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Chapter 3

Application of Microfluidics in Stem Cell Culture

Shinji Sugiura, Kohji Nakazawa, Toshiyuki Kanamori and Kiyoshi Ohnuma

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Abstract

In this chapter, we review the recent developments, including our studies on the microfabricated devices applicable to stem cell culture. We will focus on the application of pluripotent stem cells including embryonic stem cells and induced pluripotent stem cells. In the first section, we provide a background on microfluidic devices, including their fabrication technology, characteristics, and the advantages of their application in stem cell culture. The second section outlines the use of micropatterning technology in stem cell culture. The use of microwell array technology in stem cell culture is explored in the third section. In the fourth section, we discuss the use of the microfluidic perfusion culture system for stem cell culture, and the last section is a summary of the current state of the art and perspectives of microfluidic technologies in stem cell culture.

Keywords: Embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, microfluidic perfusion culture, micropatterning, microwell array

1. Introduction

This section provides a general background on microfluidic devices and explains the general microfabrication technologies applicable to stem cell culture including embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. We will discuss the importance of small-scale patterning, three-dimensional structure, and medium flow in terms of microenvironment control and the importance of small volumes, in terms of research cost, for industrial application.

1.1. Microfabrication technology available for stem cell culture

Microfabrication technology progressed rapidly with the development of semiconductor industry in the 20th century. By the end of the 20th century, the application of microfabrication

technology started to grow in different research areas including biotechnology. In biotechnology, microfabrication technology was initially used for molecular analyses of DNA and proteins and gradually its application diversified to cell culture. This technology enabled precise fabrication of structures with sizes as small as submicrometer, replication of the fabricated structure, liquid manipulation in very small volumes, portability of the devices, and usage of small amounts of expensive reagents. Owing to these advantages, microfabrication technology is expected to create new applications in the cell culture including stem cells.

Many types of materials, including inorganic materials, metals, polymers, and plastics, are applicable to microfabrication. Silicon and glass have been used to fabricate microstructures and semiconductor devices [1, 2]. Polydimethylsiloxane (PDMS), a silicone elastomer, is the most popular material used for the fabrication of microfluidic cell culture devices due to the ease of fabrication, optical transparency, gas permeability, low chemical reactivity, and inexpensiveness. In addition, microstructure of PDMS is generally fabricated by soft lithography in a few days [3]. This easy and quick process broadens the use of microfluidic devices in cell culture applications. In soft lithography process, replica of microstructure in PDMS can be repeatedly fabricated from a microstructure of photoresist that is originally made using photolithography [4, 5]. A multilayered microstructure of PDMS can also be fabricated by using multilayered photoresist pattern [6, 7]. Details of the fabrication method used for soft lithography have been described in previous studies [3, 8]. Many biologists are currently using this convenient microfabrication technology.

Soft lithography is a convenient method for fabricating microstructures on a laboratory scale. Scientists can fabricate dozens of microfluidic devices by themselves for their research. However, for industrial applications, hundreds or thousands of microfluidic devices are required. In addition, it is known that PDMS absorb small hydrophobic molecules [9]. Therefore, other materials applicable to mass production and capable of avoiding molecular adsorption are desired for the industrial application of microfluidic devices. Low-cost fabrication technologies such as injection molding [10, 11] and rapid prototyping [12, 13] are promising fabrication technologies that can address the above-mentioned issues.

In addition, cell culture often requires extracellular matrixes (ECMs). Therefore, microfabrication of biomaterials, such as hydrogel, is of interest to biologists and engineers. Photofabricated hydrogels have been studied extensively to create microstructure in the hydrogels [14]. These microfabricated hydrogels have been used for tissue engineering.

1.2. Characteristics of microfabricated cell culture device

Significant features that affect a microfluidic device are flow viscosity, interfacial tension, laminar flow, fast diffusion, etc. [15]. The characteristic flow profile enabled the formation of special microenvironment including chemical [16] and temperature gradients [17]. Also, a microfluidic perfusion culture continuously supplies nutrient and removes waste, and therefore keeps the culture condition more stable and constant compared with a static cell culture [18]. Furthermore, a microfluidic perfusion culture potentially provides new opportunities for cell culture applications because of the precise control of the microscale environment [19–22]. For example, some cell types, such as endothelial cells, are sensitive to shear

stress caused by the flow of the medium [23]. Another example, in 3D culture condition, such as spheroid culture, molecular transport in the microchannels can be controlled by convection flow and the controlled molecular transport affects the state of inner cells in the spheroids [24]. Therefore, microfluidic perfusion culture can be used for both fundamental research and drug development.

Another feature of cell culture in microfluidic device is its small volume. The miniaturized assays are expected to increase experimental throughput and reliability for drug discovery applications [25–27]. This is an important aspect for the application of microfluidic technology to stem cell culture because culturing stem cells, especially human iPSCs, is cost-prohibitive [28]. Microfluidic systems are cost-effective because these systems need small quantity of culture medium and reagents.

In addition, microfluidic device can generate many cell culture conditions using microfluidic network. For example, we have developed a microfluidic network to generate step-wise serial dilutions [29]. We also reported the method to fabricate combinatorial microenvironment array on a microfluidic device [30]. Titmarsh et al. reported a microfluidic network to generate combinatorial array of culture conditions composed of multiple soluble factors at different concentrations [31], and applied this device for the analysis of human embryonic stem cell culture conditions. We think these examples are just the beginning of the application of microfabrication technology in stem cell culture. Possibly, there are additional scopes for applications because many unknown phenomena regarding stem cell culture are yet to be understood.

1.3. Control of cell culture microenvironment

Conventionally, cell culture has been carried out in Petri dishes as static culture. In Petri dishes, the actively growing cells form monolayer sheet and culture media is placed on the cells. In this static monolayer culture, cells grow at randomly arranged positions and medium is exchanged regularly in batches. The stem cells are cultured in a similar manner. In contrast, cells in our bodies form highly ordered 3D microstructures and respond to their surrounding microenvironments including soluble factors, ECMs, contact-dependent intercellular signals, and mechanical signals. Therefore, the 3D nature of native, complex microenvironments is not accurately recapitulated in traditional cell culture on Petri dishes[32]. Microfabrication technology has the potential to control the parameters to simulate these complex 3D microenvironments.

