Evaluation of hollow fiber culture for large-scale production of mouse embryonic stem cell-derived hematopoietic stem cells

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Abstract Hematopoietic stem cells (HSCs) have the ability to differentiate into all types of blood cells and can be transplanted to treat blood disorders. However, it is difficult to obtain HSCs in large quantities because of the shortage of donors. Recent efforts have focused on acquiring HSCs by differentiation of pluripotent stem cells. As a conventional differentiation method of pluripotent stem cells, the formation of embryoid bodies (EBs) is often employed. However, the size of EBs is limited by depletion of oxygen and nutrients, which prevents them from being efficient for the production of HSCs. In this study, we developed a large-scale hematopoietic differentiation approach for mouse embryonic stem (ES) cells by applying a hollow fiber (HF)/organoid culture method. Cylindrical organoids, which had the potential for further spontaneous differentiation, were established inside of hollow fibers. Using this method, we improved the proliferation rate of mouse ES cells to produce an increased HSC population and achieved around a 40-fold higher production volume of HSCs in HF culture than in conventional EB culture. Therefore, the

HF/organoid culture method may be a new mass culture method to acquire pluripotent stem cell-derived HSCs.

Keywords Pluripotent stem cell · Hematopoietic differentiation · Three-dimensional culture · Hollow fiber

Introduction

Hematopoietic stem cells (HSCs) are a kind of adult stem cell that can differentiate into all types of blood cells, including lymphocytes and myelocytes (Seita and Weissman 2010; Schulz et al. 2012). HSCs were the first type of tissue stem cells used in human clinical trials. Currently, HSC transplantation is a treatment that facilitates reconstitution of hematogenous functions after myeloablation therapy. This therapy has contributed to the complete cure of blood disorders, including leukemia, multiple myeloma, aplastic anemia, and other disorders, that are difficult to cure by conventional treatments (Edward and Copelan 2006; Jansen et al. 2005). However, in HSC transplantation treatments, the same type of the human leukocyte antigen is required, and the shortage of donors is a serious problem (Choo 2007). In addition, transplanted HSCs have the risk of causing graft-versus-

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host disease. Therefore, establishment of a stable supply of HSCs is necessary.

To develop new methods, recent efforts have focused on differentiating HSCs from pluripotent stem cells. Pluripotent stem cells, such as embryonic stem (ES) cells (Evans and Kaufman 1981; Thomson et al. 1998) and induced pluripotent stem cells (Takahashi and Yamanaka 2006; Takahashi et al. 2007), have an infinite proliferation ability and can differentiate in vitro into all types of somatic cell. These abilities indicate that pluripotent stem cells can be a source of large quantities of HSCs. Many studies have reported a variety of methods for hematopoietic differentiation of pluripotent stem cells. Typical methods are as follows: (1) the formation of embryoid bodies (EBs) (Rungtuntakorn et al. 2009); (2) co-culture with a stromal cell line. (Nakano et al. 1994; Ledran et al. 2008); (3) transfection of several genes (Inoue-Yokoo et al. 2013; Lim et al. 2013). The formation of EBs is one of the simplest methods to differentiate pluripotent stem cells. Several investigations have demonstrated that the size of organoids, such as EBs, is closely related to differentiation efficiency (Messana et al. 2008; Mohr et al. 2010). However, EBs are three-dimensional spherical aggregates, whose size is difficult to control. Uncontrolled growth may result in insufficient supply of oxygen and nutrients to the center of EBs.

We, therefore, developed a culture method in which cells multiply within a restricted space to avoid shortages of oxygen and nutrients. We focused on a three-dimensional culture using hollow fibers (HF) in which cultured cells form cylindrical multicellular aggregates (Mizumoto et al. 2008). Using this method, cells immobilized inside HF by centrifugal force form organoids and achieve a high density of organoids in the culture space. In this study, to establish a mass culture method, we differentiated mouse ES cells into HSCs using the HF culture method.

