

## Original Article

# Hypoxia enhances proliferation through increase of colony formation rate with chondrogenic potential in primary synovial mesenchymal stem cells

Toshiyuki Ohara<sup>1)</sup>, Takeshi Muneta<sup>1)</sup>, Yusuke Nakagawa<sup>1)</sup>, Yu Matsukura<sup>1)</sup>, Shizuko Ichinose<sup>2)</sup>, Hideyuki Koga<sup>1)</sup>, Kunikazu Tsuji<sup>3)</sup> and Ichiro Sekiya<sup>4)</sup>

1) Department of Joint Surgery and Sports Medicine, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University

2) Research Center for Medical and Dental Sciences, Tokyo Medical and Dental University

3) Department of Cartilage Regeneration, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University

4) Center for Stem Cell and Regenerative Medicine, Tokyo Medical and Dental University

Synovial mesenchymal stem cells (MSCs) are an attractive cell source for cartilage and meniscus regeneration. Use of primary MSCs is the preferable because these cells are safer than cells passaged several times in terms of probability of chromosome abnormalities. The effect of hypoxia on the proliferation of MSCs is controversial and remains unknown in primary synovial MSCs. Primary synovial MSCs were cultured at normoxia or hypoxia, and colony number, cell number, surface epitopes, mitochondria activity, TEM finding, and chondrogenic potential were analyzed. To investigate the effect of hypoxia on attachment of synovial MSCs, cells were cultured at hypoxia for the first 3 days, then cultured at normoxia. To investigate the effect of hypoxia on proliferation, cells were also cultured at hypoxia for the last 11 days. Hypoxia increased colony number and cell number per dish in primary synovial MSCs. Hypoxia did not affect cell number per colony, surface epitopes, mitochondria activity, TEM finding or chondrogenic potential. Hypoxia for the first 3 days did not alter colony number per dish or cell number per dish, while hypoxia for the last

11 days increased. Hypoxia enhanced proliferation through increase of colony formation rate with chondrogenic potential in primary synovial MSCs.

**Key words:** Mesenchymal stem cells, Synovium, Hypoxia, Proliferation, Chondrogenesis.

## Introduction

Synovial mesenchymal stem cells (MSCs) are an attractive cell source for cartilage and meniscus regeneration because of their high chondrogenic potential<sup>1</sup>. Transplantation of synovial MSCs promoted cartilage regeneration in rabbits<sup>2</sup> and pigs<sup>3</sup>. Furthermore, synovial MSCs enhanced meniscus regeneration in rats<sup>4</sup>, rabbits<sup>5, 6</sup> and pigs<sup>7</sup>. Arthroscopic transplantation of autologous primary synovial MSCs improved clinical outcomes in knees with cartilage defects<sup>8</sup> and we are currently performing clinical trials for meniscus regeneration with primary synovial MSCs.

In the past 10 years, hypoxia has received attention due to its enhancing ability in cell proliferation. There are several reports describing that hypoxia enhanced proliferation of MSCs derived from bone marrow<sup>9, 10</sup>, adipose tissue<sup>11, 12</sup>, the umbilical cord<sup>13</sup>, dental pulp<sup>14</sup> and so on. However, other reports showed that hypoxia did not alter proliferation of MSCs<sup>15, 16</sup> and controversy still exists as to the effect of hypoxia on proliferation of MSCs.

Clinically, the use of primary MSCs is preferable because these cells are safer than cells passaged

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Corresponding Author: Ichiro Sekiya, M.D., Ph. D.  
Director and Professor, Center for Stem Cell and Regenerative  
Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima,  
Bunkyo-ku, Tokyo 113-8510, Japan.  
Tel: +81-3-5803-4017 Fax: +81-3-5803-0192  
E-mail: sekiya.arm@tmd.ac.jp  
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several times in terms of the probability of developing chromosome abnormalities<sup>17</sup>. In addition, the ability to prepare enough passage 0 cells in a limited time can reduce costs, compared with the need to passage cells multiple times for longer periods. Though one report showed the effect of hypoxia on proliferation of passaged synovial MSCs<sup>16</sup>, the effect of hypoxia on proliferation of primary synovial MSCs remains unknown. This is important because synovial nucleated cells consist of fibroblasts and macrophages derived from synovial membrane, and cells derived from subsynovial tissues<sup>18</sup>, contrarily passaged synovial MSCs showed the following typical surface epitope pattern of MSCs: positive for CD44 (hyaluronan receptor), CD73 (5'-nucleotidase), CD90 (Thy1), CD105 (endoglin) and negative for CD34 (hematopoietic stem cell marker), CD45 (leukocyte common antigen)<sup>19</sup>. This indicates that synovial nucleated cells are more heterogeneous than passaged synovial MSCs and surface epitope of synovial nucleated cells might be different from one of passaged synovial MSCs.

In this study, we examined whether hypoxia enhanced proliferation of human primary synovial MSCs. We also examined whether hypoxia affected properties of primary synovial MSCs and attempted to investigate the mechanisms for this effect.