2. Micropatterning technology in stem cell culture

Monolayer cultures of stem cells garnered considerable attention after human ES/iPS cells were established, because these cells are cultured as a monolayer colony and cannot survive without adhering to the surface of the culture dish. In addition, the differentiation of these cells is sensitive to cell density because cell-cell interactions affect stem cell differentiation. Thus, regulation of cell adhesion and control of shape and size of the stem cell monolayer colony are

very important for maintaining stem cell potential and for inducing these stem cells to differentiate into specific cells types. In this section, we reviewed the micropatterning technology and its application in human iPS cell culture.

2.1. Micropatterning for human ES/iPS cells: difference between human and mouse ES/iPS cells

Human ES/iPS cells can differentiate all kinds of human body cells [33, 34]. Human ES cells generated by somatic cell nuclear transfer and human iPS cells contain the donor's genetic information. Therefore, ES/iPS cells can be a good source of cells for rejection-free transplantation of tissues and disease-specific drug screening [34, 35].

Although ES and iPS cells share most of the properties, there are clear differences between mouse and human ES/iPS cells (Figure 1). Mouse ES/iPS cells can survive after single-cell dissociation, but human ES/iPS cells undergo apoptosis[1] [36] following their single-cell dissociation. Thus, the culture conditions and adhesion of human ES/iPS cells need to be carefully controlled to ensure their survival and growth.

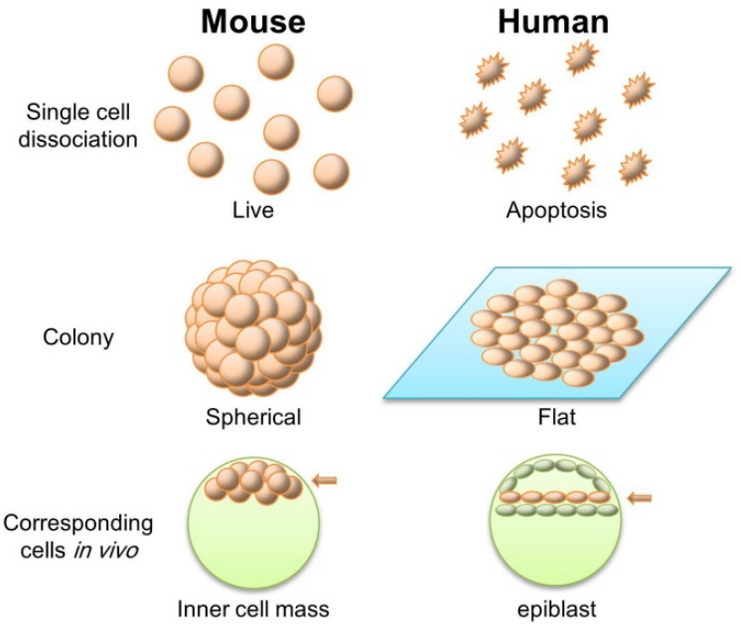

Figure 1. Difference between mouse and human ES/iPS cells.

Moreover, there are morphological differences between mouse and human ES/iPS cell colonies. Mouse ES/iPS cells form spherical aggregates [37–39]and human ES/iPS cells form flat monolayer colonies [33, 34]. Because the colony size is important for maintaining pluripotency and controlling cell differentiation in ES/iPS cells (see section 3), two-dimensional cell

1 Apoptosis caused by single cell dissociation is partially rescued by adding Y-27632, a selective inhibitor of p160-Rhoassociated coiled-coil kinase (ROCK).

patterning is especially important for human ES/iPS cells. Warmflash et al. [40] showed that the differentiation pattern of human ES/iPS cells depended on the size of monolayer colonies.

In addition, although both mouse and human ES cells are derived from inner cell mass of the blastocysts, the properties of mouse ES/iPS cells are closer to that of the inner cell mass, while the properties of human ES/iPS cells are closer to that of epiblast, which is a monolayer of cells arising from the inner cell mass [41]. The epiblast is functionally and molecularly distinct from inner cell mass, and is also pluripotent. Thus, micropatterning technology is important, especially for human ES/iPS cells.

2.2. Micropatterning technology

Although there are many cell micropatterning techniques available [42–47], two important features need to be considered while applying this technique to human ES/iPS cells. The human ES/iPS cell differentiation protocols take a few days to few months, and the cell pattern needs to be long lasting. Although there have been reports of successful patterning of ES/iPS cells, the cells have been found to escape from the pattern within a few days [43, 46]. The other feature is ease of pattern preparation. Many micropatterning methods require some special equipment and techniques that are not easy to perform routinely in cell biology laboratory.

2.3. Micropatterning technology in human ES/iPS cells

We succeeded in forming human iPS cells pattern on the PDMS surface by a simple technique using plasma[2] oxidation with perforated mask and defined culture conditions [48, 49]. As described above, PDMS is one of the most popular biocompatible materials for research and development of cell culture microdevices. Plasma treatment on PDMS oxidized ≡Si–CH3 groups to generate ≡Si–O–Si≡ groups suggests that hydrophilic and siliceous layers were formed on the surface [5, 49].

We first studied the effects of vitronectin and γ-globulin on hiPSC adhesion to plasma-treated and untreated PDMS surfaces under defined culture conditions [49]. We chose vitronectin and γ-globulin because they have contrasting properties. Vitronectin as well as fibronectin and laminin mediate hiPSC attachment, because vitronectin and fibronectin are ligands of integrin α5β1 and αvβ1, and laminin is a ligand of integrin α6β1 and αvβ5, all of which are known to be expressed on ES/iPS cells surface [50–53]. Moreover, vitronectin is especially suitable for coating on glass (≡Si–O–Si≡) [54]. On the other hand, γ-globulin is adsorbed by hydrophobic surfaces and does not mediate cell adhesion [55]. Immunostaining showed that vitronectin and γ-globulin were adsorbed on both plasma-treated and plasma-untreated PDMS surfaces when these proteins were applied separately. However, vitronectin was preferentially adsorbed on plasma-treated surfaces whereas γ-globulin was adsorbed on untreated surfaces when the mixture of vitronectin and γ-globulin was applied. Human iPSCs adhered to the vitronectin-rich plasma-treated surfaces but not to the γ-globulin-rich untreated surfaces.

