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Expansion and differentiation of human iPS cells in a three-dimensional culture using hollow fibers and separation of the specific population by magnetic-activated cell sorting

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In order to employ pluripotent stem cells in the field of regenerative medicine, it is necessary to establish a large-scale culture system for cell differentiation. We have developed a novel three-dimensional method for culturing human induced pluripotent stem (iPS) cells, using hollow fibers (HFs). The cells immobilized inside HFs can proliferate and form multicellular aggregates, capable of achieving a high cell density and promoting further spontaneous cell differentiation. We first cultured human iPS cells for 7 days under conditions that maintained their undifferentiated state and then switched the culture conditions to allow spontaneous cell differentiation. In the 7-day undifferentiated culture, a high cell density of approximately 10-fold that of the initial seeding density was achieved. The upregulation of gene markers for differentiation such as CXCR4 or SOX17 was observed in the culture of differentiated cells. Expression of the lineage-specific cell-surface marker CXCR4 was about 30% at day 5 in the differentiation culture, which was 2-fold higher than that in the traditional monolayer culture. After HF culture, we obtained the CXCR4-positive cell population and performed monolayer culture for further differentiation of the hepatic lineage. In the CXCR4-positive cell population, the expression levels of a few liver-specific gene markers tended to increase. However, there were no significant differences between the separation and non-separation groups, which indicates the need for refinement of the cell separation process and cell maturation procedure in future studies. In conclusion, the HF culture method has potential for achieving the large-scale culturing and spontaneous differentiation of human iPS cells.

[**Key words:** Induced pluripotent stem cell; Three-dimensional culture; Hollow fiber; Cell expansion; Cell differentiation; Cell sorting]

The current treatment of organ/tissue failure and loss by intact organ/tissue transplantation suffers from a limited donor supply as well as severe immune complications, but these obstacles may potentially be bypassed through the use of regenerative medicine strategies (1). Therefore, regenerative medicine is expected to be a promising strategy for patients with impaired tissue/organ functions. However, it is of utmost importance to secure cell sources for the success of regenerative medicine, where particularly, a large number of human cells are needed to make large organs. Therefore, pluripotent stem cells, such as embryonic stem (ES) cells (2,3) or induced pluripotent stem (iPS) cells (4,5), which have the potential for self-renewal, an infinite capacity for proliferation, and the capacity for pluripotent differentiation, have been used as a cell source that can supply a large number of cells. Many researchers have reported the differentiation of pluripotent stem cells into various types of functional cells. However, in order to employ pluripotent stem cells as a cell source, it is necessary to differentiate these cells efficiently and in large amounts. Several researchers have reported large-scale differentiation culture processes. In one such study, Matsuura et al. (6,7) established a mass cultivation system for human iPS cells using three-dimensional suspension culture and confirmed the differentiation of these cells into highly efficient cardiomyocytes. Three-dimensional suspension culture is a method that induces the cells to form embryoid bodies (EBs). However, it is difficult to control the size of the EBs with this culture method, and cells are damaged by the shear-stress of stirring.

Therefore, we had previously developed a culture method, in which the size of EBs can be controlled to avoid shortages of oxygen and nutrients. We focused on a three-dimensional culture using hollow fibers (HFs), in which cultured cells form cylindrical multicellular aggregates within a controlled space (8). We established an HF culture method wherein aggregates immobilized inside the HFs are able to achieve a high cell density. We have previously reported the differentiation of mouse ES cells into hepatocytes using this HF culture method. We also compared the differentiation efficiency of this culture method with that of the traditional monolayer culture method under a differentiation protocol. The results indicated that the differentiated cells from the HF culture expressed some hepatocyte-specific genes and liver-specific functions, whereas no major differentiation effects were observed in the cells grown in the traditional monolayer culture. Therefore, the HF culture method is a more effective tool for inducing the differentiation of ES cells into hepatocytes (9). We have also reported the differentiation of mouse ES cells into hematopoietic stem cells (HSCs) using the HF culture method. We evaluated the proliferation activity and hematopoietic differentiation capability of the ES cells in comparison with those of ES cells obtained by the

