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Single-cell cloning and expansion of human induced pluripotent stem cells by a microfluidic culture device

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ABSTRACT

The microenvironment of cells, which includes basement proteins, shear stress, and extracellular stimuli, should be taken into consideration when examining physiological cell behavior. Although microfluidic devices allow cellular responses to be analyzed with ease at the single-cell level, few have been designed to recover cells. We herein demonstrated that a newly developed microfluidic device helped to improve culture conditions and establish a clonality-validated human pluripotent stem cell line after tracing its growth at the single-cell level. The device will be a helpful tool for capturing various cell types in the human body that have not yet been established *in vitro*.

1. Introduction

Microfluidic culture systems have many advantages for analyzing and manipulating cells, such as a confined culture area, precise control of medium flow, low consumption of reagents, and the ability to perform high-throughput analyses by designing parallel experiments on a single chip [1]. These features allow the microenvironments of cells, which consist of cellular and non-cellular biochemical, bioelectrical, and biophysical factors, to be mimicked [2,3]. These systems have been developed over the past two decades in many fields including drug screening [4], cell engineering [5], and developmental biology [6] where they have been used to analyze underlying mechanisms. Additionally, conventional static culture systems cannot avoid an accumulation of metabolites like ammonia and lactate, and are also unable to maintain consistent levels of nutrients. Constant culture medium composition surrounding the cells is important when investigating the cellular response to a specific factor.

Although the properties of a cellular population are generally analyzed as an average, many studies have indicated that each cell in a culture exhibits different behaviors [7]. To approach such clonal differences, each single cell and the progeny should be traced and separated from others within an analysis [8]. Furthermore, it is favorable for the cells being analyzed to be recovered and expanded as a cell line. Many studies using microfluidic devices have focused on analyzing cellular responses, whereas only a few have established clonal populations [9]. Generally it is difficult to recover cells from a microfluidic device after analyzing, because of its complicated structure. Therefore, we herein attempted to construct a simple microfluidic perfusion culture device that could culture cells for more than two weeks, and that could be removed from the base plate in order to recover the clonal population. All functions to maintain proliferation of cells were designed in a simple transparent polydimethylsiloxane (PDMS)-chip, except for a pump and medium reservoir. The clonality of the cells was validated by microscopic lineage tracing. We also attempted to optimize the culture conditions in order to improve cell-survival rates.

We chose human induced pluripotent stem cells (hiPSCs) as a model of adherent cells to be cultivated in the microfluidic device. hiPSCs hold promise as tools for regenerative medicine and drug discovery because of their unique abilities to proliferate and differentiate into all cell types in the human body [10,11].

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2. Materials and methods

2.1. Cell culture

hiPSCs were established from human fetal lung fibroblasts (TIG1, JCRB Cell Bank) through the retroviral induction of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* and stably maintained in mouse embryonic fibroblast (MEFs)-conditioned medium (CM) {DMEM/F12 (Sigma–Aldrich, Madison, WI, USA) supplemented with 20% knockout serum replacement (Life Technologies, Carlsbad, CA, USA), L-glutamine, non-essential amino acids, 2-mercaptoethanol, and 10 ng/ml bFGF (Peprotech, Rocky Hill, NJ, USA) on Matrigel-coated dishes. We used hiPSC-conditioned CM (cCM) for single-cell cloning.

2.2. Fabrication of the microfluidic culture device

Microchannels were constructed by grafting a PDMS layer onto a poly-L-lysine (PLL)-coated glass slide (Matsunami Glass Ind., Ltd., Kishiwada, Japan). The PDMS layer was fabricated using molding masters made with aluminum. Thermally curable PDMS (Silpot 184; Dow Corning Toray, Tokyo, Japan) was poured onto the mold to achieve a thickness of 3 mm and cured at 80 °C for 1 h in an oven. Two turndown microchannels (width, 0.5 mm; length, 20 mm; height, 0.5 mm) were formed in the PDMS layer when it was released from the mold. The device was then clamped together with the lid using retainer plates and bolts. Tubes (PTFE tube TUF-100 series AWG-30, Chukoh Chemical Industries, Japan) were attached to the inlet holes in the PDMS layer and connected to a peristaltic pump (Aquatech Japan, Inc., Osaka, Japan). The cell isolation device was constructed by adding a PDMS layer between the microchannel-formed PDMS layer and PLL-coated glass slide. The cell isolation PDMS layer formed 27 wells and was fabricated using the same method, except at a final thickness of 500 μm. The diameter of the wells was designed to be 1 mm.