2 In this chapter, plasma refers to low-pressure plasma, not blood plasma, unless otherwise stated.

Based on the results, we succeeded in making a monolayer pattern of human iPS cells by using perforated masks to prepare plasma-patterned PDMS substrates [49]. The patterned human iPS cells expressed undifferentiated-cell markers and did not escape from the patterned area for at least 7 days. The patterned PDMS could be stored for up to 6 days before hiPSCs were plated. Furthermore, we demonstrate that not only γ-globulin but also bovine serum albumin (BSA) could be used to block human iPS cell adhesion on plasma-untreated PDMS surfaces (Figure 2) [48]. The hiPSCs proliferated without escaping from the patterned area and finally detached spontaneously from the discs to form spheroids.

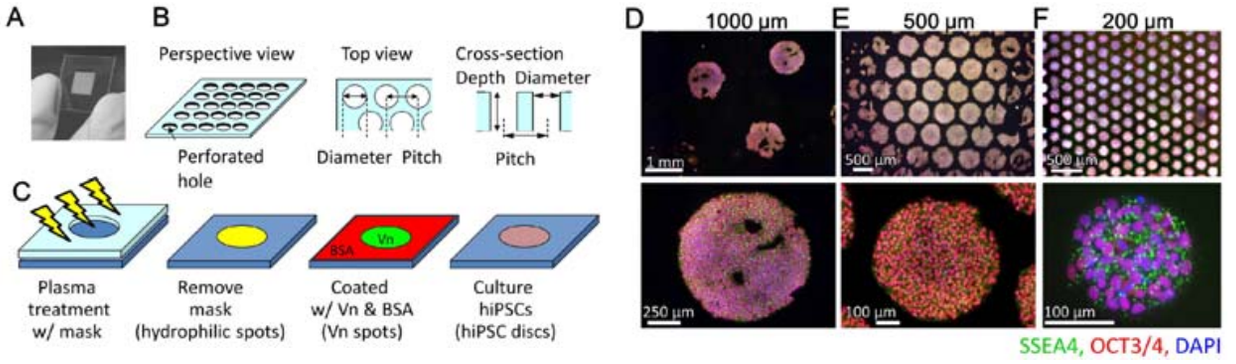

Figure 2. Micropatterned human iPS cells created by plasma patterning of PDMS surfaces and single-step coating of vitronectin and BSA. (A, B) Perforated PMMA masks. (C) Schematic of the micropatterning procedure. (D–F) Immunostaining of patterned cells with undifferentiated cells marker anti-SSEA4 (green) and anti-OCT3/4 (red). Nuclei were stained with DAPI (blue). Lower panels show high magnification images. Reproduced from Yamada et al. [48]with permission from Begell House.

Our micropatterning method presents four advantages over previously reported methods [42, 56]. (1) The plasma treatment through perforated masks enables equal patterning on a wide area, therefore a large number of homogeneously patterned cells can be created reproducibly. (2) Single-step coating of a mixture of proteins is quite simple and easy. Similar methods of producing cell patterning required additional steps, including multistep protein coatings of BSA followed by ECM [56]. (3) The cost-effectiveness and availability of γ-globulin, especially BSA is high. (4) Although there are many types of micro-fabrication tools to make equally sized spheroids, most of them are expensive and difficult to use in cell culture labs [42]. Our method is an easy and cost-effective way to fabricate hiPSC discs and spheroids.

2.4. Harvesting micropatterned human ES/iPS cells by controlling divalent cation

Cell sheets such as retinal pigment epithelium and cardiomyocytes derived from human iPS cells have been developed for applications in regenerative medicine [57, 58]. We tried to harvest micropatterned human ES/iPS cells without their splitting off. Conventionally, cell sheets are harvested using special equipments, such as a temperature-responsive surface

and magnet [59, 60]. In contrast, we focused on integrin and cadherin, which are adhesion molecules on the cell surface. Cadherins mediate cell-cell adhesion at physiological concentration of Ca^{2+}[61]. On the other hand, integrins mediate cell-ECM adhesion and depend largely on Mg^{2+} [62]. Thus, a solution containing physiological concentration of Ca^{2+}, but no Mg^{2+}, could be used to harvest cells as large cell clumps under serum-free culture condition. As expected, simple incubation in PBS with Ca^{2+} without Mg^{2+} followed by gentle pipetting enabled us to harvest the cells as sheets without cells splitting off (Figure 3) [63]. Similar results were obtained for early-differentiated cells and for hepatic progenitors derived from human iPS cells. These results suggest that the cells can be routinely and simply harvested as a large sheet by using a solution with $Ca^{2+}$and without Mg^{2+}.

Our methods introduced here can be also used practically to mimic epiblast (Figure 1) in early human embryonic development. We believe that our cell patterning method will be useful for the development of new bioengineering tools to search for effective cell differentiation methods and to test drug safety for early human embryonic development.

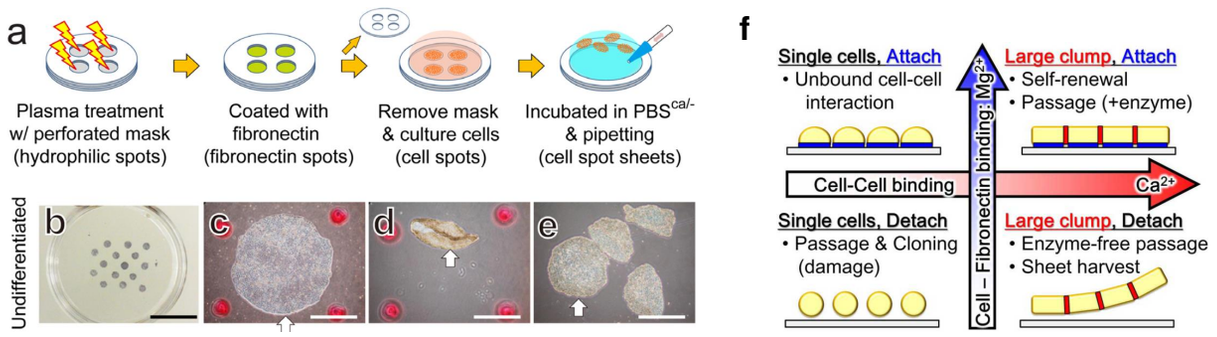

Figure 3. Human iPS cells sheet harvesting. (A) Schematics of spot sheet formation and harvest. (B) ALP staining of the hiPSCs plated on 2-mm-diameter fibronectin spots. Phase-contrast micrographs before (C) and after (D, E) 15 min in PBS with Ca^{2+} followed by pipetting. The white arrows indicate the same cellspot sheet (C–E). The red spots in (C, D) are position makers. Scale bars are 1 cm (B), 1 mm (C–E). (F) Schematics of the effects of Mg^{2+} and Ca^{2+} on hPSCs culture. Cell-cell and cell-ECM adhesion depend largely on Ca^{2+} (abscissa) and Mg^{2+} (ordinate), respectively. Large cell clumps and sheets can then be harvested by dissociating in lowMg^{2+} and high Ca^{2+} solution (lower right).Reproduced from Ohnuma et al. [63] under a Creative Commons Attribution 3.0 Unported License.