## Methods

### Isolation of primary synovial cells

This study was approved by the local institutional review board of Tokyo Medical and Dental University (reference number 2121), and informed consent was obtained from all study subjects. Human synovium was harvested during total knee arthroplasty from 33 donors diagnosed with knee osteoarthritis and some synovium were used for several experiments. The average age was  $76 \pm 9$  years old. Synovium was minced and digested in a 3-mg/mL collagenase D solution (Roche Diagnostics, Mannheim, Germany) in minimum essential medium alpha modification ( $\alpha$ -MEM: Invitrogen, Carlsbad, CA) at 37°C for 3 h. Then digested tissues were filtered with a 70- $\mu$ m nylon filter (Becton, Dickinson and Company, Franklin Lakes, NJ) to remove debris. After centrifugation, nucleated cells were plated in 60-cm<sup>2</sup> dishes.

### Colony-forming-assay and cell count

Nucleated cells from synovium were plated at 1,000 cells/60-cm<sup>2</sup> dish, plated in 12 dishes, and cultured in 10 mL  $\alpha$ -MEM containing 10% fetal bovine serum (FBS: Invitrogen), 100 unit/mL penicillin and 100 mg/ml streptomycin (Invitrogen) for 14 days as passage 0. The

cells were incubated at 21% O<sub>2</sub> (normoxia) or at 5% O<sub>2</sub> (hypoxia) in a low oxygen incubator (ASTEC, Fukuoka, Japan). Three dishes were stained with 0.5% crystal violet (Wako, Osaka, Japan) in 4% paraformaldehyde for 5 minutes for counting colony number and the other three dishes were used for cell counting.

### Analysis of surface epitope

After 14 days' culture of primary synovial MSCs, 100,000 cells were suspended in 50  $\mu$ L of FACS staining buffer (0.2% BSA fraction V and 0.09% Sodium azide in PBS) containing appropriate antibodies. After incubation for 30 minutes at 4°C, the cells were washed and resuspended in FACS staining buffer for flow cytometric analysis. Allophycocyanin (APC), Fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-Cy7 (PE-Cy7) or peridinin chlorophyll protein-Cy5.5 (PerCP-Cy5.5)-coupled antibodies against for CD45, CD73, CD90, CD105, CD140b and CD45 (Becton Dickinson) were used. For isotype controls, APC-, FITC-, PE-, PE-Cy7 or PerCP-Cy5.5-coupled nonspecific mouse immunoglobulin G (IgG; Becton Dickinson) was substituted for the primary antibody. Cell fluorescence was evaluated by flow cytometry using a FACSVerse instrument (Becton Dickinson). The data were analyzed using FACSsuite software (Becton Dickinson).

### Analysis for mitochondria activity

After 10 days' culture of primary synovial MSCs, 100,000 cells were washed with PBS and resuspended in 1 mL of FACS staining buffer containing 50nM working solution of Mitotracker Deep Red (Thermo Fisher Scientific Inc, Waltham, MA). After incubation for 15 minutes at 4°C, the cells were washed and resuspended in FACS staining buffer for flow cytometric analysis. Cell fluorescence was evaluated by flow cytometry using a FACSVerse instrument. The data were analyzed using FACSsuite software.

### Analysis of morphological differences

After 10 days' culture of primary synovial MSCs were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 2 h. Then cells were washed with 0.1M phosphate buffer, post-fixed in 1% OsO<sub>4</sub> buffered with 0.1M phosphate buffer for 2 h, dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections at 90 nm were collected on copper grids, double-stained with uranyl acetate and lead citrate, and then examined with a transmission electron microscope (TEM: H-7100, Hitachi, Tokyo, Japan)<sup>20</sup>.

### ***In vitro* chondrogenic differentiation assay**

A total of 125,000 human primary synovial MSCs precultured at normoxia or hypoxia were pelleted by centrifugation. The pellets were cultured in 400  $\mu$ L chondrogenic medium consisting of high-glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1,000 ng/mL BMP-7 (Stryker Biotech, Boston, MA), 10 ng/mL transforming growth factor- $\beta$ 3 (TGF- $\beta$ 3: R&D Systems, Minneapolis, MN), 100 nM dexamethasone (Sigma-Aldrich Corp, St. Louis, MO), 50  $\mu$ g/mL ascorbate-2-phosphate, 40  $\mu$ g/mL proline, 100  $\mu$ g/mL pyruvate, and 1:100 diluted ITS + Premix (6.25  $\mu$ g/mL insulin, 6.25  $\mu$ g/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL bovine serum albumin, and 5.35 mg/mL linoleic acid; BD Biosciences Discovery Labware, Bedford, MA). The medium was changed every 3 to 4 days for 21 days. The pellets were embedded in paraffin, cut into 5- $\mu$ m sections and stained with safranin-o and fast green. Histological sections were visualized using an Olympus BX 53 microscope (Olympus, Tokyo, Japan). The pellets were evaluated using the quantitative score based on Bern's scoring system<sup>21</sup>.

