CULTIVATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS WITH CONTROLLED AGGREGATE SIZE AND GEOMETRICAL ARRANGEMENT BY INVERTING MICROWELL ARRAY CHIP

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ABSTRACT

This paper reports a novel cell culture chip, namely "inverting microwell array chip", for cultivation of human induced pluripotent stem cells with controlled microenvironment. Our "inverting microwell array chip" comprises a lower hydrogel microwell array and an upper polystyrene (PS) culture surface. We demonstrate the formation of uniform human induced pluripotent stem cell aggregates in the microwell array, and after inversion, a culture with controlled aggregate size and geometrical arrangement on the PS surface.

KEYWORDS

iPS cells, Microwell array, Cellular aggregate, Geometry,

INTRODUCTION

The conventional method for cultivating hiPS cells involves the handling of cells as cellular aggregates; however, obtaining uniformly sized aggregates is technically difficult. Pipetting, which is generally the preferred method of handling liquids in biological laboratories, inevitably causes a broad distribution of cellular aggregate sizes, even in automated systems; the size of cellular aggregates after pipetting depends on the shear stress, the size and shape of the original aggregates, and the strength of the cell–cell and cell–extracellular matrix interactions of each individual aggregate.

Cell-assembling by using single-cell hiPS suspensions offers a solution for producing uniform cellular aggregates. Although the break-down of cellular aggregates into single cells can induce cell death, the formation of cellular aggregates promotes cell survival. Many previous studies have reported various methods for the formation of size-controlled cellular aggregates of hiPS and embryonic stem cells in studies of the effects of aggregate size on differentiation [1-9]. Especially, use of microwell structures gave successful results for formation of size-controlled cellular aggregates [1-7] In addition, some researchers reported the subsequent adherent culture after formation of uniform cellular aggregates in microwells [6, 7, 10].

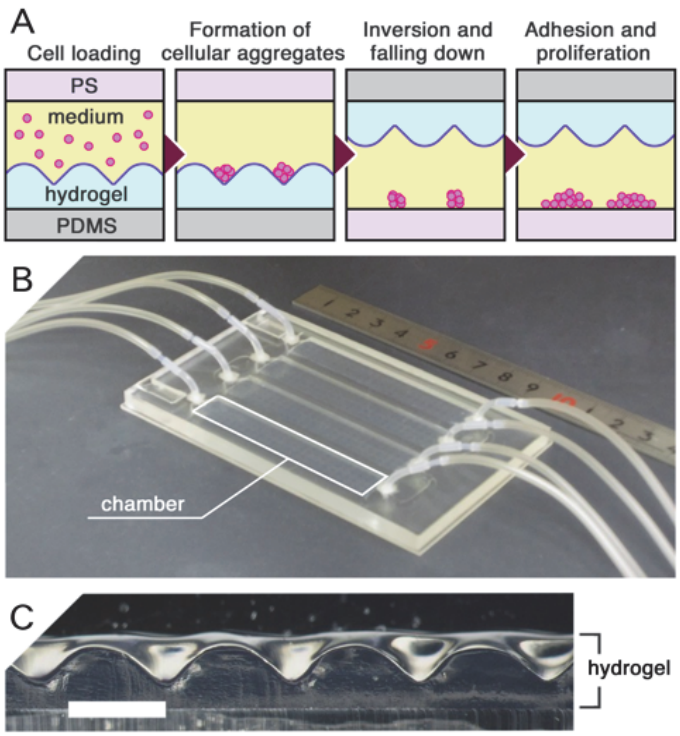

Figure 1. (A) Cultivation of hiPS cells in an inverting microwell array chip. PS, polystyrene; PDMS, polydimethylsiloxane. (B) Overview of the chip without a holder. (C) Cross-section of the microwells imprinted in the 65 hydrogel layer. Scale bar: 2 mm.

Here, we describe an inverting microwell array chip that is capable of producing uniform cellular aggregates, and of controlling the position of the cellular aggregates in both the adhesion culture and perfusion culture in a programmed manner. Figure 1A illustrates the procedure for cultivating hiPS cells in the inverting microwell array chip. A microwell array is placed in the bottom of the chamber, into which a single-cell suspension is loaded. A polystyrene (PS) surface is placed in the top of the chamber, on which the cellular aggregates adhere after the chip is inverted. The cells loaded into the chamber settle to the bottom of the microwells and form cellular aggregates. After cellular aggregates have formed, the chip is inverted, which results in the cellular aggregates being plated onto the PS culture surface in the same geometrical arrangement as the microwells. Further incubation allows the cellular aggregates to attach to the culture surface and start spreading and proliferating. Ideally, the adhered cellular aggregates will have a uniform size distribution and an array-formatted geometry. Finally, the proliferated cells are collected from the chip as a single-cell suspension by enzymatic treatment.