3. Microwell array technology in stem cell culture

The formation of three-dimensional cell aggregates called embryoid bodies (EBs) that resemble the embryo structure is the principle behind in vitro differentiation of stem cells. Microwell array is a promising platform for generating EBs, in which microwells of several hundred micrometers size are regularly fabricated on a culture substratum. It controls EB size and produces large number of homogenous EBs. An added advantage of microwell array culture is that it can influence the fate of differentiating cells in EBs. In this section, we review the architectures of microwell arrays, microwell array culture of ES/iPS cells, and the relationships between microwell conditions and EB properties.

3.1. Embryoid body (EB) culture

For in vitro differentiation of stem cells, one of the superior strategies is to imitate in vivo development processes. Three-dimensional aggregate of stem cells called embryoid body resembles early embryo. The multicellular interactions generated with the EB formation trigger cell differentiation. Thus, the EB culture has been used as a principal method for in vitro early differentiation of stem cells.

The EB is formed by the rearrangement and compaction of stem cell aggregates. Therefore, the differences in EB sizes affect the diffusion of soluble molecules, the extent of cell-ECM and cell-cell interactions, and the generation of mechanical forces (Figure 4A).Consequently, it affects the differentiation and fate of stem cells [64–66]. Thus, the culture technique capable of modulating EB size is important to regulate stem cell differentiation.

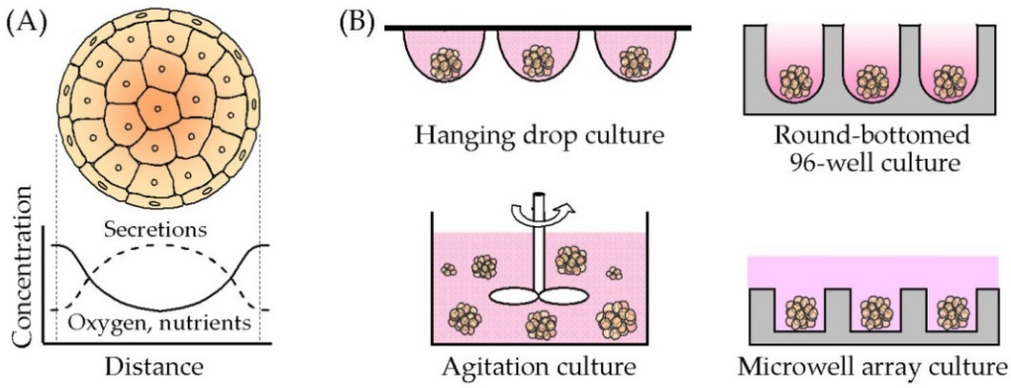

Figure 4. (A) EB characteristics and (B) methods for EB formation.

3.2. Methods for EB formation

The EB formation occurs if cell-cell adhesion is stronger than cell-surface material adhesion. Therefore, we can lead the EB formation by designing a culture environment, which promotes cell-cell adhesion. Typical methods for EB formation are hanging drop culture, round-bottomed 96-well culture, and agitation or rotational culture (Figure 4B) [67, 68]. The agitation culture can achieve mass production of EBs; however, it is difficult to control the EB size. In contrast, the hanging drop and round-bottomed 96-well cultures can control the EB size, but their scale-up is difficult. Furthermore, these methods pose difficulties in handling of the formed EBs.

Recently, microwell array has been advocated as a promising technique over the current methods. It is a culture platform in which microwells of several hundred micrometers size are regularly fabricated on a culture plate (Figure 4B). Recently, various microwell arrays have been developed by researchers [69]. Generally, the number of microwells is from tens to thousands per culture plate, and these microwells are laid as triangular or square arrangements on the plate. The microwells having various shapes such as column, square, and pyramid have been fabricated by photolithography, soft lithography mold, micromachining, etc. [70–72].

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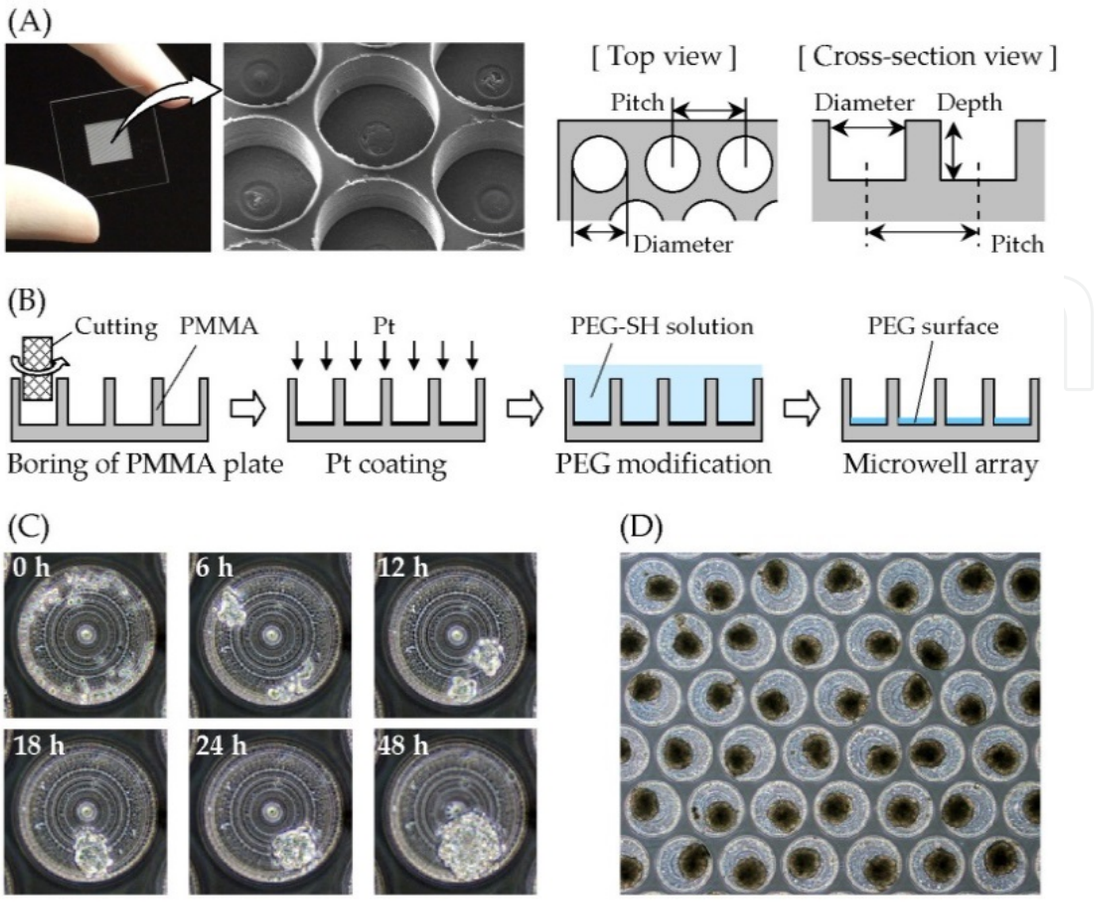