### **Statistical analysis**

We performed two experiments for each subject and all statistics were carried out using the Wilcoxon signed-rank test on the StatView 5.0 program (SAS Institute,

Cary, NC), in order to compare the data at normoxia versus hypoxia. All p values less than 0.05 were considered as statistically significant.

## **Results**

### **Effect of hypoxia on proliferation of primary synovial MSCs**

Synovial nucleated cells formed colonies 14 days after being cultured at normoxia. The colony forming cells differentiated into chondrocytes, adipocytes, and calcified when cultured in the appropriate differentiation media (Fig. 1).

To examine the effect of hypoxia, synovial nucleated cells were cultured at normoxia or at hypoxia for 14 days (Fig. 2A), and stained with crystal violet (Fig. 2B). Colony number per dish was significantly higher in the cells cultured at hypoxia than in the cells cultured at normoxia in all 8 donors (Fig. 2C). Proliferated cells were harvested from the other 3 dishes and cell number was counted. Cell number per dish was also significantly higher in the hypoxic condition than in the normoxic condition in all 8 donors (Fig. 2D). There was no significant difference of cell number per colony between the cells cultured at normoxia and at hypoxia (Fig. 2E).

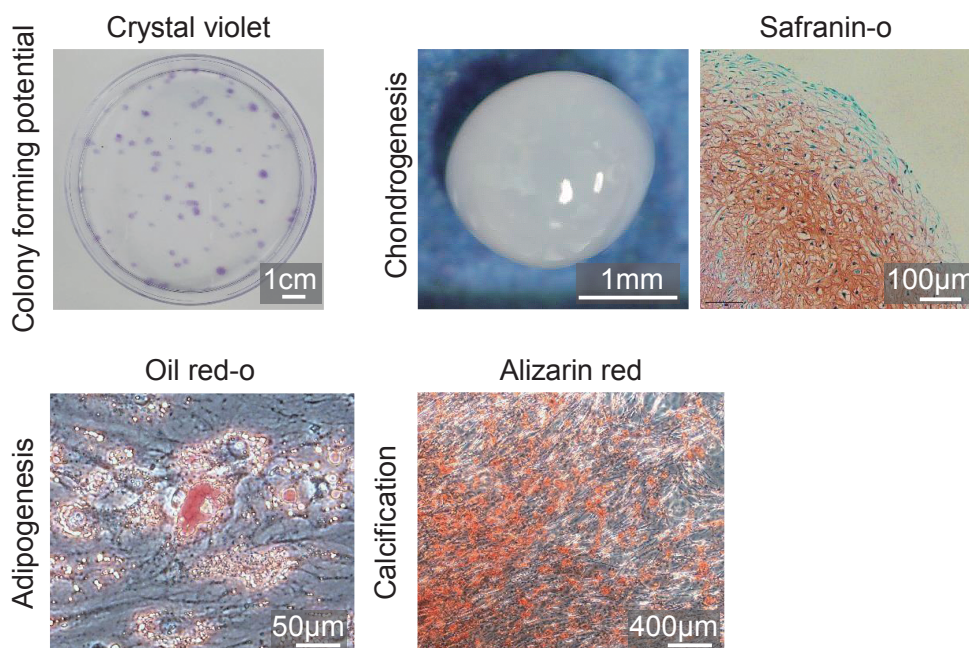


Figure 1. Representative images for colony formation and multilineage differentiation of synovial cells cultured at normoxia