2.3. Single hiPSC cloning in the microfluidic culture device or in dishes

The microchannel was coated with human recombinant laminin 521 (Veritas, Tokyo, Japan). hiPSCs were dissociated using 0.25% trypsin (Gibco, Carlsbad, CA, USA)/0.04% ethylenediaminetetraacetic acid (EDTA) and the cells were suspended in 400 μl of fresh medium at a density of 5.0×10^2 cells/ml. The cells were introduced into the inlets of the microchannels using a peristaltic pump at a flow rate of 21 μl/min. The cells were cultured in cCM at a flow rate of 5000 nl/min in a CO_2 incubator; on day 0, the cells were cultured without flow. To trace the clonal population in the dish, an adhesive tape with a grid pattern (AGC TECHNO GLASS CO., LTD, Tokyo, Japan) was placed on a laminin-coated 35 mm dish. hiPSCs were dissociated and seeded at 50 cells/cm^2 density.

2.4. Recovery and expansion of the clonal cell line

To extract the expanded colony, the PDMS layer was first removed in prewarmed DMEM from the glass plate. The single hiPSC-derived colony in the microchannel was picked up using a glass capillary tube (Drummond Scientific Co., Broomall, PA, USA) under phase contrast microscopy. The cells were reseeded on a laminin 521-coated 35 mm culture dish in CM. The cells were subcultured every 4 days by TrypLE Express (Gibco), and plated on laminin 521-coated dishes.