Figure 5. (A) Microwell array, (B) manufacturing processes, (C) EB formation, and (D) culture in the microwell array.

Furthermore, most microwell arrays are designed with cell nonadhesion surfaces to promote the EB formation. Such microwell arrays can generate a large number of homogenous EBs, with controlled size, and allow easy EB handling.

3.3. Our microwell array

We developed a microwell array comprising columnar microwells in triangular arrangement on a poly-methylmethacrylate (PMMA) plate with the surface modified by polyethylene glycol (PEG) to render cell nonadhesion (Figure 5A) [73, 74]. This was fabricated as follows (Figure 2B). The microwell structure of array was fabricated using a programmable micromilling system. Subsequently, the array surface was coated with thin layer of platinum using an ion sputter unit, and immersed in a PEG-SH solution. The PEG molecule formed covalent attachment via its thiol group to the platinum layer, thus modifying the surface. The microwell array manufactured via this process was used for the stem cell culture.

Figure 5C shows the changes in cell morphologies of mouse ES cells within the microwell. The cells that were seeded on the array began to aggregate within several hours of inoculation, and they spontaneously formed a single EB in each microwell of array within 1 day of culturing. Although none of the EBs adhered on the microwell surface, all EBs were held within the

microwells throughout the culture period. Consequently, the mass production of homogenous EBs was achieved in the single array (Figure 5D).

3.4. EB properties in the microwell array culture

The microwell array could arbitrarily vary the microwell conditions such as well number, diameter and depth of well, distance of wells, and cell density. To clarify the characteristics of microwell array culture, we evaluated the effects of microwell diameter and cell density on the EB properties of mouse ES cells [75, 76].

Four similar arrays comprising 195 microwells were fabricated with microwell diameters of 400, 600, 800, and 1000 μm to evaluate the relationship between the microwell diameter and EB property. The hanging drop (HD) culture was used as a control method. In this experiment, the inoculated cell density was at 1000 cells/well or 1000 cells/drop, and the cells were cultured in a medium without LIF and inducers.

Figure 6A shows the changes in EB sizes. The EB sizes at the initial stage were almost same (approximately 150 μm) under all culture conditions. Although the EB in the HD culture grew drastically with increasing culture time, the growth in the microwell culture was repressed compared with that in HD culture. Furthermore, the changes in EB sizes depended on the scale of microwell, and the EB growth in larger microwells was higher than that in smaller microwells. Figure 6B shows the gene expression levels of hepatic (AFP), cardiac (αMHC), and vascular (Flk1) differentiation markers. The differentiation fates of EBs were nearly the same under all conditions, but the gene expression levels varied with culture conditions. The expressions of differentiation markers were highest in HD culture, and gradually decreased in 1000, 800, 600, 400 μm arrays in that order. These results indicate that the EB growth and differentiation rate can be controlled by diameters of microwell, and that they are promoted in larger microwell conditions. These differences may be attributed to special culture environments provided by the microwell culture. The existence of micro spaces (microwell) might have facilitated accumulation of various soluble factors which were secreted from the cells. Additionally, interference effects caused by the neighboring EBs could occur because of extensive EBs on an array. Consequently, the accumulation of paracrine/autocrine factors and/or the concentrations of oxygen and nutrients in the culture medium vary by the array conditions, and the balances of these factors may regulate the EB properties.

To evaluate the relationship between the inoculated cell density and EB property, the cells at densities of 100, 1000, or 10000 cells/well were inoculated to the array that comprised 195 microwells with 600 μm diameter. Figures 7A and B show the changes in EB sizes and the gene expression levels of differentiation markers, respectively. The change in the EB size of array at 100 cells/well was higher than that of array at 1000 cells/well. In contrast, the cells of array at 10000 cells/well hardly proliferated, and the EB size that formed at the initial stage was maintained throughout the culture period. The expression levels of differentiation markers were the smallest in the array at 10000 cells/well. The expression of vascular (Flk1) differentiation was highest in the array at 100 cells/well, but the array at 1000 cells/well promoted hepatic (AFP) and cardiac (αMHC) differentiations rather than vascular differentiation, indicating that the inoculated cell density affects the differentiation fate of EBs. These differ-