Materials and methods

Mouse ES cells and cell culture

To maintain their undifferentiated state, mouse ES cells (129 Line; Chemicon, Pittsburgh, PA, USA) were cultured on a layer of mitomycin-C treated mouse embryonic fibroblasts (MEFs, isolated from

12.5 dpc embryos of ICR mice; kyudo, Co., Ltd, Saga, Japan) in a gelatinized culture dish which is coated with 0.1% gelatin solution (dissolving gelatin in deionized water; Sigma) at 37 °C in a humidified atmosphere of 5% CO₂. Knockout™ Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), nonessential amino acids (Chemicon), 2 mM L-glutamine (Chemicon), nucleosides solution (Chemicon), 100 U/mL penicillin, 100 mg/mL streptomycin, 110 mM 2-mercaptoethanol (Chemicon), 15 mM HEPES, and 1000 U/mL recombinant mouse leukemia inhibitory factor (ESGRO; Merck Millipore, Darmstadt, Germany) were added to the culture system and medium was exchanged every day. Cells were subcultured every other day to maintain their undifferentiated state.

Differentiation of mouse ES cells into HSCs

A bundle of six HF composed of cellulose triacetate for plasma separation (inner diameter: 285 µm; outer diameter: 387 µm; pore size: 0.2 µm; Toyobo Co., Ltd., Osaka, Japan) was used in this study. Subcultured mouse ES cells were recovered by trypsin treatment and resuspended at a density of 2×10^5 cells/mL in Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Sigma), 300 mM monothioglycerol (Sigma), 100 U/mL penicillin (WAKO Pure Chemical Industries, Ltd., Osaka, Japan), 100 mg/mL streptomycin (Meiji Seika Kaisha, Tokyo, Japan), and 20% FBS. A cell suspension (1 mL) was injected into the lumen of the HF using a syringe. Then, the bundle was centrifuged at $200 \times g$ for 180 s to induce organoid formation. The bundle was cut to a length of 3 cm and sealed, and then transferred to a 60-mm cell culture dish loaded with 6 mL culture medium. The bundles containing mouse ES cells were cultured on a rotary shaker at 45 rpm at 37 °C in a humidified atmosphere of 5% CO₂. Culture medium was exchanged every day.

For EB formation, subcultured mouse ES cells were resuspended at a density of 5×10^5 cells/mL in Iscove's modified Dulbecco's medium and transferred to a low attachment 96-well U-bottom plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at 200 µL per well. After culture for 2 days, culture medium was half-exchanged every day.

Morphological analysis

After culture, the HF bundle was fixed in 10% formalin neutral buffer solution and then soaked in glycerol. The glycerol penetrated the HF membrane within a few minutes, and the HF membrane became transparent. Cells inside of the HF bundle were observed using a phase-contrast microscope.

In EB culture, morphological changes were recorded every day under the same phase-contrast microscope. For each EB, diameters were measured with imaging measurement software, Micro analyzer (Nippon Poladigital Co. Ltd., Tokyo, Japan).

Cell number enumeration

HF bundles were cut into small pieces and nuclei were eluted by homogenizing the HF pieces using a polytron homogenizer (Kinematica AG, Littau/Luzern, Switzerland) in citric acid solution. Stabilizing solution (ChemoMetec, Allerød, Denmark) was then added at an equal volume to the citric acid solution to neutralize the sample.

Collected EBs were submerged in a lysis buffer (ChemoMetec) and nuclei were further eluted by pipetting. An equal volume of stabilizing solution was added to neutralize the buffer. Then, nuclei were counted using a Nucleo Counter (ChemoMetec).