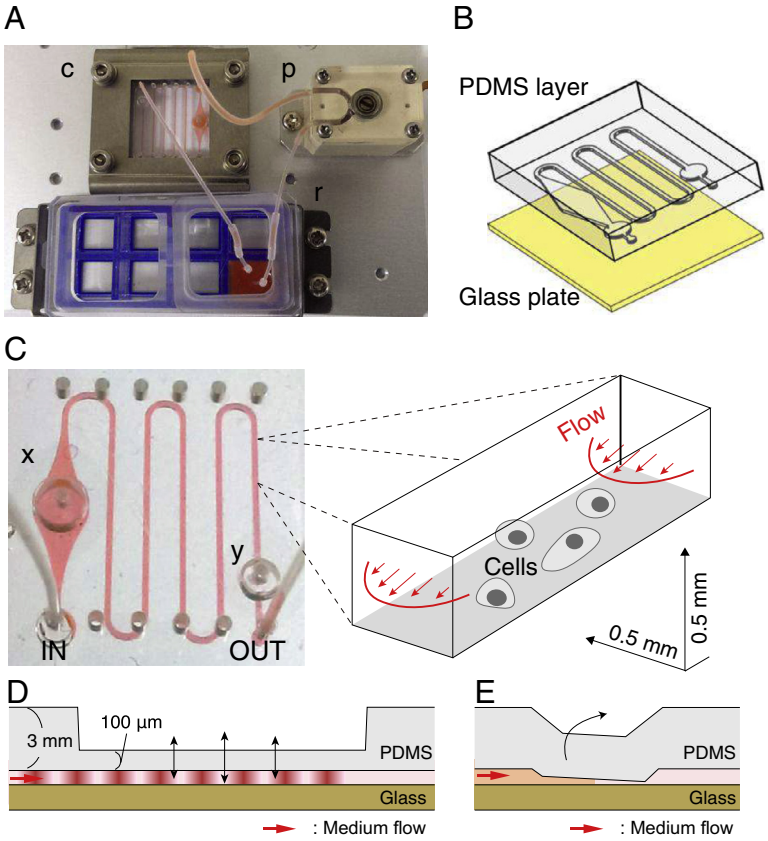

Fig. 1. Microfluidic culture device for single hiPSC cloning. (A) The microfluidic culture device contained a peristaltic pump (p), microchannel chip (c), and medium reservoir (r). (B) Structure of the microchannel chip. The PDMS layer containing the microchannel was placed on a glass plate. (C) Design of the microchannel chip. The microchannel containing the growth area was flanked by a diaphragm damper (x) and pressure valve (y). The right panel shows a scheme of microfluidic flow and the size of the microchannel. (D) Diaphragm damper. The thin PDMS layer constituting the roof of microchannel expanded and contracted to maintain a uniform flow rate. (E) Pressure valve. The PDMS pillar on the PLL-coated glass plate upregulated the pressure in the microchannel.

2.5. Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde/PBS for 10 min at room temperature, washed with PBST (0.1% Triton X-100 in PBS), and then pre-treated with blocking solution (3% BSA and 2% skim milk [Difco Laboratories Inc., Franklin Lakes, NJ, USA] in PBST) at 4 °C overnight. The cells were then stained with fluorescence-conjugated secondary antibodies (1:500; Life Technologies) following an immunoreaction with the following primary antibodies: anti-Oct-4 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-Nanog (1:200; Abcam, Cambridge, UK). The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted with a SlowFade Light Antifade Kit (Life Technologies).

2.6. Simulation of fluid dynamics

The Finite Volume Method (FVM) was employed to discretize the continuity and Navier–Stokes equations [12], which were solved in time-dependent, incompressible, and three-dimensional forms as described in Eqs. (1) and (2):

$$\frac{\partial U_i}{\partial x_i} = 0 \ (i = 1, 2, 3) \tag{1}$$

$$\rho_f \frac{DU_i}{D_t} = -\frac{\partial P}{\partial x_i} + \frac{\partial}{\partial x_j}\left(\mu \frac{\partial U_i}{\partial x_j}\right) \tag{2}$$

U_i is the velocity in the x_i direction and ρ and μ are the fluid density and viscosity, respectively. To discretize Eq. (2), a second-order central-difference scheme was employed for the diffusion terms and a first-order upwind scheme was employed for the convective terms. The semi-implicit method for pressure-linked equations (SIMPLE) algorithm was applied to solve the pressure correction. To solve the discretized algebraic equations, a linear-iterative method using the modified Tri-Diagonal Matrix Algorithm (TDMA) combined with an Alternating Direction Implicit (ADI) method was employed [13].

2.7. Quantitative PCR analysis

Real-time quantitative PCR with the Human Stem Cell Transcription Factors PCR Array (PAHS-501Z; Qiagen, Venlo, The Netherlands) was used to compare expression profiles. hiPSCs cultured in CM on a matrigel-coated dish at passage 29 were used as reference 1. hiPSCs acclimated to laminin-coated dishes by culturing for an additional 37 passages were used as reference 2. Two hiPSC clones established from the parental cell line at passages 21 and 96 by a microchannel were used as samples 1 and 2 after 13 and 10 passages on the dish, respectively.