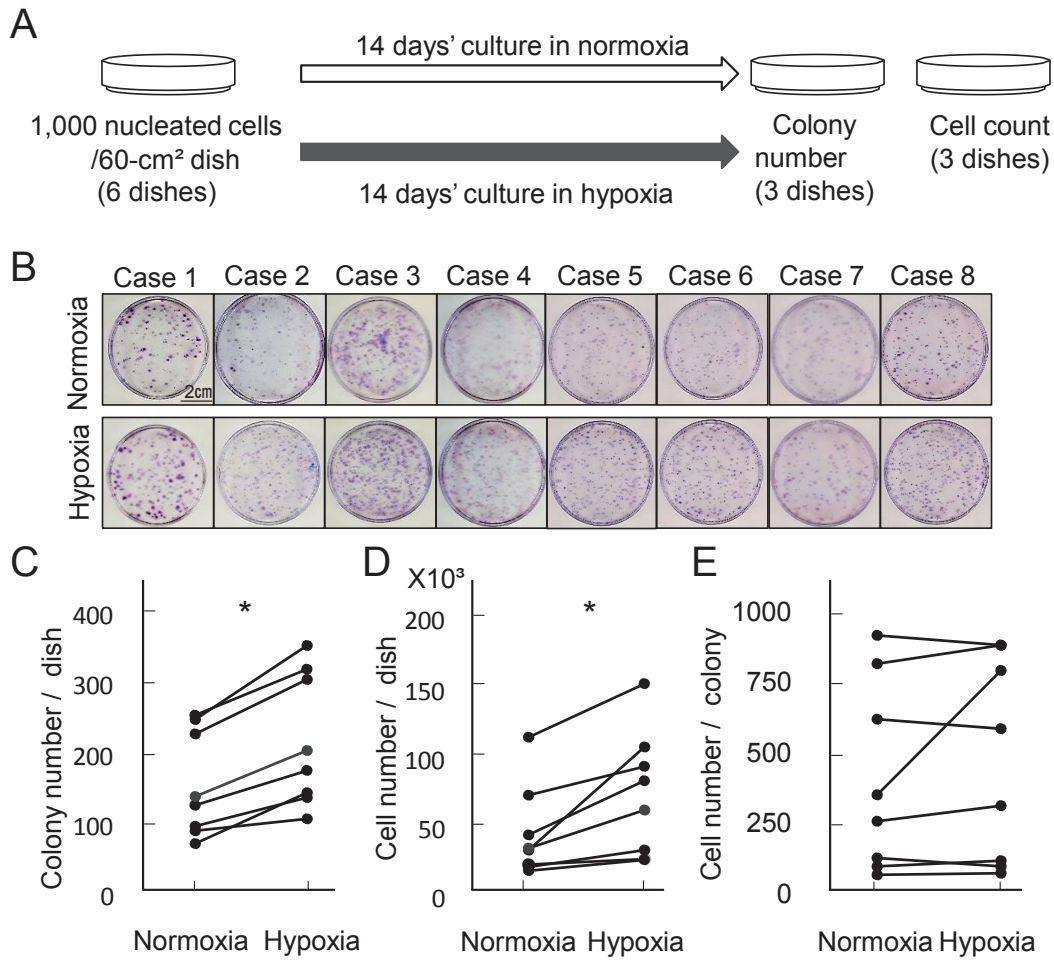


Figure 2. Comparison of proliferation of primary synovial MSCs cultured at normoxia and at hypoxia (A) Protocol. (B) Representative dishes stained with crystal violet. (C) Colony number per dish ( $n=8$ ,  $*p<0.05$  by Wilcoxon's signed rank test). (D) Cell number per dish ( $n=8$ ,  $*p<0.05$ ). (E) Cell number per colony ( $n=8$ ).

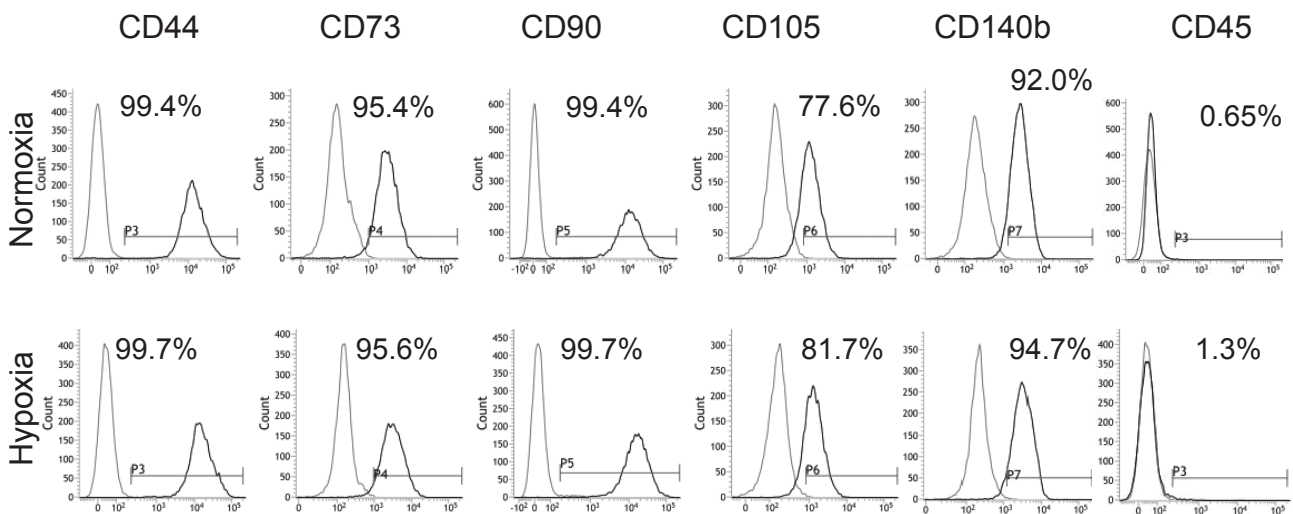


Figure 3. Epitope profile of primary synovial MSCs 14 days after being cultured at normoxia and at hypoxia



### Surface markers

Synovial nucleated cells from 2 donors cultured at normoxia and at hypoxia displayed typical surface markers as MSCs. There was no obvious difference of expressions of CD44, CD73, CD90, CD105, CD140b and CD45 between the cells cultured at normoxia and at hypoxia (Fig. 3).

