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Compartmentalized microfluidic perfusion system to culture human induced pluripotent stem cell aggregates

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Microfluidic perfusion systems enable small-volume cell cultures under precisely controlled microenvironments, and are typically developed for cell-based high-throughput screening. However, most such systems are designed to manipulate dissociated single cells, not cell aggregates, and are thus unsuitable to induce differentiation in human induced pluripotent stem cells (hiPSCs), which is conventionally achieved by using cell aggregates to increase cell–cell interactions. We have now developed a compartmentalized microfluidic perfusion system with large flow channels to load, culture, and observe cell aggregates. Homogeneously sized cell aggregates to be loaded into the device were prepared by shredding flat hiPSC colonies into squares. These aggregates were then seeded into microchambers coated with fibronectin and bovine serum albumin (BSA) to establish adherent and floating cultures, respectively, both of which are frequently used to differentiate hiPSCs. However, the number of aggregates loaded in fibronectin-coated microchambers was much lower than in BSA-coated microchambers, suggesting that fibronectin traps cell aggregates before they reach the chambers. Accordingly, hiPSCs that reached the microchambers subsequently adhered. In contrast, BSA-coated microchambers did not allow cell aggregates to adhere, but were sufficiently deep to prevent cell aggregates from flowing out during perfusion of media. Immunostaining for markers of undifferentiated cells showed that cultures on both fibronectin- and BSA-coated microchambers were successfully established. Notably, we found that floating aggregates eventually adhered to surfaces coated with BSA upon differentiation, and that differentiation depends on the initial size of aggregates. Collectively, these results suggest that the microfluidic system is suitable for manipulating hiPSC aggregates in compartmentalized microchambers.

[**Key words:** Human induced pluripotent stem cells; Defined culture conditions; Cell aggregates; Embryoid body; Spheroid; Microchamber array; Perfusion culture; Cell-based assay]

Cell–cell interactions, including direct contact and auto/paracrine signaling, are critical for differentiation during embryonic development. Thus, cell aggregation is frequently induced *in vitro* to differentiate pluripotent stem cells (1) such as human embryonic stem cells and human induced pluripotent stem cells (hiPSCs) (1–4). Notably, differentiation in aggregated pluripotent stem cells is influenced by the microenvironment, as well as by the initial number of cells (5,6). Thus, laboratory tools suitable for cultivating cell aggregates is important in cell-based drug screening and regenerative medicine.

Microfluidic perfusion culture systems were recently developed to potentially control cell fate and the microenvironment (7–9), as well as to form or trap cell aggregates in microchambers (10–12). However, these systems are elaborate in design, and are cumbersome to operate. For example, although a syringe pump is convenient to set up and to accurately control flow rate, the same number of syringes and connection tubes is needed to deliver as many types of culture media. Recently, we developed our own microfluidic perfusion culture system to control the differentiation of dissociated hiPSCs in defined culture media (13). The system uses pneumatic pressure to deliver multiple types of culture media (13–15) to be more efficiently than other microfluidic culture systems (10–12). However the flow channels of this system were so narrow that only disaggregated hiPSCs could be loaded, and were unsuitable for cell aggregates (13,14).

We have now improved our design to include wider channels that enable loading, culture, and observation of aggregated hiPSCs in compartmentalized cell-culture microchambers. By using fibronectin and BSA as coating, we established adherent and floating cultures, both of which are frequently utilized for differentiating cells from hiPSCs. We also attempted to follow and evaluate the differentiation of cell aggregates.

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MATERIALS AND METHODS

hiPSC culture Human iPS 201B7 cells (4) were obtained from RIKEN BRC Cell Bank (Tsukuba, Ibaraki, Japan) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and cultured as described (13,16,17). Briefly, cells were maintained in the undifferentiated state in dishes coated with 2 μg/mL fibronectin and containing hESF-9a medium. However, cells were passaged at least once prior to all experiments. To subculture, cells were harvested by incubation for about 10 min in 0.02% w/w ethylenediaminetetraacetic acid in PBS, and replated in hESF-9a media supplemented with 5 μM ROCK inhibitor (Y-27632, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Subsequently, media were replaced every day with fresh hESF-9a. To differentiate, media were replaced with hESF-6 medium supplemented with bone morphogenetic protein 4 (BMP4, R&D systems, Minneapolis, MN, USA).

Design and fabrication of the microchamber array chip Based on our previous devices (13,14), we designed and fabricated a microchamber array chip suitable for coating with extracellular matrix proteins, for loading hiPSC aggregates, and for perfusion cultures. Briefly, a three-layer photoresist pattern on a silicon wafer was created over 3 cycles of soft baking, exposure, and post-exposure baking (13), using multilayer photolithography and the SU-8 negative photoresists 3005, 3025, and 2075 (MicroChem, Corp., Newton, MA, USA). After development, the three-layered photoresist pattern was used as a master template for replica molding, in which polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI, USA) prepolymer was mixed with curing agent, cast between the photoresist pattern and a flat glass slide, and cured at 60°C for 2 h. Medium inlet ports and cell inlet ports were also fabricated from PDMS using an acrylic resin template and a glass plate. To assemble the microchamber array chip (Fig. 1A), these components were then bonded using O_2 plasma (PR500 plasma reactor; Yamato Scientific Co., Ltd., Tokyo, Japan) and curing adhesive (Cemedine Super-X No. 8008 Clear, Cemedine Co., Ltd., Tokyo, Japan).