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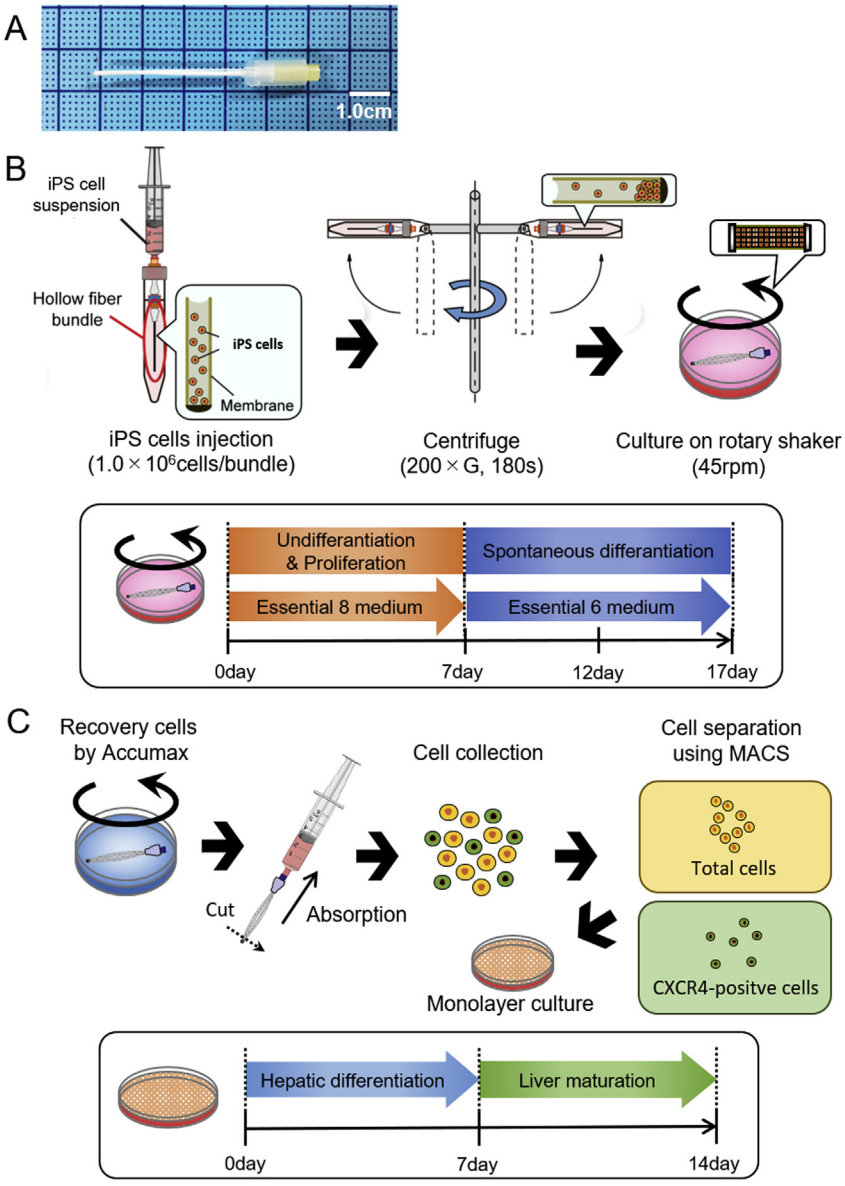

FIG. 1. Illustrations of culture methods used in this study. (A) Photograph of hollow fiber bundles. (B) The upper figures are the method of expansion and differentiation of human iPS cells in a three-dimensional culture using hollow fibers. The lower figure is the culture conditions of expansion and differentiation of human iPS cells. (C) The upper figures are the method of separation with the specific population by magnetic-activated cell sorting (MACS). The lower figure is the culture conditions of cells with specific populations after cell separation.

conventional EB culture method. As a result, the HF culture method improved the proliferation rate of the mouse ES cells to produce an increased HSC population and achieved an approximately 40-fold higher production volume of HSCs than that obtained by the conventional EB culture method (10).

Therefore, we focused on human iPS cells as a cell source for applications in regenerative medicine and developed a protocol for the large-scale culturing of differentiated human iPS cells using the HF culture method. This study utilized an original two-step culture process that shifts from undifferentiated culture to spontaneous differentiated culture in order to prepare a large number of cells. After HF culture, we isolated and evaluated cell populations labeled with CXCR4 for the purpose of selecting specific cell populations.

MATERIALS AND METHODS

Human iPS cell culture The human iPS cell line HPS0063, which was established by transducing human fibroblasts with lentiviruses carrying octamer-binding transcription factor 3/4 (*OCT3/4*), SRY-box 2 (*SOX2*), Kruppel-like factor 4 (*KLF4*), and *c-MYC*, was obtained from the Riken Bioresource Centre (Ibaraki, Japan). The iPS cells were seeded and grown on culture dishes treated with Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher Scientific, Waltham, MA, USA), at 37°C in a humidified atmosphere of 5% CO_2. Essential 8 medium (Thermo Fisher Scientific) was added to the culture system and the medium was exchanged every day. The cells were subcultured every 2 or 3 days to maintain their undifferentiated state.

HF culture of human iPS cells A bundle of six HFs (length: 3 cm) composed of polyethylene treated with ethylene vinyl alcohol for plasma separation (inner diameter: 330 μm; membrane thickness: 50 μm; pore size: 0.3 μm; Asahi Kasei Medical Co., Ltd., Tokyo, Japan) was used in this study (Fig. 1A). The volume inside the HFs, which was the effective culture volume, was 0.0154 cm^3. Fig. 1B shows the schematic diagram of the HF culture. The subcultured human iPS cells were recovered by Accutase (Merck Millipore, Burlington, MA, USA) treatment and resuspended at a density of 1×10^6 cells/mL in Essential 8 medium. The cell suspension (1 mL) was injected into the lumen of the HFs using a syringe. Then, the bundle was centrifuged at 200 ×*g* for 180 s to induce aggregate formation. The bundle was then sealed. Two bundles were transferred to a 60-mm cell culture dish loaded with 6 mL of culture medium. In order to expand the bundles containing human iPS cells, they were cultured on a rotary shaker at 45 rpm and 37°C in a humidified atmosphere of 5% CO_2. The culture medium was exchanged every day. To expand the iPS cells, they were cultured for 7 days in Essential 8 medium as the condition for maintaining their undifferentiated state. Thereafter,

the culture medium was switched to Essential 6 medium (Thermo Fisher Scientific) for 10 days to induce spontaneous differentiation of the iPS cells.