### Mitochondria activity

Synovial MSCs cultured at normoxia and at hypoxia were stained with Mitotracker Deep Red and mitochondria activity was evaluated in 10 donors by flow cytometry. There was no significant difference of fluorescent intensity for mitochondria activity between the cells cultured at normoxia and at hypoxia (Fig. 4A, B).

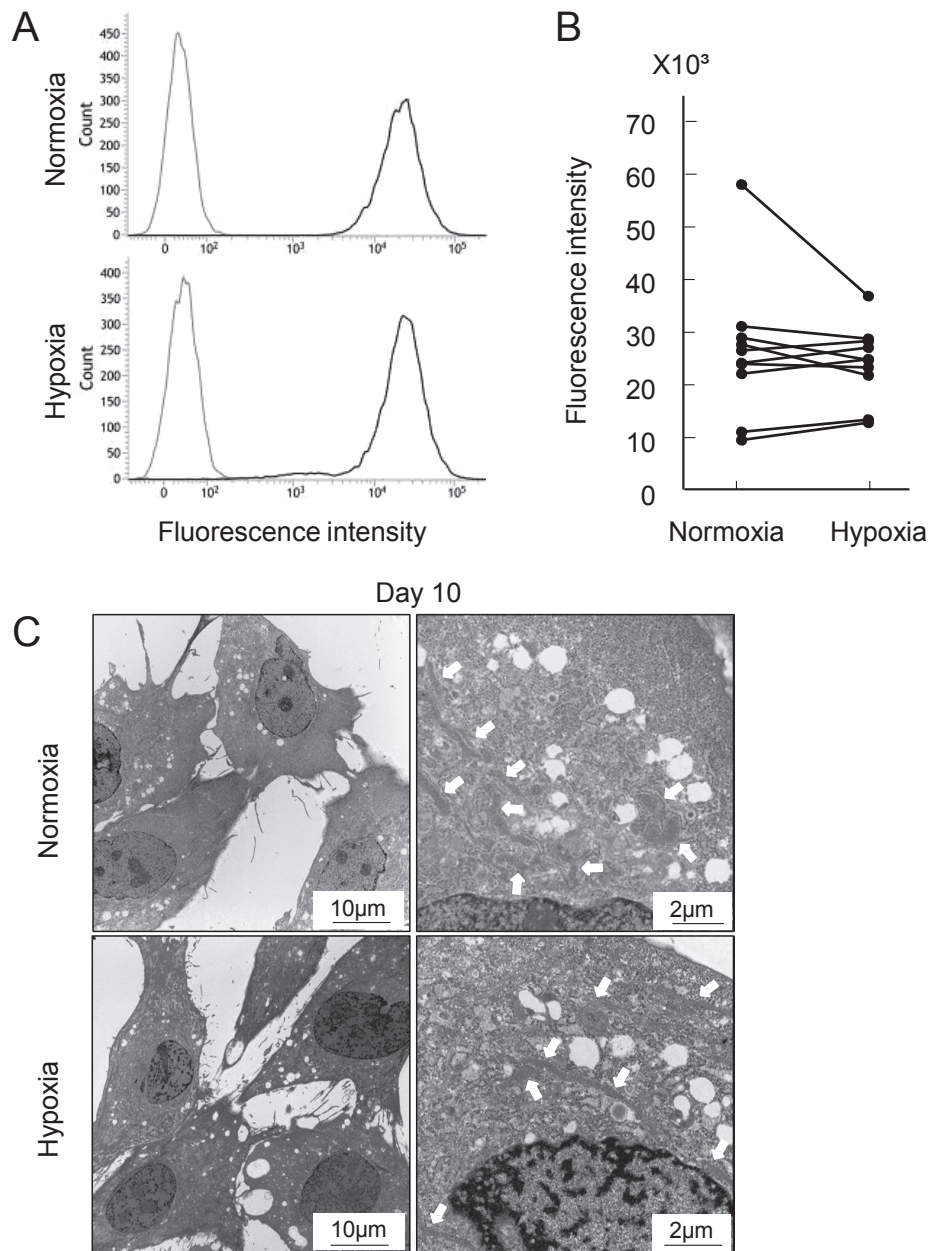


Figure 4. Analyses for mitochondria of primary synovial MSCs 10 days after being cultured at normoxia and at hypoxia (A) Representative data of fluorescence intensity of synovial MSCs stained with Mitotracker Deep Red by flow cytometry. (Gray line: unstained cells.) (B) Comparison of fluorescence intensity of the cells stained with Mitotracker Deep Red ( $n=10$ ). (C) Transmission electron microscopic features. White arrow shows mitochondria.

Morphology of primary synovial MSCs was compared by transmission electron microscope. Primary synovial MSCs cultured at normoxia and at hypoxia had an ovoid nucleus and organelles consisting of endoplasmic reticulum, ribosomes, lipid droplets, and mitochondria. There seemed to be no obvious difference of morphology and mitochondria number (Fig. 4C).