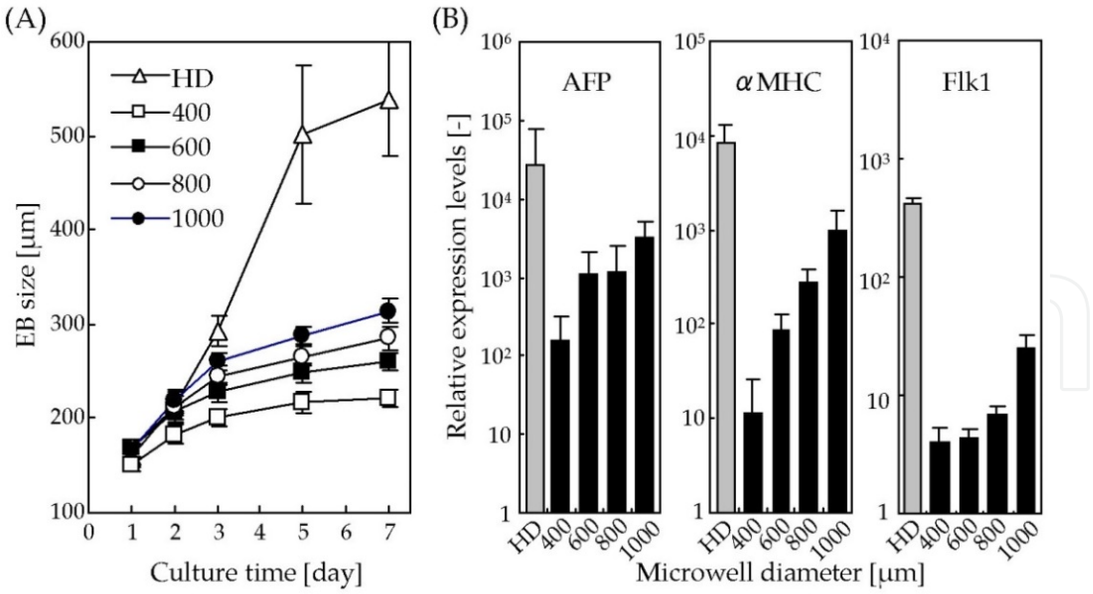

Figure 6. (A) Changes in the EB sizes and (B) the gene expression levels of differentiation markers after 7 days of culture.

ences may be caused mainly by the differences in size of EB formed at the initial stage, because the EB size affects the generation of intercellular interactions which trigger the cell differentiation. This explanation is also supported by the facts that similar results were also observed in the HD culture.

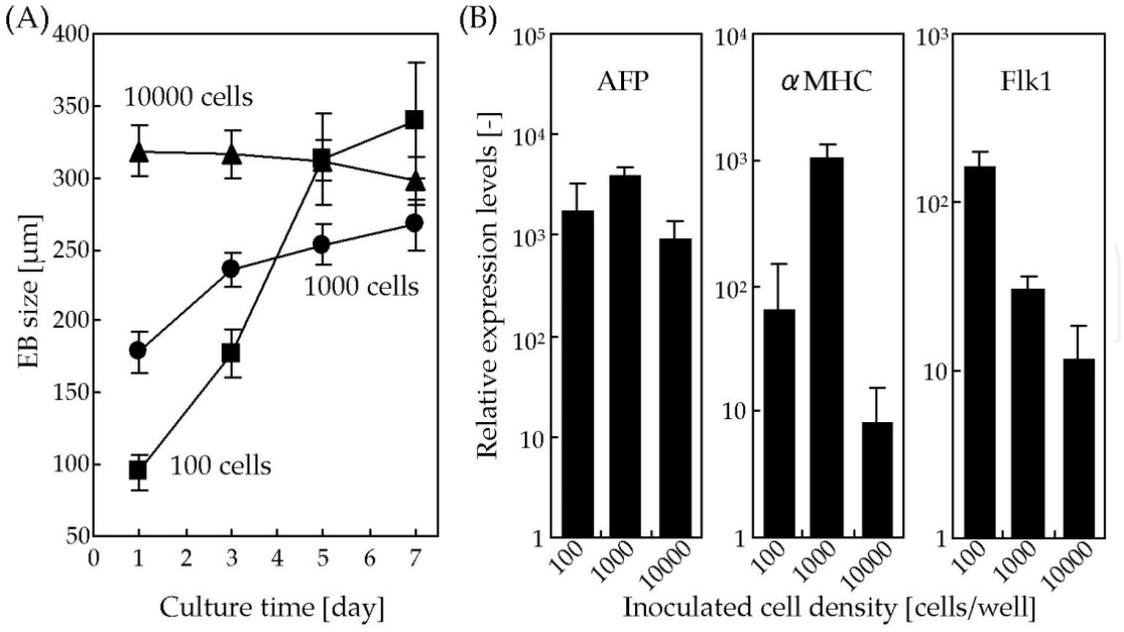

Figure 7. (A) Changes in the EB size and (B) the gene expression levels of differentiation markers after 7 days of culture.

In conclusion, the microwell array culture could control EB size and allow mass production of homogenous EBs. Furthermore, we demonstrated that differentiation fates of stem cells in the microwell array culture were similar to previous HD culture, and that the architectures of microwell array could control the EB growth and differentiation rates. These characteristics offer advantages over previous methods. Thus, the microwell array may be applicable as a cellular platform that can control the in vitro properties of EB.

4. Microfluidic perfusion system in stem cell culture

The control over microenvironment is important for controlling the stem cell fate, which is affected by various soluble factors supplied by the culture medium and by autocrine and paracrine mechanisms. Microfluidic perfusion enables the control of spatial and temporal profiles of the concentration of soluble factors. In this section, we review the use of the microfluidic perfusion culture system, and recent applications of the microfluidic perfusion culture system in the culture of human ES/iPS cells.

4.1. Microfluidic perfusion culture

Generally, microfluidic perfusion culture in a microfluidic device is carried out using syringe pumps [77, 78]. However, liquid handling using syringe pumps is cumbersome because it requires connection of many tubes from syringe pumps to the microfluidic device. To address this issue, we developed a pressure-driven perfusion culture system, in which multiple liquids could be handled by simply applying pressure in the liquid reservoir. This is a convenient system to create different culture conditions in a single microfluidic device [79]. We also developed a microfluidic network to generate serial dilution concentration profiles using this pressure-driven perfusion culture system [80].We applied this pressure-driven perfusion culture system to determine IC_{50} by using the serial dilution microfluidic network [29]. In these studies, we developed the perfusion culture microchamber array chip equipped with 12 perfusion culture microchambers. The culture microchambers were connected to a serial dilution microfluidic network that could generate 12 different stepwise concentration profiles (Figure 8A). We have successfully applied this pressure-driven microfluidic perfusion culture system to a dose-response assay of model anticancer drug, paclitaxel. The obtained IC_{50} of paclitaxel was similar to that obtained by using traditional microplate assay (Figure 8B).

