Hypoxia Promotes Differentiation of Pure Cartilage from Human iPS Cells

Seiji Shimomura

Kyoto Prefectural University of Medicine department of Orthopaedics

Hiroaki Inoue

Kyoto Prefectural University of Medicine department of orthopaedics

Yuji Arai (✉ y123arai@koto.kpu-m.ac.jp)

KPUM <https://orcid.org/0000-0003-0784-7426>

Shuji Nakagawa

Kyoto Prefectural University of Medicine Department of Sports and Para-Sports Medicine

Yuta Fujii

Kyoto Prefectural University of Medicine department of Orthopaedics

Tsunao Kishida

Kyoto Prefectural University of Medicine department of Immunology

Masaharu Shin-Ya

Kyoto Prefectural University of Medicine department of Immunology

Shohei Ichimaru

Kyoto Prefectural University of Medicine department of Orthopaedics

Shinji Tsuchida

Kyoto Prefectural University of Medicine department of Orthopaedics

Osam Mazda

Kyoto Prefectural University of Medicine department of Immunology

Toshikazu Kubo

Kyoto Prefectural University of Medicine department of Orthopaedics

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Abstract

Background

While cartilage can be formed from induced pluripotent stem cells (iPSCs), challenges such as long culture periods and compromised tissue purity remain. We aimed to determine whether cartilaginous tissue can be produced from iPSCs under hypoxia and to evaluate the effects on the cellular metabolism and purity of the tissue.

Methods

Human iPSCs (hiPSCs) were cultured for cartilage differentiation in monolayers under normoxia or hypoxia (5% O₂). We evaluated chondrocyte differentiation by real-time reverse transcription-polymerase chain reaction and fluorescence-activated cell sorting. Then, hiPSCs were cultured for cartilage differentiation in 3D culture under normoxia or hypoxia (5% O₂). We evaluated cartilage-like tissues on days 28 and 56 through histological analyses.

Results

Hypoxia suppressed the expression of immature mesodermal markers *T* (*Brachyury*) and *Forkhead box protein F1* (*FOXF1*) and promoted the expression of the chondrogenic markers aggrecan and *CD44*. Sex determining region Y-box (SOX) 9-positive cells were increased by culture under hypoxia. Percentages of safranin O-positive and type 2 collagen-positive tissues were increased under hypoxia. Moreover, upon hypoxia-inducible factor (HIF)-1 α staining, the nuclear dyeability in tissues cultured under hypoxia was greater than that under normoxia.

Conclusions

Hypoxia not only led to enhanced cartilage matrix production but also improved cell purity by promoting the expression of *HIF-1 α* . By applying this method, highly pure cartilaginous-like tissues may be produced more rapidly and conveniently.

Background

Cartilage is a type of tissue characterized by poor self-repair capacity due to the absence of blood vessels and nerve tissue, and it does not readily heal spontaneously. As such, cartilaginous tissue does not self-repair after sustained extensive damage due to trauma or similar events, potentially leading 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. Each of these treatment methods has achieved a degree of success, but problems related to the number of procedures required and the quality of regenerated tissue remain to be overcome [1]. If these problems can be solved, superior methods of treatment may be established.

In recent years, regenerative medical techniques, including transplantation of cartilaginous tissue cultured from stem cells, have been regarded as promising new therapy options. Among these, induced pluripotent stem cells (iPSCs) have attracted attention as a potential new source of cells for therapeutic uses, and clinical applications, 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 the maintenance of the ability to undergo cell division while remaining undifferentiated, even after several divisions. The formation of cartilage tissues from iPSCs has been reported in various studies [2–8]. However, regenerative medicine utilizing iPSCs also faces obstacles, such as the associated high costs, long culture periods, risk of oncogenesis, and compromised tissue purity [9].