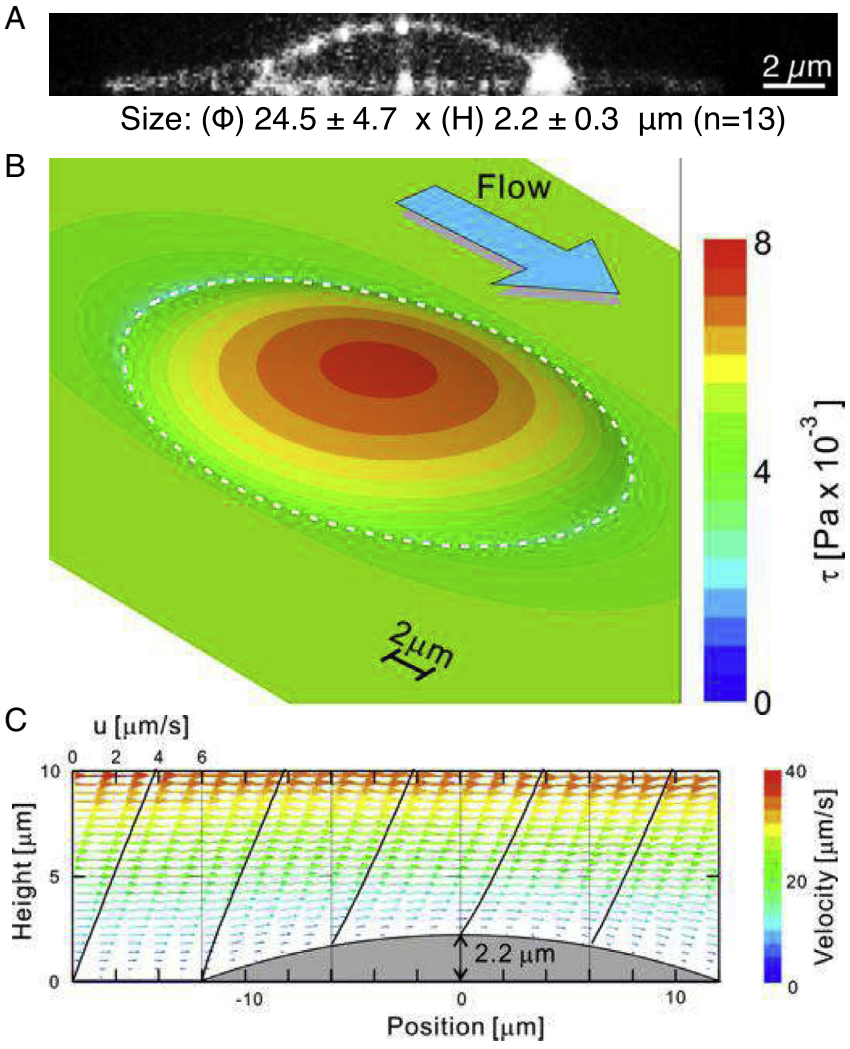

Fig. 2. Computational simulation of environments in the microchannel chip. (A) Representative confocal section of a hiPSC cultured in a microfluidic device. The size of the hiPSC was shown (Mean ± SD, $n = 13$). Φ: diameter, H: height. (B) Simulation of shear stress distribution over a channel bottom-attached hiPSC. The cell boundary is highlighted by the white dotted line. The arrow indicates the direction of fluidic flow at 5000 nl/min. (C) Simulation of the velocity vector and magnitude surrounding the bottom-attached hiPSC.

2.8. Karyotype analysis

G-band analysis was performed at Nihon Gene Research Laboratories Inc. (Sendai, Japan). After Giemsa staining, 50 cells from each sample were randomly selected and the normality of the chromosomes was analyzed.

3. Results

3.1. Establishment of a simple, flow-controllable microchannel culture device

The culture device with microchannels developed in this study is shown in Fig. 1A. The device included the following: (1) a microchannel chip (c), (2) medium reservoir (r), (3) peristaltic pump (p). The microchannel chip was composed of a PDMS layer containing an engraved microchannel and a glass plate that worked as the bottom of the channel (Fig. 1B). The PDMS layer (Fig. 1C) included a diaphragm damper (x) and pressure valve (y). The diaphragm damper, made from a PDMS thin film (diameter: 5 mm, thickness: 100 μm), eliminated the pulsing motion of pressure caused by the peristaltic pump and maintained a uniform flow rate of medium infusion (Fig. 1D). The pressure valve, made from a PDMS pillar downstream of the microchannel, maintained water pressure in the medium and prevented the generation of air bubbles caused by dissolved gases (Fig. 1E).