We have also developed an integrated microfluidic device that contains 384 microchambers in a single device. (Figure 9) [81]. In this device, 12 different drugs were diluted stepwise in the serial dilution microfluidic network into eight different concentrations. Each microchannel for each concentration is connected to four cell culture microchambers (Figure 9B). In total, 384 assays (12 drugs × 8 concentrations ×4 microchambers) could be carried out simultaneously. The culture media with 12 different drugs, the culture media without drug, and cell suspension were loaded in the liquid reservoir using a micropipette (Figure 9A). By applying appropriate pressure, serial dilution concentration profiles spanning 3 orders of magnitude were generated in six dilution steps. Theoretically, IC_{50} of 12 drugs could be determined in the single-experiment setup.

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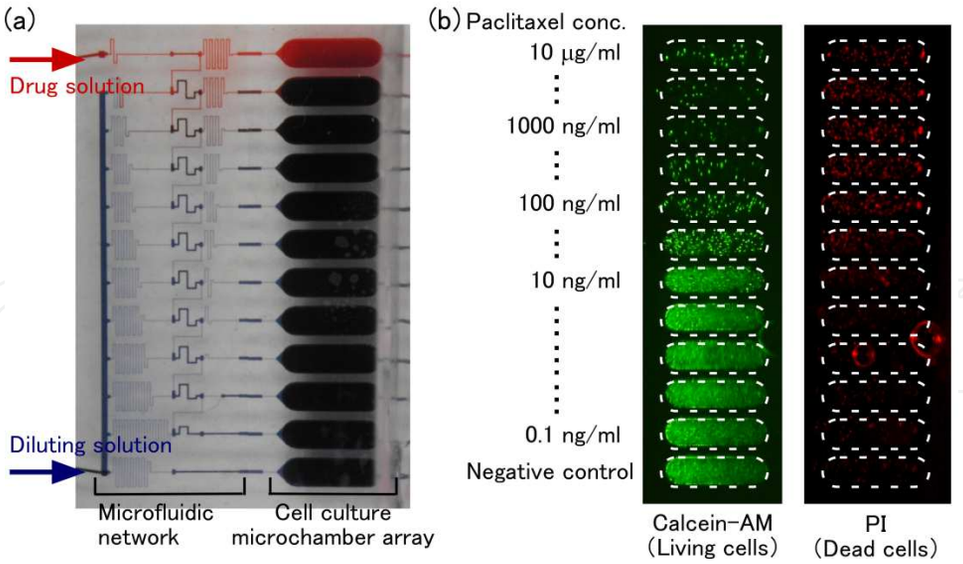

Figure 8. Photographs of the perfusion culture microchamber array chip equipped with a serial dilution microfluidic network. (A) Serial dilution microfluidic network and the cell culture microchambers. (B) Fluorometric cell growth measurement for drug cytotoxicity assay. Reprinted with permission from Sugiura et al. [29]. Copyright (2010) American Chemical Society.

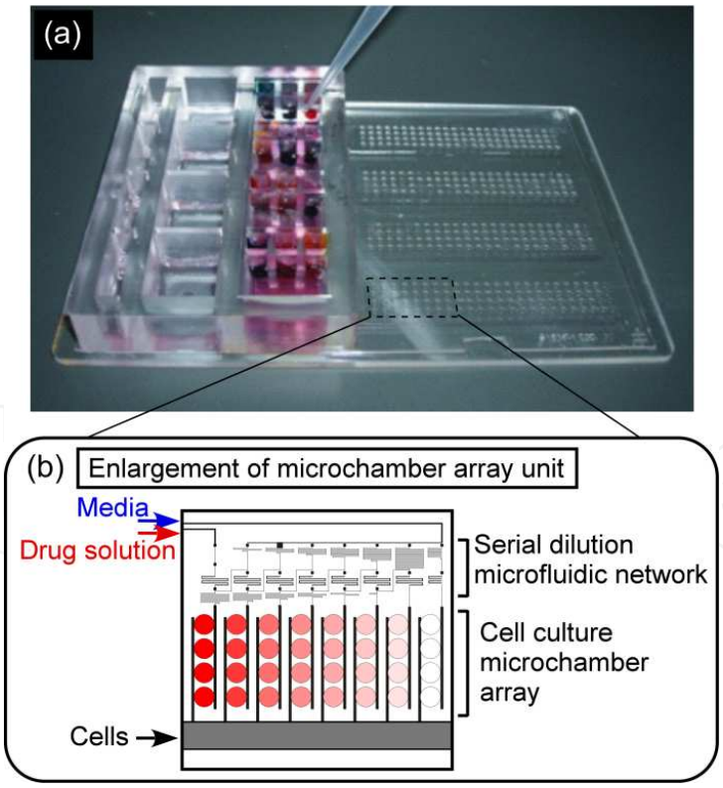

Figure 9. Microplate-sized integrated perfusion culture microchamber array chip. (A) Photographs of the integrated perfusion culture microchamber array chip. (B) Enlarged view of a microchamber array unit. Reproduced from Hattori et al. [81] with permission from CBMS.

4.2. Microfluidic perfusion system for human iPS cells under defined culture conditions

We tried to control the state of human ES/iPS cell cultures by using microfluidics perfusion system and defined the culture conditions [82]. Microfluidics perfusion system enables us to control spatial and temporal application of the soluble factors to the cells. On the other hand, defined culture conditions enable us to control the kinds of factors that are applied to the cells. Thus, the combination of microfluidic perfusion system and defined culture conditions enable us to control microenvironment and replicate stem cells niche in vitro.

Conventional culture methods for human ES/iPS cells use many undefined supplements including liquid additives such as knockout serum replacement (KSR; Life technologies, Grand island, NY, USA) and coating matrix such as Matrigel (BD Biosciences, Mississauga, Canada) [83]. These undefined supplements contain unknown quantity of growth factors, hormones, and integrin receptors, all of which affect human ES/iPS cells states. Thus, they are not suitable for being used to control state of human ES/iPS cells. Therefore, culture systems, which consist of defined supplements including hormones and cytokines and a defined coating matrix such as fibronectin or laminin, have been developed [84–86]. These defined culture systems enabled us to assess the direct responses of added factors without masking by undefined factors and to control differentiation of human ES/iPS cells.