A common method for inducing tissue differentiation from stem cells involves the application of biochemical stimulation in conjunction with recombinant proteins. However, in recent years, it has been revealed that mechanical signaling via physical stimulation is also important in the process of morphogenesis/differentiation. Chondrocytes exist in a physiologically hypoxic environment, and this hypoxic environment is essential for their growth, differentiation, and survival [10]. A previous report found that chondrocyte differentiation was promoted by culture under hypoxic conditions during the production of cartilaginous tissues from human embryonic stem (ES) cells [11]; thus, it is possible that a hypoxic environment may also promote differentiation of cartilaginous tissue from iPSCs.

In light of this evidence, we hypothesized that, if cartilaginous tissues could be prepared from iPSCs in a hypoxic environment, it would be possible to produce tissues more quickly than under a stable oxygen environment. The objective of this study was to investigate whether cartilaginous tissue could be produced from iPSCs under hypoxic conditions and to evaluate the effects of such an environment on the cellular metabolism and purity of the tissue produced.

Methods

Chondrogenic differentiation of human iPSCs (hiPSCs) in a monolayer culture

The established hiPSC line Toe was maintained in feeder-free medium that included StemFit AK-02N (Reprocell Inc., Yokohama, Japan) in 6-cm dishes coated with laminin 511 (Nippi, Inc., Tokyo, Japan). The hiPSCs were transferred and then maintained in StemFit AK-02N in 6-well dishes coated with laminin 511. The hiPSCs formed high-density cell colonies consisting of $1-2 \times 10^5$ cells at 10–15 days after starting maintenance under feeder-free culture conditions. Then, chondrogenic differentiation of the iPSCs was started. First, the hiPSCs were differentiated into mesendodermal cells cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma-Aldrich, St. Louis, MO, USA) with 10 ng/mL Wnt3A (R&D Systems, Minneapolis, MN, USA), 10 ng/mL activin A (R&D Systems), 1% Insulin, Transferrin, and Selenium (ITS) (Thermo Fisher Scientific, Rockford, IL, USA), 1% fetal bovine serum (FBS), and Penicillin and Streptomycin (P/S) for 3 days. On day 3, the medium was changed to chondrogenic medium [DMEM with 50 mg/mL ascorbic acid (Nacalai Tesque), 10 ng/mL BMP2 (PeproTech, Rocky Hill, NJ, USA), 10 ng/mL TGF- β 1 (PeproTech), 10 ng/mL GDF5 (PeproTech), 1% ITS, 1% FBS, 2 mM L-glutamine (Thermo Fisher

Scientific), 1×10^{-4} M nonessential amino acids (Nacalai Tesque, Kyoto, Japan), 1 mM Na pyruvate (Thermo Fisher Scientific), and P/S. bFGF (1 ng/mL; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to the medium from day 3 to day 14. Two groups of cells were assessed, consisting of hiPSCs cultured under normoxic or hypoxic (5% O₂, 5% CO₂, and 90% N₂) conditions in a multigas incubator (MODEL 9200; Wakenyaku Co., Ltd., Kyoto, Japan) (Fig. 1a).

Total RNA extraction and real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the cells using ISOGEN (Nippon Gene Co., Ltd., Osaka, Japan). The extracted RNA was reverse transcribed using PrimeScript™ RT Master Mix (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer’s direction. We performed Quantitative real-time RT-PCR using Step One Plus™ (Applied Biosystems, Carlsbad, CA, USA) with a primer probe. Each 20-μL reaction mixture contained 1 μL of cDNA (100 ng) and 9 μL TaqMan™ Fast Advanced Master Mix (Applied Biosystems), as well as 0.33 μL of target gene primers (Table 1) and probes from the Universal Probe Library (Roche, Basel, Switzerland). The amplification protocol was denaturation at 95 ° C for 15 seconds and annealing and extension at 60 ° C for 1 minute for 40 cycles. Relative changes in gene expression were calculated according to the comparative Ct method and normalized to the internal control gene 18S ribosomal RNA gene. Results are shown as the average of 3 samples in which each sample was assayed in duplicate.

Table 1
Primers used for real-time RT-PCR.