Flow cytometric analysis

After differentiation, organoids were dissociated by enzymatic treatment. A total of 2×10^5 dissociated cells were washed with phosphate-buffered saline (PBS) containing 5% mouse serum (Cedarlane, Ontario, Canada) and resuspended in 10 μ L of the solution described above. Cells were then incubated with PE-labeled anti-mouse Sca-1 (MBL, Nagoya, Japan) and PE-Cy5-labeled anti-mouse CD117 (Cedarlane) monoclonal antibodies in the dark at room temperature for 30 min. After incubation, cells were washed with PBS containing 5% mouse serum (Cedarlane) three times. Cells were resuspended in PBS containing 10% BSA Diluent/Blocking Solution (KPL: 50-61-01, Milford, MA, USA) and analyzed using a Guava PCA cytometer (Guava Technologies, Inc., Hayward, CA, USA).

Statistical analysis

To test the difference between HF and EB culture methods, we performed Welch's *t* test using software R version 3.1.2 (R Core Team 2014). $P < 0.05$ was considered statistically significant.

Results

Changes in morphology using EB and HF methods

Figure 1a shows the morphology of EBs in each culture period. The cultured cells formed an EB in each well by the first day. The diameter of EBs was about 1000 μ m, which remained nearly constant during the culture period. However, the shape of the EB collapsed gradually after 7 days of culture. These results indicated that about 1000 μ m was the maximum allowable size for EBs to grow because of the depletion of oxygen and nutrients in the core of EBs.

Figure 1b shows the morphology of cells cultured inside HF bundles. After seeding, cell aggregates were formed at the tip of the bundle by centrifugal force. The aggregates grew continuously into a cylindrical form along the shape of the HF lumen throughout the culture period. It appeared that the length of the HF bundle restricted the organoid size, indicating that the length of the tissue organoid can be changed by the inner diameter and length of the HF.

Changes in cell numbers using EB and HF culture methods

Figure 2 shows a comparison of the cell numbers in EB and HF cultures. In EB culture, the cell number was almost the same throughout the culture period. Conversely, in HF culture, the cell number increased with the culture time, and an approximate 80-fold increase was observed after 14 days of culture.

Expression of HSC markers c-kit and Sca-1

Figure 3 shows the hematopoietic population in each culture system. The hematopoietic population was determined by double-staining of cell surface antigens c-kit and Sca-1. A similar tendency for HSC differentiation was observed in both culture systems. At days 10 and 14, the HSC population in the EB culture

Fig. 1 Organoid morphology in each three-dimensional culture system. **a** Representative images of EBs consisting of mouse ES cells in suspension culture on days 3, 5, 7, 10, and 14. Scale bar = 500 μ m. **b** Representative images of cylindrical organoids inside transparent zed HFs formed by HF/organoid culture on days 0, 5, 7, and 14. Scale bar = 200 μ m. *ES* embryonic stem, *EB* embryoid body, *HF* hollow fiber

was significantly higher than that in the HF culture. However, the largest population of HSCs, which was about 15% of total cells, was observed on day 5 in both culture systems (no significant differences were observed between culture systems).

Based on the cell number and HSC population, we calculated the estimated number of HSCs by multiplying the HSC population by the cell number. Figure 4 shows a comparison of the estimated numbers of HSCs between EB and HF cultures. Because of

Fig. 2 Cell number variation in each three-dimensional culture system. Data are presented as the mean $\pm$ SD. Asterisk (*) indicates significant difference between EB and HF culture systems at the same time point ($P < 0.05$). In each culture system, cell numbers were evaluated on days 3, 5, 7, 10, and 14. Square: EB culture; diamond: HF/organoid culture

Fig. 3 HSC populations in each culture system. The hematopoietic population was determined by double staining for cell surface antigens, c-kit and Sca-1, followed by flow cytometric analysis. In each culture, hematopoietic populations were measured on days 3, 5, 7, 10, and 14. Data are presented as the mean $\pm$ SD. Asterisk (*) indicates significant difference between EB and HF culture systems at the same time point ($P < 0.05$). HSCs, hematopoietic stem cells

the difference in cell proliferative activity, HF culture achieved 40-fold higher production of HSCs compared with traditional EB culture on day 5 (Fig. 4).