### Chondrogenesis

To investigate the effect of hypoxia on chondrogenesis of synovial MSCs, synovial MSCs derived from 7 donors were precultured at normoxia or at hypoxia for 14 days, and then pellets of the cells were cultured at normoxia in the chondrogenic medium for further 21 days (Fig. 5A). Pellets of the cells precultured at normoxia and at hypoxia formed a cartilage mass (Fig. 5B). There was no

significant difference of pellet weight between the cells precultured at normoxia and at hypoxia (Fig. 5C). Sections stained with safranin-o showed glycosaminoglycans in their matrix (Fig. 5D). There was no difference of Bern score for histology in the cells precultured at normoxia and at hypoxia (Fig. 5E).

### Effect of hypoxia on attachment of synovial MSCs

To investigate the effect of hypoxia on attachment of synovial MSCs to culture dishes, synovial nucleated cells from 6 donors were cultured at normoxia or at hypoxia for the first 3 days, then cultured at normoxia for 11 days (Fig. 6A). There was no significant difference of colony number per dish (Fig. 6B, C), cell number per dish (Fig. 6D), and cell number per colony (Fig. 6E) between the cells cultured at normoxia and at hypoxia.

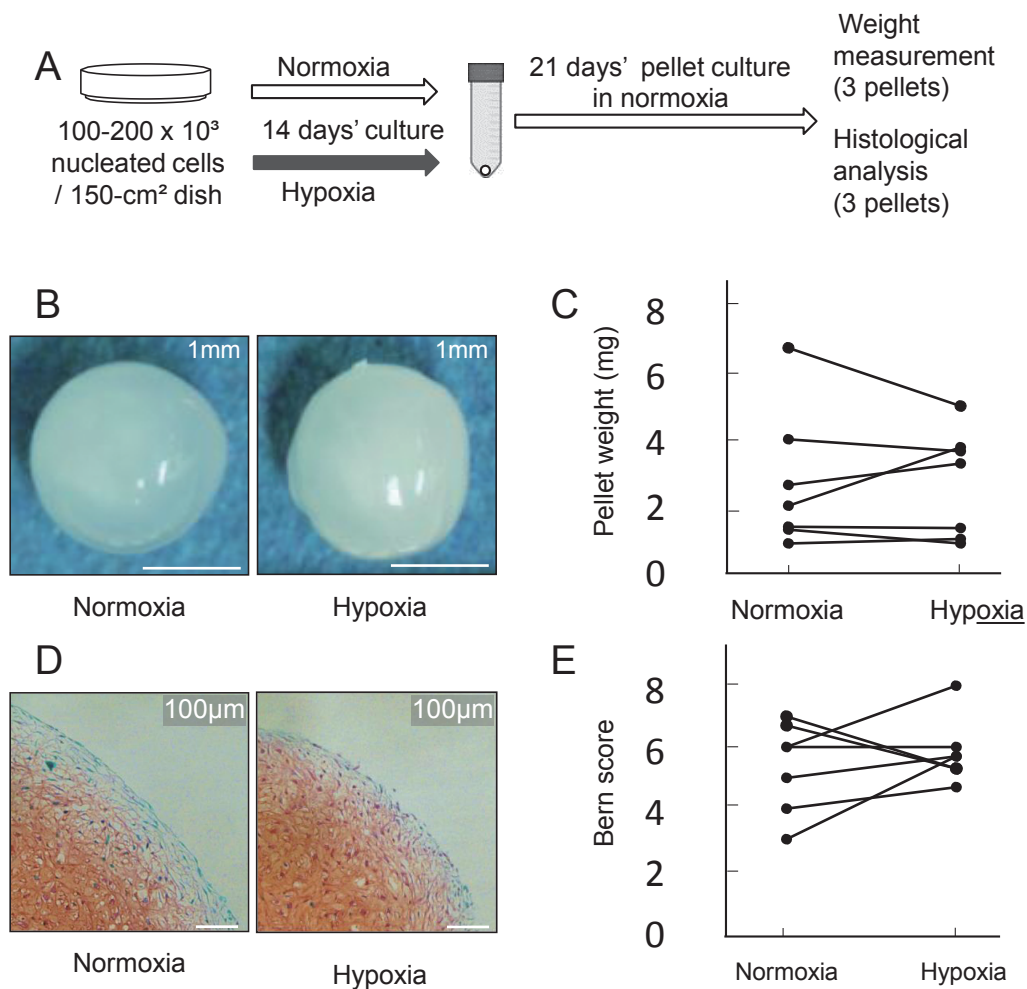


Figure 5. Comparison of chondrogenesis of primary synovial MSCs precultured at normoxia and at hypoxia (A) Protocol. (B) Representative macroscopic features. (C) Pellet weight ( $n=7$ ). (D) Representative histological pictures stained with safranin-o. (E) Bern score for histology ( $n=7$ ).

