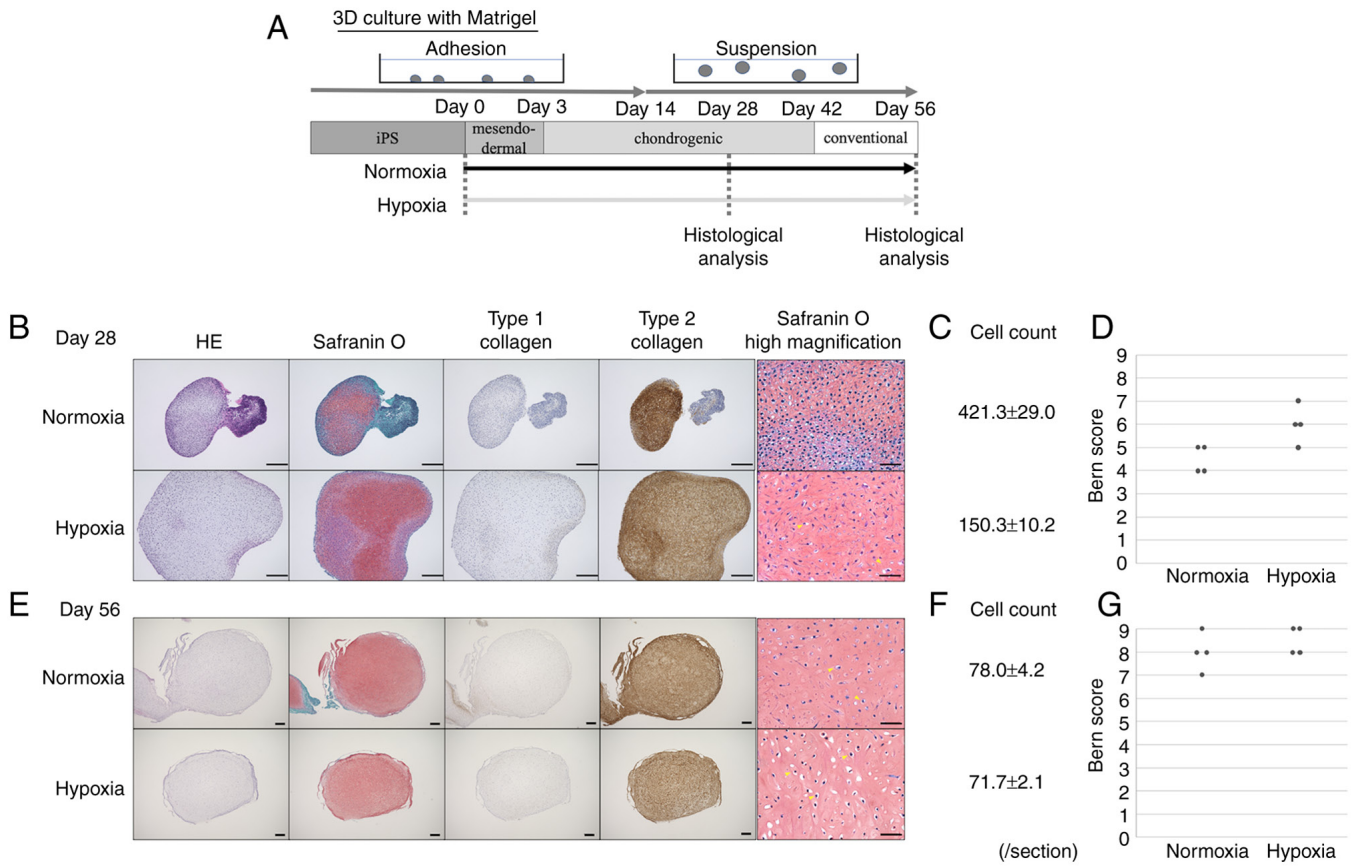

Figure 2. Hypoxic conditions promotes purity of cartilage-like tissue. (A) hiPSCs cultured in 3D culture under normal oxygen or 5% O_2 hypoxic conditions during cartilage differentiation for 28 or 56 days. (B-F) Histological analysis and quantification of mid-sagittal sections using HE and safranin O, and immunohistochemical analysis using anti-type 1 or 2 collagen antibodies to visualize chondrogenic differentiation on (B) day 28 (magnification, x40) and (E) day 56 (magnification, x20), [HE, Safranin, Type 1 and 2 collagen scale bar, 500 μm; safranin O high magnification scale bar, 50 μm (magnification, x400)]. The yellow arrows indicate chondrocyte-like cells and the pericellular matrix. Number of cell nuclei in the high magnificent section (magnification, x400) on (C) day 28 or (F) 56 was counted. The mean value was calculated (n=4). Cartilage-like tissue stained with Safranin O on (D) day 28 or (G) 56 was qualitatively evaluated using the Bern Score system (n=4). HE, hematoxylin and eosin; hiPSCs, human induced pluripotent stem cells.

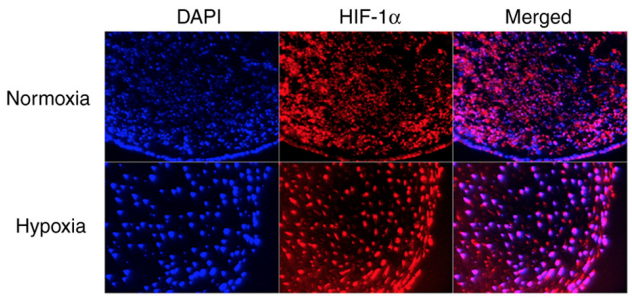

Figure 3. Culturing under hypoxia promotes the dyeability of HIF-1α in cartilage-like tissues. 3D culture of hiPSCs under normoxia or hypoxia during cartilage differentiation for 28 days. Immunofluorescence staining with DAPI counterstaining to demonstrate HIF-1α positivity and visualization of nuclear uptake (magnification, x400). HIF-1α, hypoxia-inducible factor-1α; hiPSCs, human induced pluripotent stem cells.