Cell number counting The HF bundles were cut into small pieces and the nuclei were eluted by homogenizing the pieces in citric acid solution, using a Polytron homogenizer (Kinematica AG, Luzern, Switzerland). A stabilizing solution (ChemoMetec, Allerod, Denmark) was then added at a volume equal to that of the citric acid solution, to neutralize the sample. Finally, the nuclei were counted using a Nucleo Counter (ChemoMetec).

Flow cytometric analysis After differentiation, cells were collected and dissociated by enzymatic treatment. A total of 1×10^6 dissociated cells were washed with phosphate-buffered saline (PBS) and resuspended in 100 μL of a buffer containing PBS (pH 7.2), 0.5% bovine serum albumin (BSA), and 2 mM ethylenediaminetetraacetic acid (EDTA). The cells were then incubated with 10 μL of phycoerythrin (PE)-conjugated human CD184 (CXCR4) antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) in the dark at 2–8°C for 10 min. A set of cells was treated in the same way with PE-conjugated mouse IgG2a isotype antibody (Miltenyi Biotec) as a negative control. After the incubation, the cells were washed two to three times with the buffer. The cells were then resuspended in the buffer and analyzed using a Guava PCA flow cytometer (Guava Technologies, Inc., Hayward, CA, USA).

Cell harvest and separation After expansion and differentiation in the HF culture, cells were harvested from the HFs; we then separated and further cultured the specific population. The schematic diagram of the protocol is shown in Fig. 1C. A total of 1×10^7 dissociated cells were washed with PBS and resuspended in 100 μL of a degassed magnetic-activated cell sorting (MACS) buffer containing PBS (pH 7.2), 0.5% BSA, and 2 mM EDTA. The cells were then incubated with 10 μL of PE-conjugated human CD184 (CXCR4) antibodies (Miltenyi Biotec) in the dark at 2–8°C for 10 min. After the incubation, the cells were washed two to three times with MACS buffer. The cells were then resuspended in 80 μL of MACS buffer and incubated with 20 μL of Anti-PE Microbeads (Miltenyi Biotec) in the dark at 2–8°C for 15 min. After the incubation, the cells were washed two times with 1–2 mL of MACS buffer and then resuspended in 500 μL of the same buffer. To separate the cells, we used a MACS separator (Miltenyi Biotec) with a LS column (Miltenyi Biotec) that was prepared by rinsing with 3 mL of MACS buffer. The cell suspension was applied onto the column and unlabeled cells were collected by washing the column with 3 mL of MACS buffer. This washing step was performed three times. The column was then removed from the MACS separator and placed on a suitable collection tube. Immediately thereafter, 5 mL of MACS buffer was added to the column and the magnetically labeled (CXCR4-positive) cells were collected by firmly pushing the plunger into the column.

Monolayer culture of the separated cells After the cell separation process, two cell populations (viz., the separated CXCR4-positive population and the total cell population) were subjected to monolayer culture for further differentiation into the hepatic lineage. Each cell population was seeded on a Growth Factor-Reduced Matrigel (Corning Inc., Corning, NY, USA)-coated dish at 7×10^4 cells/cm^2. During the first 7 days, KnockOut Dulbecco's modified Eagle's medium (Invitrogen, Thermo Fisher Scientific) supplemented with 2% KnockOut Serum Replacement (Thermo Fisher Scientific), 1 mM nonessential amino acids (Merck Millipore), 2 mM L-glutamine (Merck Millipore), 10 ng/mL recombinant human hepatocyte growth factor (PeproTech, Rocky Hill, NJ, USA), and 10 ng/mL recombinant human fibroblast growth factor 4 (PeproTech) was used for hepatic differentiation. After that, during the next 7 days, the culture medium was exchanged every day with the above-described medium components in addition to 10^{-7} M dexamethasone (Sigma–Aldrich, St. Louis, MO, USA) and 10 ng/mL recombinant human oncostatin M (R&D Systems, Minneapolis, MN, USA). All cultures were carried out at 37°C in a humidified atmosphere of 5% CO_2 (Fig. 1C).