We designed a perfusion culture microchamber array chip that was suitable for ECM coating, cell loading, and human iPS cells perfusion culture based on our previous reports [30, 79]. In this system[82], ECM-coating solution and cell suspensions were loaded in all microchambers through a cell-inlet port (Figure 10A, right) via cell-inlet main channels (Figures 10Band C). The culture media are supplied from a medium-inlet port (Figure 10A, left) via medium-inlet main channels (Figures 10B and C). By applying air pressure to these channels, four different culture conditions can be generated (Figure 10B).

To coat microchambers with purified ECM-coating matrix, we applied two modifications to our system. First modification was the reduction of medium flow resistance at the medium-inlet channel[3] and the second was intermittent pressure application for medium perfusion. ECM-coating solution was compulsorily loaded in dried microchambers prior to cell suspension loading. Because the flow resistance of the liquid was much higher than that of the air, this extracoating step decreased the cell loading flow-rate at the same pressure compared with our previous system, in which the cells were loaded into dried microchambers without an ECM-coating solution.[4] Thus, the medium-inlet channels were modified to be thick to increase the flow rate of cell suspension, resulting in an increase in the medium perfusion flow rate at the same pressure. As a result, intermittent pressure was applied during perfusion culture to keep the average flow rate almost the same as in previous systems. Based on the coating experiment, we chose fibronectin as ECM for coating microchambers.

3 The medium-inlet branch channel is much more shallow than other microchannels; therefore, the flow rate in each microchamber is determined by the maximum fluidic resistance of the medium-inlet branch channel.

4 The microchambers were coated unintentionallybecause serum contains ECM such as fibronectin, laminin, and vitronectin.

We found that the growth rate of human iPS cells under pressure-driven perfusion culture conditions was higher than under static culture conditions in the microchamber array. We also applied our new system to self-renewal and differentiation cultures of human iPS cells. Immunocytochemical analysis showed that the state of the human iPS cells was successfully controlled (Figure 9). Moreover, the effects of three antitumor drugs on human iPS cells were comparable between microchamber array and 96-well plates.

We believe that our system will be a platform technology in future for large-scale screening of fully defined conditions for differentiation cultures on integrated microfluidic devices.

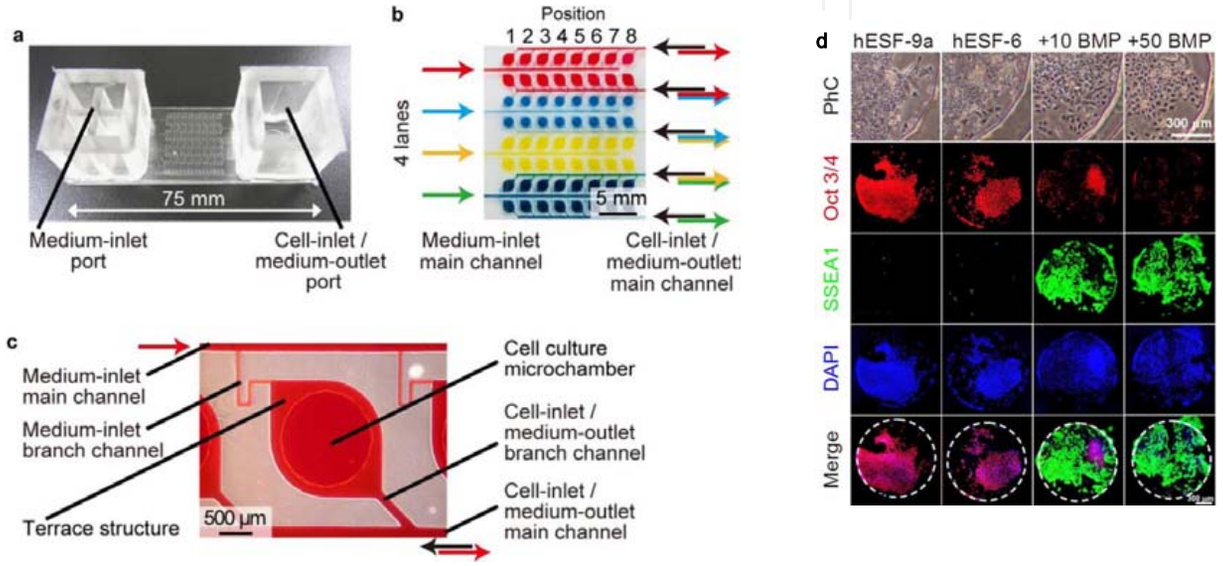

Figure 10. Structure of the perfusion culture microchamber array chip. (A) Overview of the perfusion culture microchamber array chip. (B) Enlarged view of the array with four dye solutions. (C) Enlarged view of the microchamber. (D) Undifferentiated and differentiated human iPS cells in the microchamber array chip. The four lanes of the array were perfused with four types of defined culture medium: hESF-9a (including a growth factor to keep the cells in undifferentiated state), hESF-6 medium (without growth factor), and hESF-6 medium supplemented with 10 ng/mL BMP4 (+10 BMP) or 50 ng/mL BMP4 (+50 BMP). BMP4 induces early differentiation. Microphotographs were taken at day 3. PhC: the top panels show phase-contrast micrographs. The lower panels show immunocytochemistry of the self-renewal marker, Oct 3/4 (red), the early differentiation marker, SSEA1 (green), nuclear staining with DAPI (blue), and the merged image. The white dotted lines represent the edges of cell culture microchamber. Reproduced from Yamada et al. [82]with permission from John Wiley and Sons.

5. Summary and perspectives

In this chapter, we introduced the current state of the art of microfabrication technologies used in stem cell culture. As described above, this technology has already been applied to micropatterning, EB formation, and microfluidic perfusion culture. The microenvironment controlled by these microfabrication technologies provided sophisticated culture conditions compared to that of conventional static monolayer culture. As a result, stem cell cultures could be carried out in a controlled manner. So far, some obvious advantages of the use of micro-

fabrication technology in stem cell culture include control of stem cell fate, easy cultivation method, and reduction in culture volume.

Although methods discussed achieved certain success to control the culture microenvironment, the use of microfluidics in human ES/iPS cell studies is still limited [77, 87–91]. For example, the induction of fully functional organs and the fabrication of complicated 3D structure mimicking an intact organ is still difficult. Moreover, culture conditions available in current microfluidic technology are basically limited to conventional 2D cultures. Even cultivation of cellular aggregate in microfluidic device is still challenging. We believe that these challenges will be addressed gradually in the future by interdisciplinary approaches including mechanical engineering, material science, device design, and cell biology.

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