EXPERIMENTAL

The inverting microwell array chip is composed of a polydimethylsiloxane (PDMS) block with four individual chambers, a PS plate as the culture surface, and hydrogel layers in which the microwell arrays are imprinted (Fig. 1B). Each chamber has a hydrogel layer with a 4 × 24 array of 96 microwells each with a depth of 0.8 mm and pitch of 2.7 mm. The bottom of the microwell forms a reverse cone shape with a top edge angle of 90°, meaning that each microwell wall has a slope angle of 45° (Fig. 1C).

We made the microwells out of poly(ethylene glycol) (PEG)-based hydrogels because of its low cell-adhesive property, easy method of preparation, and transparency. The hydrogel layer was prepared by means of photoinduced polymerization from an aqueous solution of PEG diacrylate, PEG methacrylate, and sodium acrylate, with Irgacure 2959 as the initiator. The microwell structure was imprinted in the hydrogel layer during the polymerization process by using a PDMS template that had a 4 × 24 array of cones. The PDMS chambers were temporary filled with acetone solution of benzophenone (10%) for 1 min to form covalent bonding of the PDMS and a hydrogel. The negative PDMS templates were placed in the PDMS chambers; the tops of cones were set 0.5 mm apart from the chamber surfaces. A mixture solution of PEGDA (4.40%), PEGMA (3.08%), AANa (1.32%), and Irgacure 2959 (0.5%) in water was filled into opening spaces between the PDMS chambers and negative templates. The solutions were irradiated with an UV light at 90 mJ/cm2 for 300 sec. Prepared hydrogels were soaked three times in PBS to wash and were then exposed to an UV light for sterilization.

The PS plate was coated with Matrigel according to the supplier's protocol. We assembled the PDMS block with the hydrogel layers and the Matrigel-coated PS plate, and then fixed them together with an aluminum holder and screws.

RESULTS AND DISCUSSION

We demonstrated the cultivation of hiPS cells in the inverting microwell array chip. Single cell suspensions were prepared by accutase treatment of hiPS cell colonies that had been cultivated in a feeder-free condition. A cell suspension corresponding to 31 to 2000 cells/microwell was loaded into the chambers of the chip. The cells were incubated for 4 hours with intermittent tapping in order to gather the cells falling to the bottom of the microwells. Uniform cellular aggregates were observed after further 16 hours of static cultivation (Fig. 2). Culture medium was temporarily removed from the chambers with keeping a part of medium remaining in the microwells, and the chip was vibrated to release the cellular aggregates from the bottom of the microwells. Fresh medium was then filled into the chambers again. The chip was inverted, i.e. turned over and placed upside down, then the formed cellular aggregates fell onto the cultivate surfaces in array formatted positions corresponding to the geometry of the microwell array (Fig. 3A and 3B). The cellular aggregates were

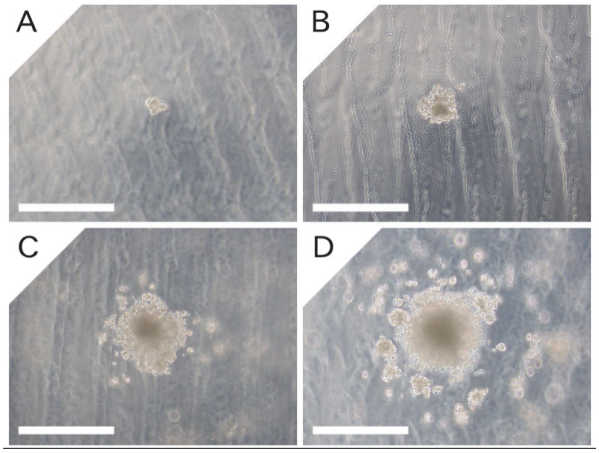

Figure 2. Microscopic images of cellular aggregates formed after a 20 h incubation at concentrations of (A) 31, (B) 125, (C) 500, and (D) 2000 cells/microwell. Satellite-like aggregates were observed around major one at the higher concentrations (C and D). Scale bars: 400 µm. A striped-pattern on a PDMS surface, which resulted from machining marks on a template, is seen through a hydrogel layer.