By applying air pressure to medium inlet ports, four dyes were delivered into cell-culture microchambers without leakage (Fig. 1B), suggesting that four different cultures can be established at the same time. The detailed structure of a microchamber (depth 488.9 ± 11.1 μm, n = 16; diameter 1007.3 ± 19.7 μm, n = 45) and connecting channels is shown in Fig. 1C. The channels consist of a medium inlet branch (depth 15.1 ± 0.6 μm, n = 21; width 50 μm) and a cell inlet/medium outlet branch (depth 62.4 ± 2.4 μm, n = 24; width 300 μm). The detailed dimensions and fluidic parameters, including estimated flow rate and fluidic resistance, are listed for each channel in Table S1. The critical design element is that the shallow microchannels, including the flow rate control channel and medium-inlet branch channel, essentially determine the flow rate. Accordingly, the flow rate was easily calculated based on the applied pressure to the medium-inlet port and the fluidic resistance of the flow rate control channel, because the pressure drop in the latter was 94% of the former as shown in Table S1. In addition, the difference in flow rate through chamber 1 and through chamber 8 (Fig. 1B) was approximately 6%, considering the fluidic resistance in the medium-inlet branch channel, medium-inlet main channel, and cell-inlet/medium-outlet main channel. Thus, the 40-fold higher pressure drop in the medium-inlet branch channel than in medium-inlet main channel essentially determines the flow rate through the chamber. As in our previous designs (13), surface coating solutions and cell suspensions are loaded in reverse flow from a cell inlet/medium outlet port via a cell inlet/medium outlet main channel, while culture media are supplied in forward flow from a medium inlet port via four medium inlet main channels (Fig. 1A,C).

Hydrodynamic performance To test forward and reverse flow, 300 μL deionized water (Direct-Q 3 UV, Roche-Millipore, Billerica, MA, USA) was added to the medium inlet port or to the cell inlet port, and air pressure was applied at 5 kPa for 60 min and at 20 kPa for 20 min, respectively. The weight of water lost from the inlet port and recovered at the other end of the port was measured and converted to volume assuming a specific gravity of 1.0 g/cm^3. Similarly, latex beads (Count Bright Absolute Counting Beads, diameter 7 μm, C36950, Life Technologies) were added to the cell inlet port, which was then pressurized at 20 kPa for 4 min in reverse flow. The number of beads loaded into microchambers was quantified under a TS-100 microscope (Nikon, Tokyo, Japan). Subsequently, the distribution of the number of beads loaded was fit to the Poisson distribution

$$P(X = k) = \frac{\lambda^k e^{-\lambda}}{k!} \quad (1)$$

where X is the number of beads in a microchamber, k is a non-negative integer (0, 1, 2, 3, …), and λ is a positive real number equal to the expected value of X and to its variance.

Preparation of cell aggregates A uniform mix of PDMS precursor and curing agent was cast between two polyethylene terephthalate films separated by 1 mm silicone rubber spacers, and cured at 120°C for 2 h to generate 1 mm PDMS sheets. Square 8 mm × 8 mm holes were cut out of the film to generate frames (Fig. 3A),

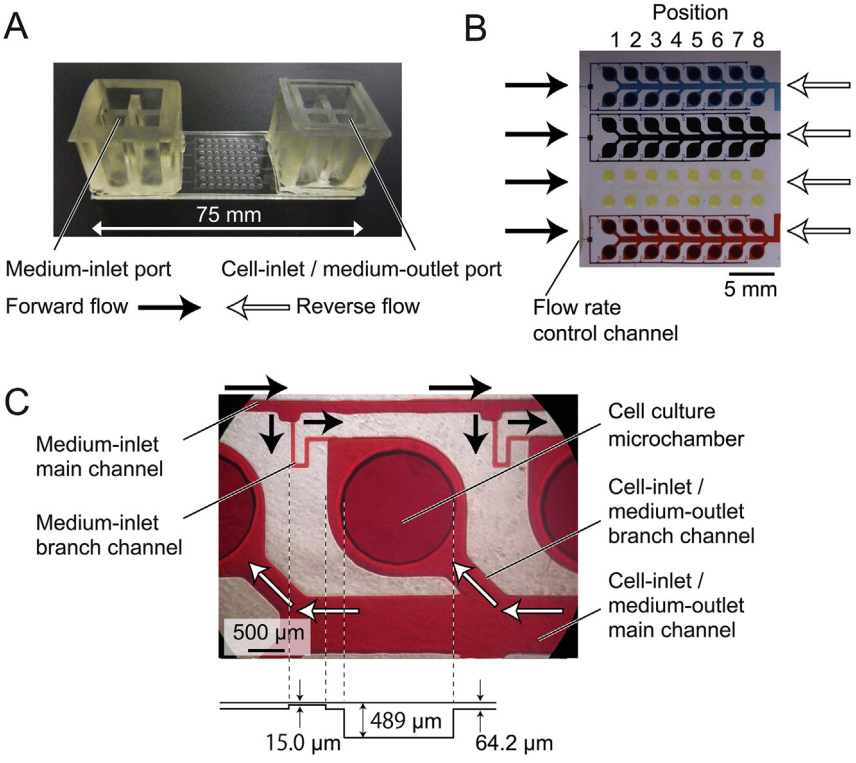

FIG. 1. A compartmentalized microchamber array chip with wide flow channels that can accommodate cell aggregates. (A) Overview and (B) enlarged top view of the microchamber array chip. In panel B, dyes were loaded to visualize the microfluidic networks, and microchamber positions are indicated. Positive air pressure is applied from the top of the medium inlet port for forward flow (perfusion) and from the top of the cell inlet/medium outlet port for reverse flow (coating and cell loading). (C) Top view (photograph) and side view (illustration) of a cell culture microchamber. The wide flow channels allow loading and culture cell aggregates, while compartmentalized microchambers enable fixed-point observation.

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