Quantitative real-time reverse-transcription polymerase chain reaction analysis Total RNA from the differentiating cells was prepared using NucleoSpin RNA II (Nippon Genetics, Bunkyo, Japan). Then, cDNA synthesis was performed using the High-Capacity cDNA Achieve Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. We confirmed the expression levels of the selected genes using the quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR), which was performed using the Applied Biosystems 7300 Real-Time PCR System and the following primer sets: CXCR4 (Applied Biosystems; assay ID: Hs00607978_s1), SOX17 (Applied Biosystems; assay ID: Hs00751752_s1), hepatocyte nuclear factor 4alpha (HNF4α) (Applied Biosystems; assay ID: Hs00230853_m1), albumin (ALB) (Applied Biosystems; assay ID: Hs00609411_m1), tryptophan 2,3-dioxygenase (TDO2) (Applied Biosystems; assay ID: Hs00194611_m1), and carbamoyl phosphate synthetase 1 (CPS-1) (Applied Biosystems; assay ID: Hs00157048_m1), cytochrome P450 family 3 subfamily A member 4 (CYP3A4) (Applied Biosystems; assay ID: Hs00604506_m1). The amplification protocol was as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and finally 60°C for 1 min. Glyceraldehyde 3-phosphate dehydrogenase expression was also determined using specific primers (Applied Biosystems; assay ID: Hs02786624_g1) and was used as the endogenous control.

Statistical analysis Results are presented as the mean ± standard deviation. Statistical analysis was performed using the two-tailed unpaired or paired Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Changes in human iPS cell density using the HF culture method Fig. 2A shows the changes in density of the human iPS cells in HF culture. After 7 days under the conditions for culturing undifferentiated cells, the cell density reached 6.3×10^8 cells/cm^3-lumen volume, which was about 10 times the initial seeding density. Then, under the spontaneous differentiation culture condition, slow cell growth was confirmed throughout 10 days. At the end of the entire culture period, we had achieved a high cell density of 1.4×10^9 cells/cm^3-lumen volume, which was about 23 times the initial seeding density.

Fig. 2B shows the comparison of morphology inside the HF before and after cell culture. On day 7 of culture, the inner space of HFs was almost entirely filled with cells. Fig. 2C shows the cell

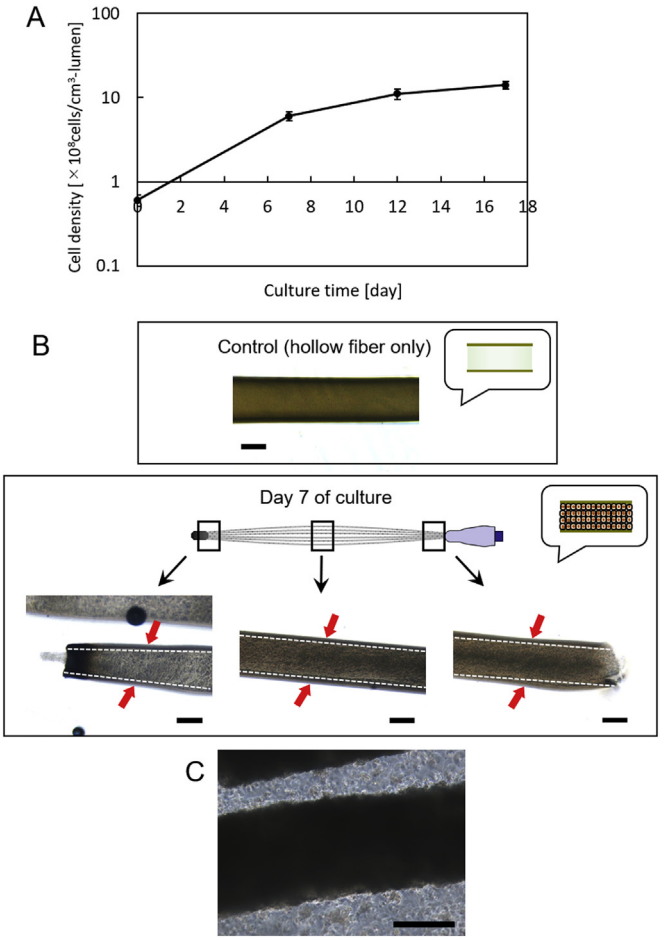

FIG. 2. (A) Changes in the cell density of human induced pluripotent stem (iPS) cells in hollow fiber culture. Data are presented as the means ± standard deviation. n = 4. In the first 7 days, human iPS cells were cultured under conditions that maintained their undifferentiated state. Thereafter, the culture conditions were switched to allow the iPS cells to differentiate spontaneously for 10 days. (B) Morphological changes inside a hollow fiber. Upper photographs are the state without cells inside the hollow fiber as control. Lower photographs are cell morphology inside a hollow fiber on day 7 of culture under conditions that maintained their undifferentiated state. Red arrows indicate hollow fiber membranes. Cell aggregates are present inside white dashed lines (inside the hollow fiber membrane). (C) The morphology of a cell population removed from a hollow fiber. Scale bar: 200 μm.

morphology after harvest by aspirating cells from an HF. The cells formed large cylindrical aggregates inside the HFs and maintained their shape even after harvest. These results indicated that the human iPS cells proliferated and formed an aggregate in HF culture.

Expression of differentiation-related genes in iPS cells in the HF culture To examine the gene expression of human iPS cells inside the HFs during culture, we extracted total RNA from the spontaneously differentiating cells. The cDNA was reverse transcribed from the total RNA, and the expression of several differentiation-related marker genes was examined by qRT-PCR using several commercially available human-specific DNA primers. Fig. 3 shows the changes in the gene expression levels of the differentiation-related markers over the spontaneous differentiation culture period. The expression levels of all differentiated markers are shown as relative expression intensity compared to that of the initial day of differentiation culture (day 7).