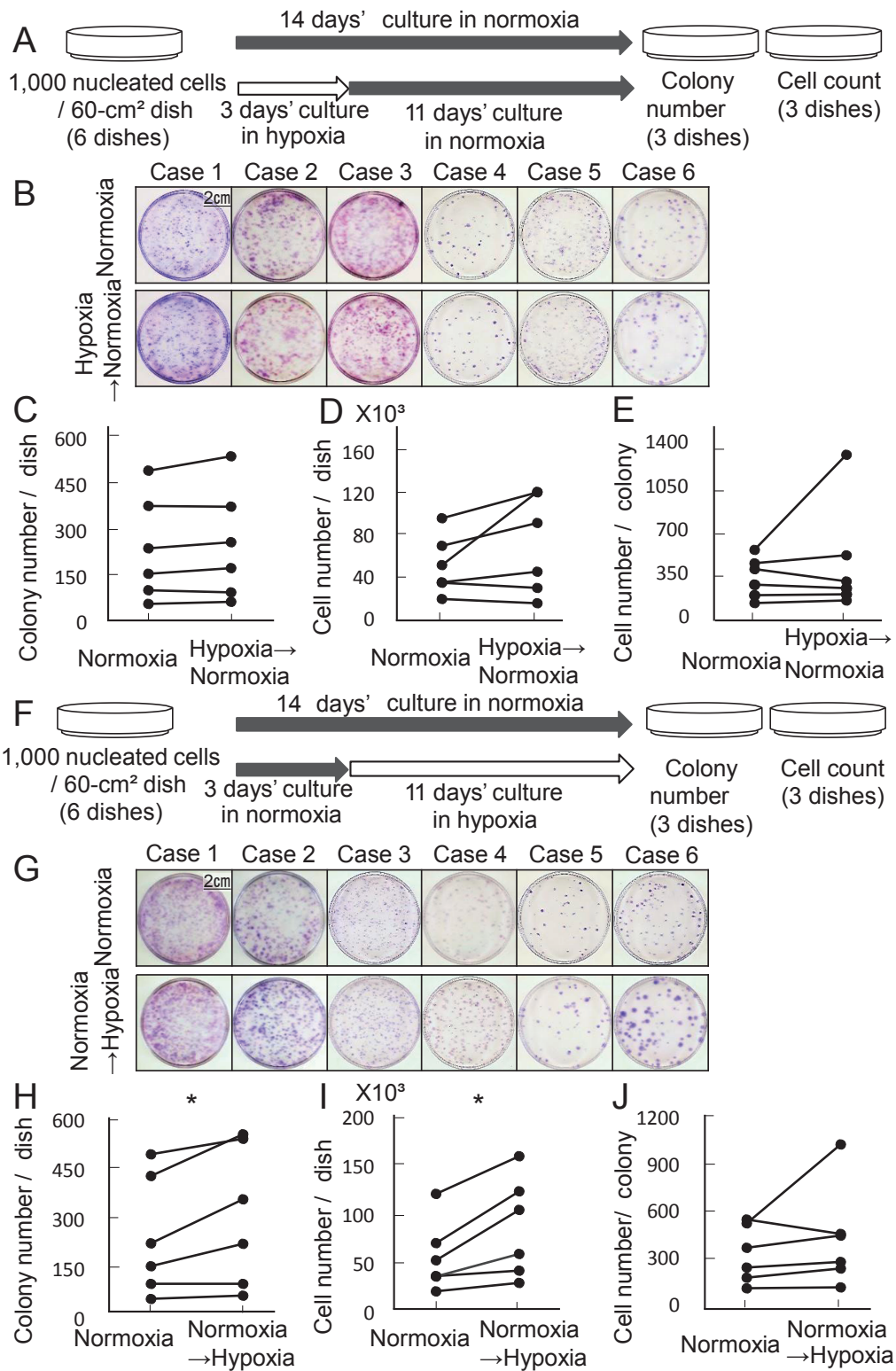


Figure 6. Comparison of cell attachment of primary synovial MSCs cultured at normoxia and at hypoxia

(A) Protocol. (B) Representative dishes stained with crystal violet. (C) Colony number per dish ( $n=6$ ). (D) Cell number per dish ( $n=6$ ). (E) Cell number per colony ( $n=6$ ). (F) Protocol to investigate the effect of hypoxia on cell proliferation with the exclusion of the effect of cell attachment. (G) Representative dishes stained with crystal violet ( $n=6$ ). (H) Colony number per dish ( $n=6$ ,  $*p<0.05$  by Wilcoxon's signed rank test). (I) Cell number per dish ( $n=6$ ,  $*p<0.05$ ). (J) Cell number per colony ( $n=6$ ).