Fig. 4 Production of HSCs in each culture system. HSC production was calculated from total cell numbers and HSC populations on days 3, 5, 7 and 10. Data are presented as the mean $\pm$ SD. Asterisk (*) indicates significant difference between EB and HF culture systems at the same time point ($P < 0.05$)

Discussion

The aim of this study was to establish a method for stable and mass production of HSCs from pluripotent stem cells. In recent years, several different strategies have been used for mass production of HSCs, such as proliferation of HSCs in vitro (Zhang et al. 2006; Huynh et al. 2008; Schuster et al. 2012) and direct reprogramming from somatic cells (Batta et al. 2014; Riddell et al. 2014). Nevertheless, mass cultivation technology for HSCs remains imperfect. Therefore, it is necessary to establish a more efficient in vitro process for HSC production.

In this study, we focused on efficient differentiation of pluripotent stem cells into HSCs. Several inspiring results in this field have been reported by other groups (Lim et al. 2013; Chen et al. 2015). Therefore, we focused on acquiring HSCs in large amounts.

To obtain differentiated cells from pluripotent stem cells, two-dimensional culture, also called monolayer culture, has been widely used. However, two-dimensional culture might not be suitable for scaling up to a mass production scale. Therefore, three-dimensional culture of pluripotent stem cells, such as culture of EBs in suspension, is required (Matsuura et al. 2012; Kempf et al. 2016). However, the growth of EBs in

suspension culture is inhibited by the limited diffusion of molecules such as oxygen and nutrients, which is a major challenge for the stable production of differentiated cells in large quantities.

To overcome these limitations, we established an effective new method using HF for large-scale production of pluripotent stem cell-derived HSCs. Our study indicates that HF/organoid culture is far more efficient than conventional EB culture.

Remarkable proliferation of mouse ES cells observed in the HF culture method

In EB culture, EBs were formed on the first day and their diameter did not increase, which was maintained at approximately 1000 μm , despite the gradual collapse of their shape from day 7. Generally, there is a limit on mass transfer during EB culture. Van Winkle et al. (2012) reported that the oxygen concentration in the centers of large EBs (400 μm radius) was 50% lower than that of smaller EBs (200 μm radius). There is a similar trend for cytokine concentrations (Hirschhaeuser et al. 2010). Moreover, waste products, such as lactase, accumulate. These phenomena contribute to cell death inside EBs, which is indicated by structural collapse and detachment of cells from EBs. In addition, collapse of the spherical structure of mouse EBs can be attributed to the possible differentiation of ES cells into various cell types, which occurs inside EBs of different sizes and morphologies (Keller 1995). Despite possible cell death and structural collapse, the total cell number of EBs remains constant, which can be attributed to the balance between cell proliferation and death. This finding indicates that the proliferation potential of mouse ES cells may be restricted by mass transfer throughout the organoid.

Mouse ES cells in HF culture showed a robust proliferation activity with 80-fold expansion throughout the culture period. The possible reason for this more robust proliferation in HF culture compared with EB culture could be related to the difference in the shape of the constructed organoid. A cylindrical organoid with a diameter of around 300 μm , which is restricted by the size of the HF itself, can be obtained via HF culture, instead of an approximate 1000 μm spherical organoid obtained via EB culture. This cylindrical tissue could proliferate from the tip along the HF lumen without depletion of oxygen or

nutrients, possibly because of the improvement of mass transfer, until the lumen of the HF was completely filled. A pause in proliferation was observed from day 5 in the HF culture. A possible reason could be insufficient space for further proliferation due to high cell density, which could be resolved by simply elongating the HF.

Similar production efficiency of HSCs from mouse ES cells observed in the HF culture method

The expression rates of cell surface antigens, c-kit and Sca-1, were examined by flow cytometry in both culture systems. A similar trend was observed in both culture methods. A peak of about 15% was observed on day 5 of culture, followed by decreases as the cultures progressed. There was little difference between the two culture systems, indicating the considerable production efficiency of HF culture.