3.2. Computational simulation of the shear stress caused by medium flow

We used hiPSCs as a model cell line established from the retroviral transduction of TIG1, a human fetal lung cell line, by pluripotency-associated genes (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*). A preliminary culture revealed that cells could not survive without the flow of medium (Fig. S1). Therefore, computational simulations of fluid dynamics were performed to set the flow rate of medium before starting the perfusion culture. A simulation was conducted with a numerical code according to the finite volume method, and the immersed boundary method was used to estimate the force of shear stress against each single cell. Shear stress was calculated with two two-dimensional (2D) and one three-dimensional (3D) models (Fig. S2A and B). The 3D model was configured from the results of the confocal microscopic analyses. A typical hiPSC in the microchannels was approximately 550 μm^3 in volume, 24.5 μm in diameter, and 2.2 μm in height (Fig. 2A). The results of the simulation at a flow rate of 5×10^3 nl/min are shown as an example. The simulation indicated that shear stress was the greatest at the top of the cell surface (Fig. 2B). The results obtained also revealed that the highest shear stress on a cell (τ, [Pa]) and flow rate (Q, [nl/min]) in a channel (height = 500 μm × width = 500 μm) showed a close linear correlation ($\tau = 1.61 \times 10^{-6}\ Q$) (Fig. S2C). A nano-fluidic flow was generated at a velocity of nearly 5 μm/s in magnitude and approximately 250 nm from the hiPSC surface (Fig. 2C). Based on these results, we set the flow rate to 5×10^3 nl/min at approximately ~0.01 Pa (0.1 dyn/cm^2). A similar set point was used as a minimum in previous studies that assessed shear stress [14].

3.3. Investigation of appropriate culture conditions for hiPSCs in the microchannel

Single-cell suspensions of hiPSCs were prepared using trypsin and introduced to a microchannel. When a Matrigel-coated microchannel was used to maintain parental cells, many single hiPSCs were found to have adhered to the channel the next day; however, they could not proliferate and died within 3 days after their introduction ($n > 1000$). With laminin-coating, which was previously shown to support the single-cell survival of human pluripotent