Next, to investigate the effect of hypoxia on cell proliferation with the exclusion of the effect of cell attachment, synovial nucleated cells from 6 donors were cultured at normoxia for the first 3 days, then cultured at normoxia or at hypoxia for 11 days (Fig. 6F). Hypoxia after normoxia for the first 3 days significantly increased colony number per dish and cell number per dish (Fig. 6G, 6H, 6I), but did not alter cell number per colony (Fig. 6J).

## Discussion

Though the definition of MSCs is still controversial, a minimum criterion for MSCs was advocated in 2006, in which MSCs were defined by adherence to plastic, colony formation, trilineage differentiation, and surface markers<sup>19</sup>. In this study, colony forming cells derived from synovium could differentiate into chondrocytes, adipocytes, and could calcify when cultured in the appropriate differentiation media. Furthermore, colony forming cells derived from synovium were positive for CD44, 73, 90, 105, 140b and negative for CD45. Therefore, we defined the cells used in this study as MSCs.

Hypoxia increased colony number per dish and cell number per dish, but did not alter cell number per colony (Fig. 2). This suggests that hypoxia increased cell number through increase of colony formation rate, not through expansion of colonies. To examine the effect of hypoxia on attachment of synovial MSCs to culture dishes, synovial nucleated cells were cultured at hypoxia only for the first 3 days, because we thought that cell attachment was completed within the first 3 days (Fig. 6A-E). Hypoxia only for the first 3 days did not affect colony formation rate. To investigate the effect of hypoxia on cell proliferation with the exclusion of the effect of cell attachment, synovial nucleated cells were cultured at normoxia for the first 3 days, then cultured at hypoxia for 11 days (Fig. 6F-J). Hypoxia after normoxia for the first 3 days significantly increased colony number per dish and cell number per dish, indicating that hypoxia still increased cell number through increase of colony formation rate.

Hypoxia did not affect surface markers of primary synovial MSCs and primary synovial MSCs were positive for CD44, CD73, CD90, CD105 and CD140b and negative for CD45. These are similar to the MSC results derived from the umbilical cord<sup>22</sup>, adipose tissue<sup>23</sup>, and bone marrow<sup>24, 25</sup> except one paper in which hypoxia decreased CD90 expression in bone marrow MSCs at passage 2<sup>26</sup>. We previously reported that CD90

expression was correlated with chondrogenic potential of synovial MSCs<sup>27</sup>. In the current study, the positive rate of CD90 in synovial MSCs was not decreased by hypoxia, indicating that hypoxia does not decrease chondrogenic potential of synovial MSCs, which was indeed confirmed as shown in Fig. 5.

Some reports described that hypoxia decreased mitochondrial activity of MSCs derived from bone marrow, in which MSCs were exposed to hypoxia for several weeks after several passages<sup>24, 28, 29</sup>. In our current study, hypoxia did not alter mitochondrial activity of synovial MSCs. To account for the discrepancy, 3 different conditions can be listed: we used MSCs derived from synovium, we used primary MSCs, and the period of exposure to hypoxia was limited to only 10 days in our condition.

The effect of hypoxia on chondrogenesis of MSCs is still controversial. Hypoxia promoted chondrogenesis of MSCs derived from bone marrow<sup>26, 30</sup>, adipose tissue<sup>31</sup> and synovium<sup>16, 32</sup>, while hypoxia did not alter chondrogenesis of MSCs derived from bone marrow<sup>28, 33</sup> and synovium<sup>16</sup>. Concerning previous reports of the effect of hypoxia on chondrogenesis of MSCs derived from synovium, Li et al. showed that hypoxia enhanced Sox9 and Col2 mRNA expressions in passage 7 MSCs derived from human fetal synovium<sup>32</sup>. Furthermore, Li et al. showed that hypoxia did not alter chondrogenesis of passage 4 MSCs derived from pig synovium<sup>16</sup>. Our current study is the first report describing the effect of hypoxia on chondrogenesis of passage 0 MSCs derived from human synovium, which is important for clinical applications of cartilage<sup>3</sup> and meniscus regeneration<sup>34</sup>.

Lennon et al. previously reported that hypoxia increased colony formation in primary bone marrow MSCs<sup>10</sup>. In this report, we demonstrated the positive effect of hypoxia on colony formation rate of primary synovial MSCs for the first time. We could not clarify why hypoxia enhanced colony formation rate of primary synovial MSCs. According to previous reports, we propose 2 possibilities; hypoxia increases the number of G2/S/M period cells<sup>35</sup> and hypoxia increases sensitivity of MSCs to growth factors in serum<sup>24</sup>.

Does hypoxia affect the genomic stability of MSCs? According to Bigot et al., 1% O<sub>2</sub> did not affect the genomic stability in bone marrow MSCs, but affected slightly in adipose derived MSCs<sup>36</sup>. Influence of hypoxia on the genomic stability may be due to microenvironment of original tissues for MSCs. The effect of hypoxia on the genomic stability of synovial MSCs is unknown but careful examinations are needed before clinical applications.



## Conclusions

Hypoxia enhanced proliferation through increase of colony formation rate with chondrogenic potential in primary synovial MSCs.

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