Primer		Sequence
18S rRNA	forward	ATGAGTCCACTTTAAATCCTTTAACGA
	reverse	CTTTAATATACGCTATTGGAGCTGGAA
T (Brachyury)	forward	AGACACGTTCACCTTCAGCA
	reverse	GCTCACCAATGAGATGAYCG
FOXF1	forward	CAGCCTCTCCACGCACTC
	reverse	CCTTTTCGGTCACACATGCT
Aggrecan	forward	CTGGAAGTCGTGAAAGG
	reverse	TCGAGGGTGTAGCGTGTAGA
CD44	forward	GCAGTCAACAGTCGAAGAAGG
	reverse	TGTCCTCCACAGCTCCATT

Flow cytometry

Cells were detached and digested using trypsin to form a single-cell suspension. For labeling of intracellular antigens, cells were fixed with 4% paraformaldehyde for 30 min at 4 °C and further permeabilized by incubation with 1% (wt/vol) bovine serum albumin (BSA) and 0.2% (vol/vol) TritonX-

100 in phosphate buffered saline (PBS) for 15 min at room temperature. Cells were incubated with primary Alexa Fluor® 488-conjugated rabbit anti-SOX9 antibody (EPR14335, ab196450, Abcam, Cambridge, UK) 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, Franklin Lakes, NJ, USA) with CellQuest software (Becton, Dickinson and Company).

Chondrogenic differentiation of hiPSCs in 3D culture

The hiPSCs were transferred and then maintained in StemFit AK-02N in 6-well dishes coated with Matrigel GFR (Thermo Fisher Scientific). The hiPSCs formed high-density cell colonies that consisted of $1-2 \times 10^5$ cells at 10–15 days after the start of maintenance. Subsequently, chondrogenic differentiation of the 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, particles were transferred to suspension culture in 3.5-cm non-attachment culture dishes (Prime surface®; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) on day 14. On day 42, the medium was changed to conventional medium (DMEM with 10% FBS and 50 U and 50 mg/mL of penicillin and streptomycin, respectively). The medium was changed every 2–7 days.

Two groups of cells were assessed, consisting of hiPSCs cultured under normoxic or hypoxic (5% O₂, 5% CO₂, and 90% N₂) conditions in a multigas incubator.

Histological and immunohistochemical analyses

Pellets were fixed in 4% paraformaldehyde (Wako Pure Chemical Industries, Ltd.), embedded in paraffin, and cross-sectioned (5-µm thick sections). The sections were stained with hematoxylin and eosin or safranin O. For immunohistochemistry analysis of type 1 collagen and type 2 collagen, paraffin-embedded sections were de-paraffinized in xylene, rehydrated by graded alcohol series, and immersed in PBS. Endogenous peroxidase activity was blocked by incubating the sections in 3% H₂O₂ in methanol for 5 min. The sections were incubated at 4 °C with mouse polyclonal anti-type 1 collagen antibody (ab6308; Abcam) at 1:150 or anti-type 2 collagen antibody (F-57; Kyowa Pharma Chemical Co., Ltd., Takaoka, Japan) at 1:50 overnight. After extensive washing with PBS, the sections were incubated in Histofine Simple Stain Rat MAX-PO (Nichirei Biosciences Inc., Tokyo, Japan) for 30 min at room temperature. Immunostaining was detected by 3,3'-Diaminobenzidine (DAB) staining. Counterstaining was performed with Mayer's hematoxylin.

Immunofluorescent staining

For immunohistochemistry of hypoxia-inducible factor (HIF)-1α, paraffin-embedded sections were de-paraffinized in xylene, rehydrated in a graded alcohol series, and immersed in PBS, followed by application of protein block (Dako, Carpinteria, CA, USA) for 10 min. The sections were incubated at 4 °C with rabbit polyclonal anti-HIF-1α antibody (ab2185, Abcam) at 1:250 for 1 h. After extensive washing with PBS, the sections were incubated in goat anti-rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 568

(A-11036, Thermo Fisher Scientific) at 1:500 for 30 min at room temperature. After extensive washing with PBS, counterstaining was performed with VECTASHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories, Burlingame, CA, USA).