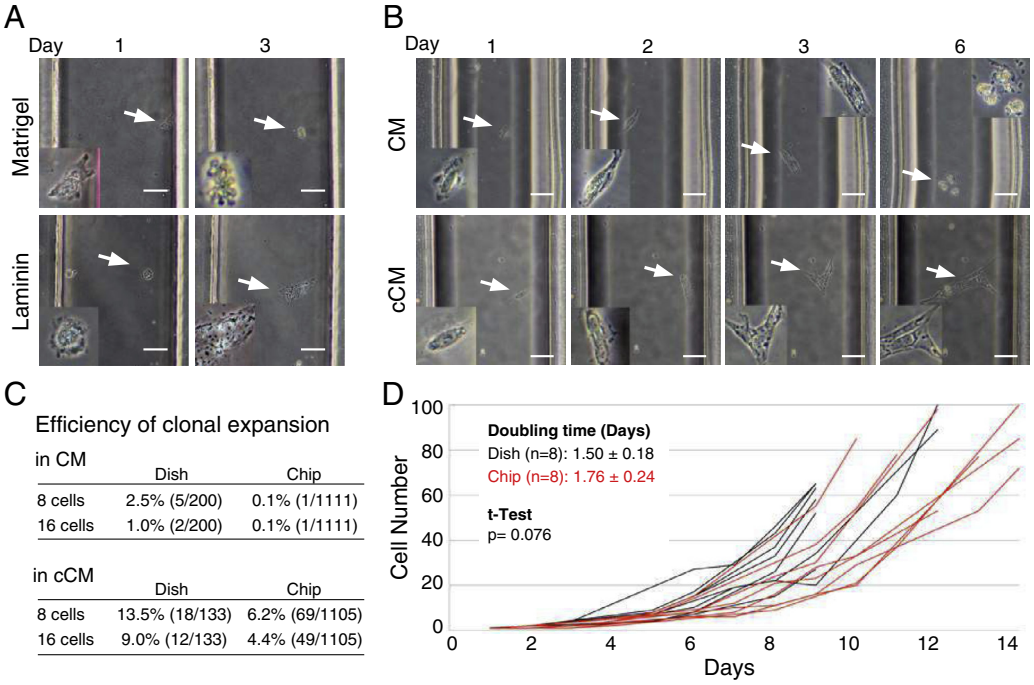

Fig. 3. Appropriate culture conditions for culturing a single hiPSC in the microchannel. (A) Determination of the basal matrix. Pictures of hiPSCs cultured on the matrigel- or laminin 521-coated microfluidic culture device on days 1 and 3 are shown. Insets are enlarged images of adhered cells (arrow). Scale bar: 100 μm. (B) Determination of the culture medium. Pictures of hiPSCs cultured with the culture medium (CM) or iPSCs-conditioned CM (cCM) on days 1, 2, 3, and 6 are shown. Insets are enlarged images of adhered cells (arrow). Scale bar: 100 μm. (C) The viability of a single hiPSC in CM or cCM. The rates of expansion from an adhered single cell to 8 or 16 cells in the conventional culture dish (Dish) or in the microfluidic culture device (Chip) are shown. (D) Growth curves of 8 clones expanded by Dish (black lines) or Chip (red lines). The doubling time was estimated from an exponential approximation of the growth rate. $p = 0.076$, using the two-sided Student's t-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