The expression levels of *CXCR4* and *SOX17* (markers of endodermal differentiation) were upregulated. In particular, the gene expression level of *CXCR4* significantly increased with increase in culture time. The expression level of *HNF4α* (a hepatocyte nuclear factor) was also upregulated. Although the expression levels of the hepatocyte-specific markers *ALB*, *TDO2*, and *CPS-1* were likewise upregulated, they were lower than those of the other differentiation markers. The gene expression level of *TDO2* was significantly increased at day 17.

Proportion of CXCR4-expressing iPS cells in the HF culture In order to quantitatively evaluate the proportion of endodermal cells inside the HFs during the spontaneous differentiation culture process, flow cytometric analysis was performed using the endodermal marker CXCR4 as a label. As indicated in Fig. 4A, the proportion of CXCR4-expressing cells was about 30% at day 12 under the spontaneous differentiation culture condition, and decreased to about 12% at day 17.

Additionally, Fig. 4B compares the proportion of CXCR4-expressing cells in the HF culture with that in the monolayer culture. The data indicated that the maximum level of CXCR4 expression in the HF culture, which was approximately 30%, was 2-fold that in the traditional monolayer at day 12 under the spontaneous differentiation culture condition, and the difference was significant ($P < 0.05$).

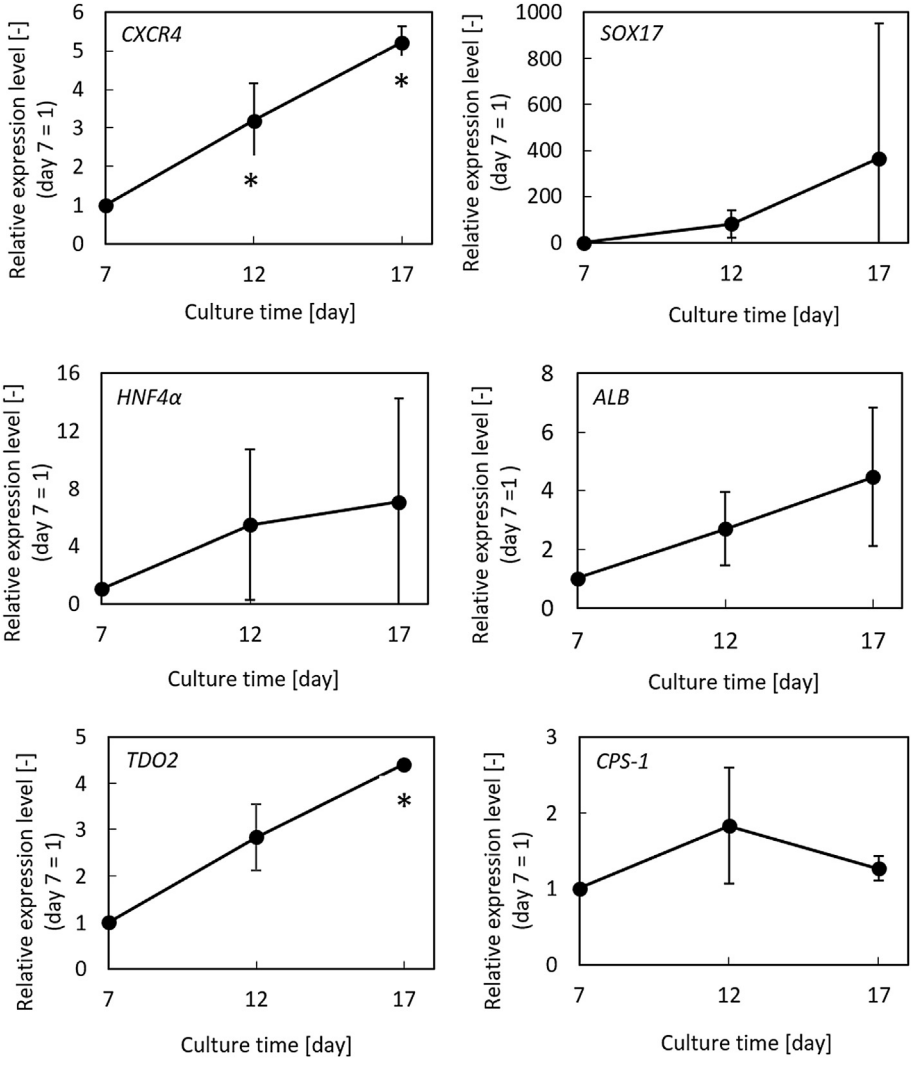

FIG. 3. Real-time reverse-transcription polymerase chain reaction analysis of the mRNA expression of endodermal markers (CXCR4, SOX17), hepatocyte nuclear factor 4alpha (HNF4α), albumin (ALB), tryptophan 2,3-dioxygenase (TDO2), and carbamoyl phosphate synthetase 1 (CPS-1) by spontaneously-differentiated human induced pluripotent stem cells in hollow fiber culture. The expression levels of all differentiated markers were shown as relative expression intensity compared with that of the initial day of differentiation culture (day 7). Data are presented as the means ± standard deviation. n = 4. CXCR4, C-X-C motif chemokine receptor 4; SOX17, SRY-box 17. Asterisk denotes a significant difference between day 7 and day 12 or day 17.