Statistical analysis

All duplicate and triplicate experiments gave almost identical results. All data in this study are expressed as mean $\pm$ standard deviation. We used a parametric one-way analysis of variance to test for differences between groups. The Tukey-Kramer test was used to determine certain differences between groups when the results were considered significant. For all analyses, differences of $p < 0.05$ were considered statistically significant in all analyses.

Results

Effects of hypoxic stimulation on the purity of differentiated cartilage

We measured the number of cells comprising the tissue specimen on day 14 in order to confirm the effects of hypoxic stimulation on cell viability. Hypoxic stimulation at 5% O₂ did not adversely affect cell viability during differentiation of cartilaginous tissue from iPSCs. In the 2% O₂ hypoxic environment, cell viability decreased by about 80% (data not shown). Thus, a hypoxic environment of 5% O₂ was used for subsequent analyses. The effect of a hypoxic environment on cartilage differentiation was investigated through real-time RT-PCR performed during plate culturing. Cultivation 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% O₂ hypoxic environment on day 14 after the start of culture, a point at which cartilage differentiation had advanced to some extent and gene expression could be 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 aggrecan, a marker of the presence of 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, the master regulator related to chondrocytes, fluorescence-activated cell sorting (FACS) targeting SOX9 was performed on days 10 and 14 in both the group cultured under steady oxygen conditions and the group cultured in a 5% O₂ hypoxic environment. The results showed that the proportion of SOX9-positive cells had increased by day 10 under hypoxic culture conditions, and this number increased further by day 14 (Fig. 1c).

Effect of hypoxic stimulation during cartilage differentiation on substrate production

In the same manner, 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.

According to the results of the histological examination performed on day 28, only a portion of the cells grown under normoxic conditions was 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 significant 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.

Investigation of the promotive effects of HIF-1 α on cartilage differentiation under hypoxia

Next, we investigated the mechanism promoting cartilage differentiation in a hypoxic environment. The expression of HIF-1 α is known to be promoted under hypoxia and to accelerate cartilage differentiation. We also performed 3D culture using Matrigel according to the differentiation protocol up to day 28, followed by immunostaining for HIF-1 α . The expression of HIF-1 α was observed under both normoxia and hypoxia (Fig. 3). However, the nuclear dyeability in tissues cultured under hypoxia was greater than that under normoxia.

Discussion

With the industrialization of tissue graft materials in the area of regenerative medicine, quality assurance and standardization have been recognized, in recent years, as having critical importance. As ES cells and iPSCs maintain their ability to differentiate, they are excellent cell sources. However, because differentiation is induced in undifferentiated cells, it is necessary to improve the level of cell purity. Various studies have been conducted regarding improving 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 [12]. In addition, Hwang et al. increased the purity of cardiomyocyte differentiation from iPSCs by adding a small molecule compound to the culture environment [13]. 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. Chondrocytes are surrounded by a thick extracellular matrix, enabling them to remain viable in increasingly hypoxic environments, and chondrocytes present in articular cartilage are generally found in environments containing 1–5% O₂ [14]. 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 [15]. In this study as well, the expression of the genes *T* 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 had increased. A previous report found

that HIF-1 α expression under hypoxic conditions induced differentiation into articular cartilage via SOX9, a master regulator related to cartilaginous tissue [16]. Based on this observation, it was reported that, in hypoxic environments, cell differentiation was promoted via HIF-1 α expression, accompanied by decreasing levels of undifferentiated cell markers and an increase in the population of SOX9-positive chondrocytes.

HIF-1 α also has an anabolic effect on the metabolism of cartilaginous tissue. It is known that HIF-1 α translocates to the nucleus in hypoxic environments and regulates the expression of SOX9, a master regulator in chondrocytes [10, 16]. Furthermore, a prior study found that the production of substrates, such as aggrecan and type 2 collagen, can be promoted via HIF-1 α expression and by culturing chondrocytes in a hypoxic environment[17]. In this study, the histological examination performed on culture day 28 revealed that safranin O staining and substrate production were both increased in the group cultured under hypoxic conditions. Then, by day 56, it was possible to produce tissue specimens similar to those cultured under a stable oxygen environment. These findings indicate that hypoxic culture may be used to produce high-quality tissue more rapidly.