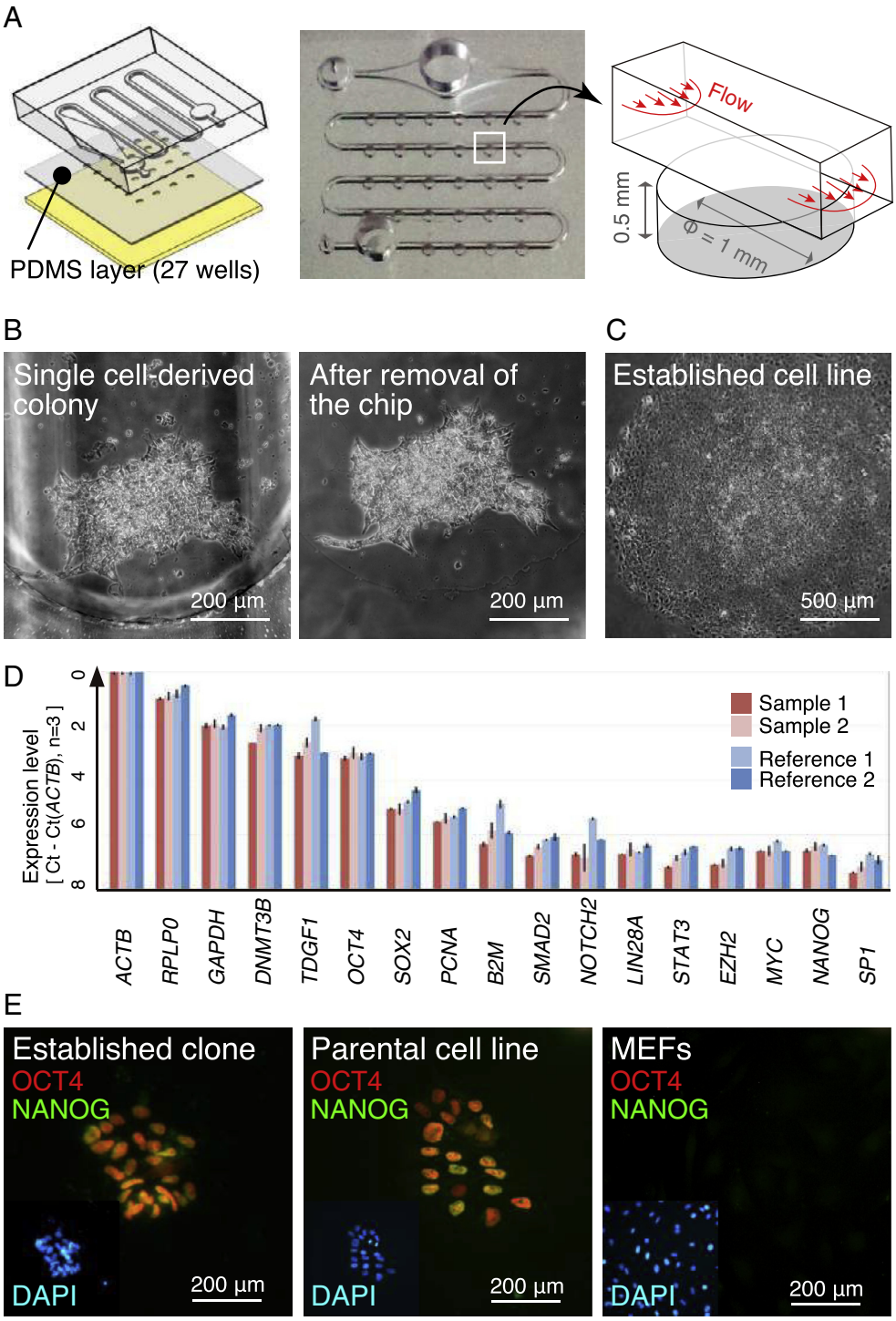

Fig. 4. Clonality-validated hiPS cell line established by the microchannel chip. (A) The single cell isolation microchannel chip. An additional PDMS layer containing 27 wells was placed between the microchannel layer and glass plate (left). An image of the whole chip (middle) and design of a single cell isolation-well (right) are shown. *Φ*: diameter. (B) Recovery of expanded cells from the chip. The expanded colony was recovered by removing the PDMS layers containing the microchannel and isolation well from the glass plate. Phase contrast images of the hiPSCs colony before (left) and after (right) the removal are shown. (C) Phase contrast image of a colony of the established clonal hiPSC line. Recovered and expanded cells showed the conventional hiPSC-like morphology. (D) Gene expression profile of the single cell-derived and their parental cell lines. The results of quantitative PCR from single cell-derived clones established by the microchannel chip (Sample 1, 2) and parental hiPSCs (Reference 1, 2) are shown. $n = 3$, Error bars represent the standard deviation. (E) Immunocytochemistry of pluripotency-associated proteins. The results showed that the clone established by the microchannel chip expressed NANOG (green) and OCT4 (red) proteins similar to the parental cell line. Cell nuclei were counterstained with DAPI (blue).

stem cells [15], the survival and proliferation of the introduced cells could be observed on day 3 (Fig. 3A). However, the efficiency of cell survival/proliferation in the microchannel was approximately 10-fold lower than that of a conventional culture. To further optimize culture conditions, the culture medium (CM) was preconditioned with hiPSCs to complement the niche provided

by the hiPSC itself in the dish culture. hiPSC-conditioned CM (cCM) was capable of supporting survival/proliferation efficiencies at similar levels to those of the conventional culture (Fig. 3B and C). No significant differences were observed in the doubling time of surviving cells between the microchannel and in dishes ($p = 0.076$) (Fig. 3D).

3.4. Clonality-validated hiPSC line established by the microchannel device

Although a single-cell culture of a hiPSC could be achieved with the microchannel, flanked populations may still have made contact with each other within the proliferation process. To eliminate the possibility of contact, a small well-like structure was introduced into our device. A multiwell layer composed of 27 thin PDMS-containing punched wells (diameter, 1 mm) was placed under the microchannel layer so that a single hiPSC was segregated and cultured in a well clonally (Fig. 4A). Other cells in the microchannel layer were kept in the channel or flushed out by the initial medium-flow and did not attach to the glass plate. A hiPSC was successfully observed and subsequently cultured at a flow rate of 5000 nl/min with cCM. The absence of contamination from other wells was validated everyday under a microscope. After the clonal population was adequately expanded (day 10–15), PDMS layers were peeled from the glass plate (Fig. 4B). The clonal colony was picked manually and propagated in a conventional manner in order to establish a cell line using CM (Fig. 4C).