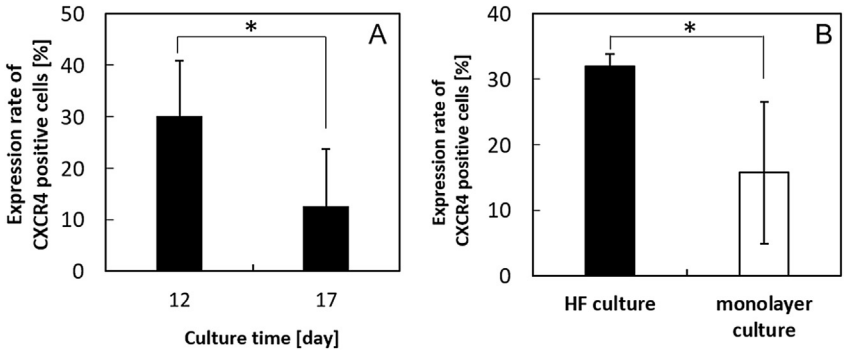

FIG. 4. Flow cytometric analysis of the proportion of CXCR4-positive cells, using the lineage-specific cell-surface endodermal marker CXCR4. Data are presented as the means ± standard deviation. (A) Proportion of CXCR4-positive cells in the hollow fiber (HF) culture under spontaneous differentiation conditions. Asterisk denotes significant difference in each culture day under spontaneous differentiation conditions ($P < 0.05$). n = 4. (B) Comparison of the proportions of CXCR4-positive cells between the HF culture and the traditional monolayer culture at day 12 under spontaneous differentiation conditions. Asterisk denotes a significant difference between the HF culture and the traditional monolayer culture ($P < 0.05$). n = 6.

Evaluation of differentiation-related gene expression after cell separation In order to examine whether the spontaneously differentiated cells in the HF culture have the ability to further differentiate into mature cells, we separated the cells into two populations and respectively cultured them on monolayers. Cell separation was performed with CXCR4 as a label using the MACS separator system, which was able to separate the cells into a CXCR4-positive cell population and a total cell population. CXCR4 is an endodermal marker that differentiates the digestive organs, such as the liver and intestine. In this study, we focused on the liver and differentiated the two cell populations into hepatocytes.

We then performed qRT-PCR on the two cell populations using human-specific DNA primers. Fig. 5 shows the changes in the expression levels of several differentiation-related marker genes. The expression levels of all differentiated markers are shown as relative expression intensity compared to undifferentiated (day 0) human iPS cells in hollow fiber culture. The CXCR4-positive cell population showed a higher relative expression of the gene compared to the total cell population although there is no statistically different. The gene expression level of *CXCR4* in the total cell population did not change much throughout the culture period, whereas it decreased in the CXCR4-positive cell population. The gene expression level of *HNF4α* decreased temporarily at day 7, but increased at day 14 in both cell populations. The gene expression level of *ALB* typically increased in the CXCR4-positive cell population throughout all 14 days of culture. Similarly, the gene expression level of *CYP3A4* also tended to increase in the CXCR4-positive cell population. The gene expression level of *TDO2* in both cell populations showed increasing tendencies for the first 7 days and was then maintained thereafter. The gene expression level of *CPS-1* in both cell populations showed a decreasing trend throughout the culture period. However, there was no significant difference between the CXCR4-positive cell population and the total cell population in the expression levels of all differentiated markers.

DISCUSSION

Changes in cell density using the HF culture method Human iPS cells inside the HFs proliferated well throughout the entire culture period. In particular, the density of the undifferentiated cells reached 6.3×10^8 cells/cm^3-lumen volume after 7 days of culture, which was about 10 times the initial seeding density. We confirmed that three-dimensional structure similar to EB is formed in the HFs by utilizing the proliferation property of iPS cells in this study (Fig. 2B). Another group of researchers reported the successful expansion of human iPS cells in a three-dimensional culture using a stirred tank. They reported that the iPS cells proliferated to about 5-fold after 4 days in an undifferentiated state (6). A different group reported that undifferentiated maintenance culture of hiPS cells using 3D sphere culture could promote hiPS cell proliferation approximately 10 times for up to 5 days (11). Also, yet another group reported that undifferentiated cultures of hiPS cells using their original suspension culture showed about 5-fold proliferation in 4 days (12). Therefore, it was suggested that cells cultured with the HF culture method could proliferate at the same level as those in other three-dimensional culture methods. The culture methods for forming cell aggregates, such as suspension culture, cannot control the size of the aggregates and may cause cell death due to the depletion of oxygen or nutrients inside the aggregates. It was reported that the oxygen concentration in the center of large EBs (400 μm radius) was 50% lower than that in smaller EBs (200 μm radius) (13). A similar trend was observed for the cytokine concentrations (14). In our present study, it was possible not only to control the size of the aggregates but also to allow them to grow until the interior of the HF was completely filled, without depletion of oxygen or nutrients, because the cells proliferated inside HFs having a diameter of 330 μm. Therefore, the HF culture has the advantage of achieving a high cell density over a longer culture period to generate cells on a large scale.