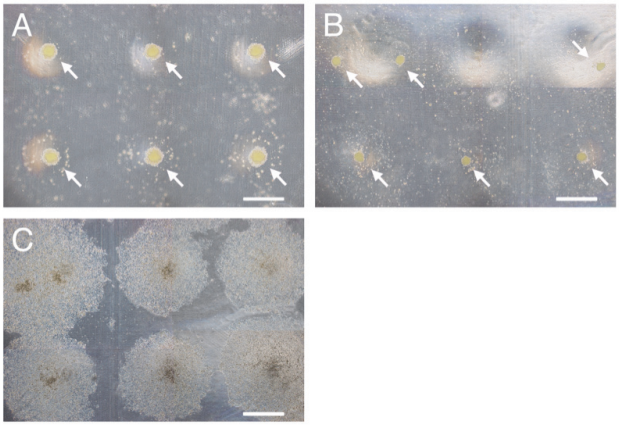

Figure 3. Cultivation of hiPS cells in the inverting microwell array chip. (A) 75 Cellular aggregates formed in the microwells (arrows). (B) Cellular aggregates on the culture surface after inversion (arrows). (C) Proliferated cellular aggregates 5 days after inversion. Scale bars: 1 mm.

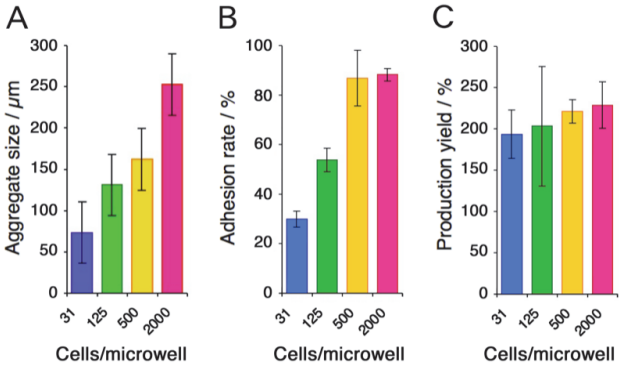

Figure 4. (A) Average diameters of hiPS cellular aggregates (n = 8). (B) Average adhesion rates of the aggregates (n = 3). (C) Average production yields in the inverting microwell array chip after cultivation for 6 days, which included the period of aggregate 5 formation (n = 3).

cultivated with a semi-continuous medium perfusion, and then adhered on to the PS surface and formed cell colonies. Fig. 3C shows the proliferated hiPS cell colonies at 5 days after the plating. We observed the colonies spread with keeping the array formatted positions. After 5 days of cultivation, the colonies were treated with accutase, and then most of the cultivated hiPS cells were collected as single cell suspension. These results demonstrate the feasibility of the passage culture of hiPS cells in the inverting microwell array chip.

We investigated the effects of cell concentration on the cultivation in the inverting microwell array chip. When cell suspensions were loaded at the concentrations of 31, 125, 500, and 2000 cells/microwell, diameters of the formed cellular aggregates were 70, 130, 160, and 250 μm, respectively (Fig. 4A). Fig. 4B shows the adhesion rate of the cellular aggregates that had settled on the culture surface after inversion. Adhesion rate was calculated from the number of adhered cellular aggregates and of settled aggregates. We observed high adhesion rates at the high cell concentrations of 500 and 2000 cells/microwell. Increased numbers of cells capable of contacting the culture surface probably enhanced the interactions of the cells with the Matrigel at these higher cell concentrations. Cells were collected 5 days after inversion. Fig. 4C shows the production yields for the four cell concentrations assessed, which was calculated as the number of cells collected divided by the number of cells loaded. The production yields were around 200% for the four cell concentration conditions. Production yields are likely affected by the following parameters: (i) aggregate formation yield, (ii) settling rate, (iii) adhesion rate, (iv) aggregate proliferation, and (v) collection yield after Accutase treatment.

CONCLUSION

In summary, we successfully demonstrated the cultivation of hiPS cells in an inverting microwell array chip. We were able to control aggregate size and produce an array-formatted arrangement. Cultivation using the inverting microwell array chip is very simple and includes the formation of the cellular aggregates and adhesion to the culture medium. Aggregate size was controlled in the microwell array and was dependent on initial cell concentration. The cellular aggregates formed adhered to the culture medium in an array pattern and proliferated uniformly. Production yields were approximately 200% after 6 days of cultivation, which included the period of aggregate formation. The inverting microwell array chip permits controlled and reproducible cultivation of hiPS cells and this will provide new opportunities for controlled maintenance cultivation, efficient differentiation, and automated cultivation in basic laboratory studies and industrial applications.

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