3.5. Microchannel chip-derived cell lines were equivalent to parental cell lines

To determine whether cellular properties were retained, the expression profiles of human stem cell-associated genes (genes related to housekeeping: *ACTB, RPLP0*, *GAPDH,* and *B2M*, epigenetics: *DNMT3B* and *EZH2*, pluripotency: *TDGF1, OCT4, SOX2, SMAD2, NOTCH2, LIN28A, STAT3, MYC, NANOG,* and *SP1*, and proliferation: *PCNA*) were assessed by PCR arrays. The results obtained revealed no significant changes in gene expression profiles within clonal expansion (Fig. 4D). The retention of cellular properties was further validated by the immunofluorescence of the pluripotency-associated proteins, OCT4 and NANOG (Fig. 4E). The expression levels of those proteins were similar to those of the parental cell lines. To confirm that single hiPSC-derived clones were normal, we examined karyotype abnormalities (Fig. S3). The expanded clones isolated by the microchannel chip showed no chromosomal abnormalities by G-band analysis.

4. Discussion

In the present study, we developed a simple microfluidic device that could stably and precisely control the flow of culture medium in channels and could also culture and trace a single cell and its progeny. The dumper element, simply formed by a thin layer, effectively removed the oscillations in flow caused by the peristaltic mechanism of the pump, and achieved a precisely controlled flow-rate (Fig. 1D). Although PDMS-based microfluidic technologies have been used widely, the undesirable accumulation of air bubbles sometimes becomes an obstacle especially in long-term cultures [16]. We added an air-trap structure to the inlet (data not shown) and pillar-like resistance downstream of the microchannel to remove or prevent bubbles (Fig. 1E). Therefore, our device successfully supported a perfusion culture for more than 15 days without stacking. The wells integrated in the device helped to keep each adhered cell isolated (Fig. 4A and B), and clonality was validated by tracing cell proliferation at the single-cell level under a microscope. On the other hand, unwanted cells were segregated from the glass plate; therefore, only the clonal population of interest could be recovered easily by manually removing the PDMS layers for further analyses. Cellular morphologies, gene expression profiles (Fig. 4C–E), and karyotypes (Fig. S3) showed that single cell-derived hiPSCs had no obvious abnormalities and maintained the characteristics of its parental cell line.

The combination of matrigel-coated dishes and mouse embryonic fibroblast (MEF)-conditioned medium is widely used to practice feeder-free cultures [17]. However, a previous study reported that a human pluripotent stem cell underwent apoptotic cell death with single-cell dissociation under these conditions [18]. We used laminin 521, which was shown to support the survival and self-renewal of human embryonic stem cells as the basement membrane [15]. Although the laminin coating could improve cell survival as expected, cell death was still prominent when the conventional culture medium was used in the present study. The results obtained showed that the conditioning of medium by hiPSCs greatly supported clonal survival (Fig. 3C), which indicated that a niche was provided by the hiPSC itself to survive and also that conditioning by MEFs was not sufficient as essential extracellular stimuli. A previous study reported that human pluripotent stem cells formed heterogeneous colonies, and a subset of cells provided an IGF signal to control the FGF stimuli in order to maintain pluripotency [19]. hiPSCs may not be able to provide adequate amounts of niche signals including IGF in a small population. This insufficiency may be one of the reasons why the growth rates of hiPSCs were slower in our study (approximately 40 h) than previous findings (~30 h) [20]. These issues regarding the microenvironment are not apparent when handling cells as a population because of sufficient cell density. Single-cell-based assays using a microfluidic device will be helpful for elucidating the mechanisms involved in producing and maintaining microenvironments. The cells examined in the device have to be expanded in order to analyze their characteristics as well as the appropriateness of the culture conditions used. Many types of microfluidic devices that can achieve various conditions in a single chip have been described previously. With this knowledge, devices that can simultaneously assay many culture conditions (including basements, growth factors, and chemical compounds) and establish a clonal cell line from each condition will be realized. Various types of cells in the human body that have not been maintained *in vitro* can be cultured by analyzing/mimicking the appropriate physiological conditions with these devices.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.081.

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