Expression of differentiation-related genes in iPS cells in the HF culture The gene expression of all differentiation-related markers was upregulated ever since the start of the spontaneous differentiation culture condition. In addition, as a preliminary experiment, we confirmed up-regulation of *MSX1*, a mesodermal gene, as well as *PAX6* and *Otx2*, which are ectodermal genes, similar to upregulation of endodermal genes under spontaneous differentiation culture conditions (Fig. S1). Therefore, this suggested that cells inside the HFs had differentiated spontaneously. On the other hand, we also evaluated the undifferentiated state of cells for the first 7 days of culture. The expression of the undifferentiated marker genes *Oct-3/4* and *Nanog* were examined by qRT-PCR. During the expansion for the first 7 days, expression levels of *Oct-3/4* and *Nanog* were almost maintained at initial levels (Fig. S2). After 7 days of culture, we evaluated the expression of cell surface marker SSEA-4. The expression of SSEA-4 was 97.2 ± 1.8% (Fig. S3). These results indicated that the iPS cells maintained their undifferentiated state during the first 7 days of culture.

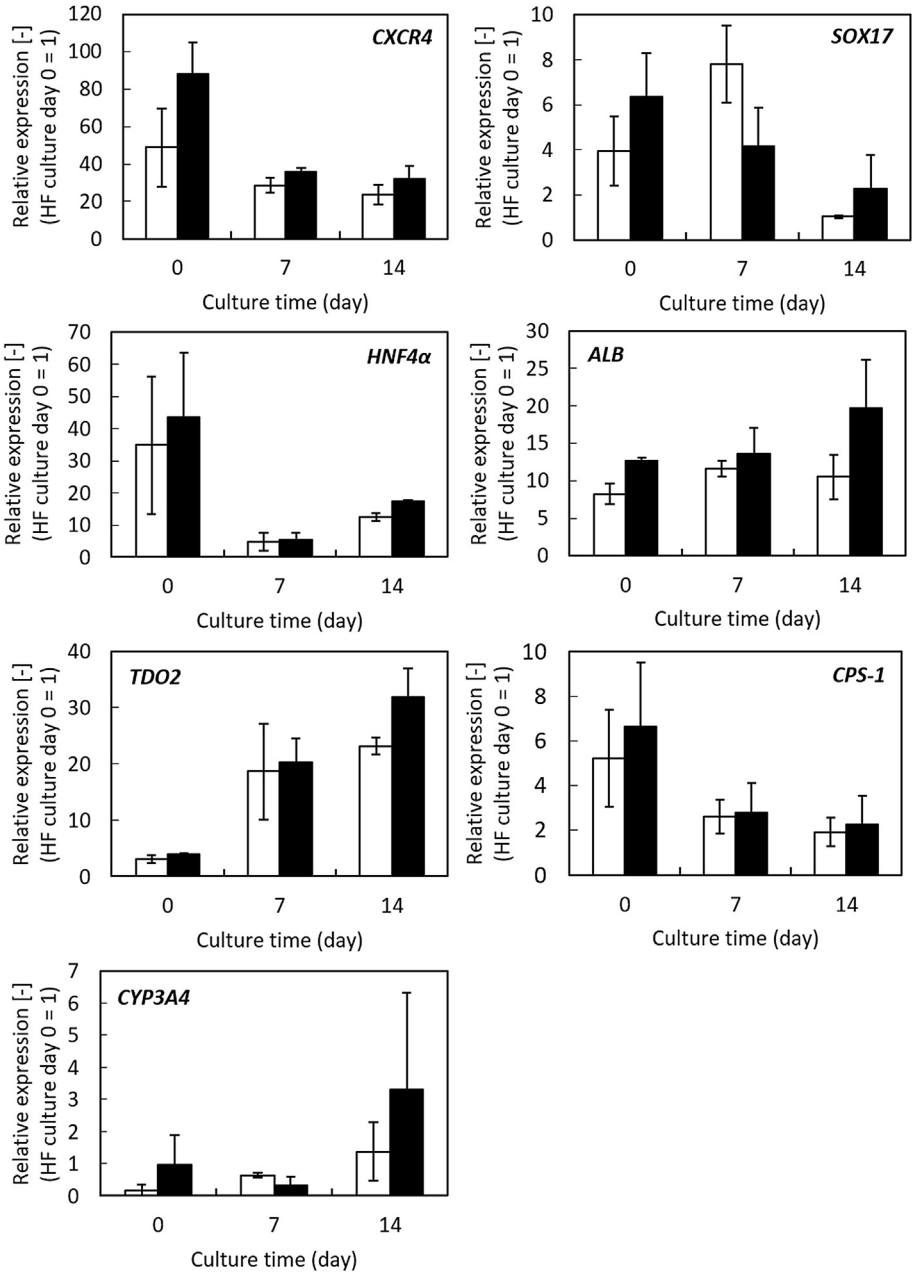

FIG. 5. Real-time reverse-transcription polymerase chain reaction analysis of the mRNA expression of endodermal markers (CXCR4, SOX17), hepatocyte nuclear factor 4alpha (HNF4α), albumin (ALB), tryptophan 2,3-dioxygenase (TDO2), carbamoyl phosphate synthetase 1 (CPS-1), and cytochrome P450 family 3 subfamily A member 4 (CYP3A4) in the total cell population and CXCR4-positive cell population (separated by magnetic-activated cell sorting with CXCR4 as a label) after their hepatic differentiation on monolayer culture. The expression levels of all differentiated markers are shown as relative expression intensity compared with undifferentiated (day 0) human iPS cells in hollow fiber culture. Data are presented as the means ± standard deviation. n = 2. Open bars, total cell population; closed bars, CXCR4-positive cell population.

In a previous study, we reported that HF cultures of primary rat hepatocytes maintained hepatic function for a long period of about 5 months (8). Moreover, in another previous study, we reported that HF culture could differentiate mES cells into hepatocytes more efficiently than monolayer culture and maintained hepatic function for about 1 month (9). Therefore, according to the results of this study, it is suggested that spontaneous differentiation is promoted in HF culture by changing the culture conditions, and the HF culture method induces differentiation more efficiently than the traditional monolayer culture method.

Proportion of CXCR4-expressing iPS cells in the HF culture In order to quantitatively evaluate the proportion of endodermal cells inside the HFs during the spontaneous differentiation culture process, we performed flow cytometric analysis using CXCR4 as a label. The proportion of CXCR4-positive cells was about 30% at day 5 under the spontaneous differentiation culture condition (day 12 including the period for culturing undifferentiated cells), and it decreased thereafter. On the other hand, the proportion of CXCR4-positive cells at day 5 in the traditional monolayer culture under the same spontaneous differentiation condition was 15%. In our previous study on the differentiation of mouse ES cells into hepatocytes, the gene expression level of hepatocyte markers was higher in the HF culture than in the monolayer culture (9). This is consistent with a previous report indicating that the formation of cellular aggregates, such as embryoid bodies, promotes cell differentiation (15).