Keller et al. (1993) reported that EBs mimic the mouse hematopoietic differentiation process. They also found the frequency of hematopoietic precursors increased from day 4 to 5 in EB culture using methylcellulose. Meanwhile, Krassowska et al. (2006) showed that expression of HSC markers in EBs differentiating on collagen gel reached a maximum after 6 days of culture, with expression rates of c-kit and Sca-1, 8.14 ± 6.5 and $4.42 \pm 4.8\%$, respectively. These results indicate that the expression of HSCs by spontaneous differentiation in our study was consistent with that reported in previous studies. Furthermore, the decrease of HSC marker expression after the peak value suggests the further spontaneous differentiation of HSCs inside of the organoid into various kinds of mature blood cells (Keller 1995; Seita and Weissman 2010). These results support organoids inside HFs mimicking hematopoietic differentiation.

We then considered the effect of initial EB size. We aligned the initial cell number of both culture systems and as a result, the initial diameter of organoids was different between the culture systems because of the difficulty in controlling the size of EBs. Therefore, we evaluated the cell growth and differentiation of EBs with an initial size, which was set closer to the inner HF diameter ($251.8 \pm 14.68 \mu\text{m}$; data not shown). We found that these smaller EBs showed high proliferation activities, which were similar to those of the HF culture (50.8-fold; data not shown), whereas the expression of hematopoietic stem cell-specific

markers was very low ($3.2 \pm 2.2\%$, on day 5; data not shown). We found in these preliminary evaluations that HSC differentiation was enhanced with increasing EB diameter. We, therefore, choose similar initial cell numbers instead of similar size of the constructed tissue to control the comparison with the HF culture method.

Furthermore, we calculated the number of HSCs by multiplying the HSC population by the cell number (Fig. 4). HF culture was able to produce a large number of HSCs throughout the entire culture period, especially on day 5, at around 40-fold higher efficiency than EB culture. As a result, the HSCs generated inside HFs corresponded to an approximately 6-fold increase over the initially immobilized mouse ES cells, indicating that HF culture is an effective method for in vitro generation of HSCs.

In this study, a major objective was to acquire a large amount of HSCs from pluripotent stem cells. To this end, the HF/organoid culture method is suitable. In addition, studies have reported that the differentiation fate of pluripotent stem cells is influenced by the size of organoids (Bauwens et al. 2008; Miyamoto and Nakazawa 2016). To control the size of organoids, an advantage of the HF culture method is formation of tissue bodies in the form of HFs, which is extremely useful. For clinical use, approximately 2.5×10^6 HSCs/kg of recipient weight are required for peripheral blood transplantation (Hatzimichael and Tuthill 2010). To achieve this number of cells based on the HSC marker expression rate in this study, a cellular organoid of about 2 cm^3 volume is required, which, for a 50 kg recipient, is about 150 times the volume of the HF bundle used in this study. As a simple scale-up method, it is conceivable to increase the number and length of the HFs fixed as bundles. Also, our technique can be applied to a conventional HF bioreactor. However, to optimize the HF culture conditions, it will be necessary to evaluate the effect of HF diameter on cell proliferation and differentiation efficiency. Moreover, in our study, spontaneous differentiation was assessed; therefore, further differentiation efficiency may be possible by the addition of suitable cytokines.

Conclusion

We obtained the following results from our experiments. (1) HF/organoid culture achieves robust cell

proliferation (approximately 80-fold higher expansion) compared with conventional EB culture. (2) The production of HSCs depends on cell proliferation activity, and the HF culture method achieved a 40-fold higher production volume of HSCs compared with the EB culture method.

Based on the above results, this study indicates that our HF/organoid method can be developed as a new large-scale culture method to acquire pluripotent stem cell-derived HSCs. However, some key issues remain to improve differentiation methods of pluripotent stem cells, such as in vitro functional studies and molecular studies to assess the effect of growth factors. In future studies, we will try to improve the differentiation conditions to produce functional HSCs, and the findings in mice will be adapted to human pluripotent stem cells for clinical use.

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