Discussion

In this study, we found that hypoxic culture conditions not only led to enhanced cartilage matrix production but also improved cell purity of cartilaginous tissue differentiated from iPSCs. Using this method, highly pure cartilage-like tissues may be produced more rapidly and conveniently.

With the industrialization of tissue graft materials in the field of regenerative medicine, quality assurance and standardization have been recognized as critical aspects in recent years. As ES cells and iPSCs maintain their ability to differentiate, they are excellent cell sources for regenerative medicine. However, because differentiation is induced in undifferentiated cells, it is necessary to improve the level of cell purity. Various studies have been conducted to improve the degree of purity of tissue differentiation from iPSCs. Hirano *et al* were able to increase the purity of islet cell differentiation from iPSCs by applying a unique culture method referred to as a closed-channel culture system (13). In addition, Hwang *et al* increased the purity of cardiomyocyte differentiation from iPSCs by adding a small molecule compound to the culturing environment (14). Various methods for differentiating iPSCs with a high degree of purity are being studied in similar ways.

The intra-articular cavity, where chondrocytes are present, is physiologically hypoxic. Generally, chondrocytes present in the articular cartilage are found in environments containing 1-5% O_2. Further, they are surrounded by a thick extracellular matrix, enabling them to remain viable in increasingly hypoxic environments (15). Reports have also stated that changes in oxygen concentration are important when preparing cartilaginous tissue from ES cells (11) and that applying hypoxic

stimulation during cartilaginous tissue differentiation from mesenchymal stem cells can promote cartilage matrix production in the resulting chondrocytes (16). Consistent with these reports, we also observed that the expression *T* (*Brachyury*) and *FOXF1*, which serve as markers of undifferentiated mesodermal tissue, declined by day 14 as a result of hypoxic stimulation during cartilaginous tissue differentiation from iPSCs. Moreover, FACS performed on days 10 and 14 revealed that the proportion of SOX9-positive cells increased. A previous report found that HIF-1α expression under hypoxic conditions induced chondrogenic differentiation into articular cartilage via SOX9, a master regulator related to cartilaginous tissue (17). Therefore, it has been considered that, in hypoxic environments, cell differentiation is promoted via HIF-1α expression, accompanied by decreased levels of undifferentiated cell markers and an increase in the population of SOX9-positive chondrocytes.

HIF-1α also has an anabolic effect on the metabolism of cartilaginous tissue. It is known that HIF-1α translocates to the nucleus in hypoxic environments and regulates the expression of SOX9 (10,17). Furthermore, a previous study found that the production of substrates, such as aggrecan and type 2 collagen, can be promoted via HIF-1α expression and by culturing chondrocytes in a hypoxic environment (18). In this study, HIF-1α expression increased as chondrogenic differentiation progressed from day 3 to day 14. The histological examination performed on culture day 28 revealed that safranin O staining and substrate production were both increased in the cells cultured under hypoxic conditions. By day 56, it was possible to produce tissue specimens similar to those cultured in a stable oxygen environment. These findings indicate that hypoxic cultures may be used to produce high quality tissue more rapidly. However, HIF-1α expression decreased on day 3 compared to day 0. It has been reported that HIF-1α expression decreases after prolonged culture under hypoxic conditions in various cell types (19). HIF-1α expression might have decreased till day 3, as has been reported in other cells. Further, HIF-1α expression might have increased due to the change to a chondrogenic medium and the progress of chondrogenic differentiation.

Although the study has several merits, there are a few limitations as well. First, we chose a 5% O_2 culture environment because it is easier to maintain cell viability at this concentration than in a 2% O_2 environment; however, evaluations of other oxygen concentrations are currently lacking and are therefore needed. In addition, the period for which hypoxia should be administered is also a subject that requires further investigation. Second, we did not evaluate these conditions with respect to SOX9-negative cells, and therefore the possibility of contamination by undifferentiated cells cannot be ruled out. Third, we could not evaluate the role of HIF-1α in chondrogenic differentiation, including suppression experiments. However, HIF-1α is a key gene for chondrocytes, and chondrogenic differentiation did not proceed when HIF-1α was suppressed (10). In addition, we have previously reported that HIF-1α expression was increased by culturing chondrocytes under hypoxic conditions, which in turn increased the production of the cartilaginous matrix (20). These results suggest that HIF-1α may be involved in the increase in cartilaginous matrix production. Fourth, as we did not conduct an *in vivo* transplantation experiment, we were unable to evaluate the risks of tumorigenesis or tissue deformation after transplantation.

We believe that the results of this study can be applied to create high-quality cartilage tissue from iPSCs more easily and at a lower cost, and more, leading to the advancement of transplantation medicine using iPSCs.

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Availability of data and materials

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

Authors' contributions

SS, HI, SN, SI and ToK conceptualized the study. SS, HI, ST, and YA organized the data for paper. SS, YF, TsK and OM performed formal analysis. SS, YF, SI and ToK examined the study data. SS, SN, YF, ST, MS and YA designed and performed the experiments. OM, YA and ToK administrated the project. OM supervised the study. ToK acquired funding and validated the study design. SS wrote the original draft of the manuscript. All authors contributed to manuscript reviewing and editing, and have read and approved the final manuscript. SS and YA confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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