On the other hand, it has been reported that hiPS cells maintain undifferentiation by appropriate interaction with cell–cell contacts via E-cadherin and cell–substrate interactions via integrin (16). In addition, it has been reported that aggregates are formed as cell–substrate interactions decrease, and thus are destined to differentiate into early endoderm (17). In this study we have not reported the relationship between the properties of the HF membranes and hiPS cells, but there is a possibility of influencing cell aggregate formation ability, proliferation and differentiation depending on the material of the HF membranes. In future studies, it is necessary to optimize HFs to select an appropriate HF membrane according to the purpose.

Evaluation of differentiation-related gene expression after cell separation It was shown that the cell separation using *CXCR4* had been performed successfully, because the gene expression level of *CXCR4* was highest in the CXCR4-positive cell population at day 0. However, the expression of the other differentiation marker genes did not change significantly during the 14 days of differentiation culture. In this study, a magnetic cell separation method was used for cell isolation, but not all cells labeled with CXCR4 were attached to magnetic beads in this method. Actually, the ratio of CXCR4 expression of the CXCR4-positive cell population after cell separation by flow cytometry was around 75% (data not shown). Therefore, even though it was shown that the cell separation using CXCR4 had been performed successfully, the total cell population and CXCR4-positive cell population were not necessarily 100% separated. It is necessary to devise measures to increase the separation efficiency including the use of FACS in future studies.

Moreover, in general hepatic differentiation, a definitive endoderm differentiation-inducing factor, such as activin A, is almost always added for the initial differentiation culture period (18,19). In our study, however, such factor was not added for HF culture before cell separation, and culture conditions for spontaneous differentiation were instead applied. However, it could be considered necessary to definitively fate cells from the endodermal stage into hepatocytes in the early differentiation stage, because there was no difference in gene expression between the total cell population and the CXCR4-positive cell population. For that, it may be necessary to add a definitive endoderm differentiation-inducing factor. From this result, it is expected that the gene expression levels of the other mature hepatic markers will be increased by the addition of factors such as Activin A under spontaneous differentiation conditions in the HF culture, and differences may then be observed between the total cell and CXCR4-positive cell populations. Furthermore, it is expected that the proportion of CXCR4-positive cells will be higher than 30% and the efficiency of hepatic differentiation consequently increased.

In conclusion, we found that the HF culture method could achieve the high-cell-density culture of human iPS cells. We also found that the maximum proportion of endodermal cells in the HF culture was 2-fold that in the traditional monolayer culture. These results indicate that the HF culture method is more efficient for the differentiation of human iPS cells. The endodermal cells, which were sorted from the differentiating population, were further differentiated into hepatic lineages in monolayer culture. The cells showed signs of initial maturation into the hepatic lineage. Although we need to investigate the further maturation of these cells in an optimal condition, we were successful in obtaining a population that had potential to differentiate into a specific lineage using the efficient HF culture method. In conclusion, the HF culture method has potential for the large-scale preparation and differentiation of human iPS cells.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.03.014.

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