This study has some limitations. First, a 5% O₂ culture environment was evaluated during this study. This oxygen concentration was chosen because, at this concentration, it is easier to maintain cell viability than in a 2% O₂ environment; however, evaluations of other oxygen concentrations are currently lacking and are needed. In addition, the period for which hypoxia should be administered is also a subject requiring further investigation in the future. Second, we have not yet evaluated these conditions with respect to SOX9-negative cells, and the possibility of contamination by undifferentiated cells cannot be ruled out. Third, as we did not conduct a transplantation experiment in an animal model, we were unable to evaluate the risks of tumorigenesis or tissue deformation after transplantation.

Conclusions

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

Abbreviations

iPSCs: Induced pluripotent stem cells; hiPSCs : Human iPSCs; *FOXF1*: FOXF1 gene; FOXF1: Forkhead box protein F1; *SOX9*: SOX9 gene; SOX9: SRY (sex determining region Y) - box 9; *CD44*: CD44 gene; HIF-1 α : Hypoxia-inducible factor-1 α ; *HIF-1 α* : HIF-1 α gene; ES cells: Embryonic stem cells; DMEM: Dulbecco's modified Eagle's medium; ITS: Insulin, transferrin, and selenium; FBS: Fetal bovine serum; P/S: Penicillin and streptomycin; RT-PCR: Reverse transcription-polymerase chain reaction; BSA: Bovine serum albumin; PBS: Phosphate buffered saline; DAB: Diaminobenzidine; DAPI: 4',6-diamidino-2-phenylindole; FACS: Fluorescence-activated cell sorting

Declarations

Ethics approval and consent to participate

All experimental animals in our study were approved by Committee for Animal Research, Kyoto Prefectural University of Medicine (number: M25–29).

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Author contributions: Conceptualization, SS, HI, and SN; data curation, SS, HI, ST and YA; formal analysis, SS and TsKi; funding acquisition, ToKu.; investigation, SS, YF, and SI; methodology, SS, SN, and MS; project administration, YA and ToKu; supervision, OM; validation, ToKu; writing – original draft, SS; writing – review & editing, all authors.

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Authors' information

The principle author consents to the inclusion of personal information in the journal's database.

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Figures

Figure 1

Hypoxia promoted chondrogenic differentiation. (a) hiPSCs were cultured in monolayer under normal oxygen or 5% O₂ hypoxic conditions during cartilage differentiation for 14 days (n = 3). (b) On day 14, gene expression levels of *T* and *FOXF1* (immature mesodermal markers), and aggrecan and *CD44* (chondrogenic markers) were determined by real-time RT-PCR. (c) On days 10 and 14, the percentage of SOX9-positive cells was determined by FACS analysis. *p < 0.05, **p < 0.01.

a 3D culture with Matrigel

b: day 28

c: day 56

Figure 2

Hypoxic conditions promoted purity of cartilage-like tissue. (a) hiPSCs were cultured in 3D culture under normal oxygen or 5% O₂ hypoxic conditions during cartilage differentiation for 28 or 56 days. (b, c) Histological analysis of mid-sagittal sections using hematoxylin and eosin (HE), and safranin O, and immunohistochemical analysis using anti-type 1 or 2 collagen antibodies were performed to visualize chondrogenic differentiation on (b) day 28 and (c) day 56. Scale bar: 200 μ m.

Figure 3

Culturing under hypoxia promoted the dyeability of HIF-1 α in cartilage-like tissues. hiPSCs were cultured in 3D culture under normoxia or hypoxia during cartilage differentiation for 28 days. Immunofluorescence staining with DAPI counterstaining demonstrating HIF-1 α positivity was used to visualize nuclear uptake. This is shown at a magnification of 400x.