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Differentiation of Neural Progenitor Cells in a Microfluidic Chip-Generated Cytokine Gradient

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ABSTRACT

In early embryonic development, spatial gradients of diffusible signaling molecules play important roles in controlling differentiation of cell types or arrays in diverse tissues. Thus, the concentration of exogenous cytokines or growth factors at any given time is crucial to the formation of an enriched population of a desired cell type from primitive stem cells in vitro. Microfluidic technology has proven very useful in the creation of cell-friendly microenvironments. Such techniques are, however, currently limited to a few cell types. Improved versatility is required if these systems are to become practically applicable to stem cells showing various plasticity ranges. Here, we built a microfluidic platform in which cells can be exposed to stable concentration gradients of various signaling molecules for more than a week with only minimal handling and no external power source. To maintain stability of the gradient concentration, the osmotic pumping performance was optimized by balancing the capillary action and hydraulic pressure in the inlet reagent reservoirs. We cultured an enriched population of neural progenitors derived from human embryonic stem cells in our microfluidic chamber for 8 days under continuous cytokine gradients (sonic hedgehog, fibroblast growth factor 8, and bone morphogenetic protein 4). Neural progenitors successfully differentiated into neurons, generating a complex neural network. The average numbers of both neuronal cell body clusters and neurite bundles were directly proportional to sonic hedgehog concentrations in the gradient chip. The system was shown to be useful for both basic and translational research, with straightforward mechanisms and operational schemes. STEM CELLS *2009;27:2646–2654*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Stem cells can both self-renew and differentiate into multiple cell types. Such cells have thus attracted great interest in the fields of both regenerative medicine and biopharmaceuticals. In particular, embryonic stem cells (ESCs) have been shown to offer higher potential for proliferation and differentiation than do adult stem cells, and ESCs recapitulate early embryonic development. Therefore, to obtain large numbers of clinically relevant cell types, it is essential to develop methods encouraging ESCs along desired developmental paths under conditions similar to those of normal organogenesis.

Many studies of vertebrate development show that a multiplicity of signaling molecules passes between different tissues and organs, fulfilling important roles in linkage of cell differentiation to organogenesis [1]. A cell is believed to recognize its position in a concentration gradient of extracellular signaling molecules, and the cell's developmental fate is determined accordingly [2]. Therefore, understanding the effects of both the concentration gradient and the absolute concentration of signaling molecules that influence cell fate commitment may provide useful information for controlling and optimizing the differentiation of human ESCs into specific cell types in vitro. Recently, technological advances in microelectromechanical systems (MEMS) have opened the possibility of the use of miniaturized systems for many different biological applications. Previous studies demonstrated that on-chip-based microfluidic devices could be used for microscale cultures, creating specific cellular microenvironments [3–5] as well as analysis, processing, and manipulation of biological samples [6–9]. Therefore, BioMEMS technologies are expected to allow for more effective investigations in a broad range of biomedical applications, ranging from microscale cultures to drug screening on a high-throughput scale. In this respect, the microfluidic gradient chip is a powerful tool for studying the effects of different concentrations of signaling molecules compared with a traditional cell culture system.

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However, as listed in the following paragraph, current microfluidic systems pose many limits that must be solved before chip applications to the study of stem cell differentiation for the successful rganogenesis.

Some requirements of microfluidic systems used for stem cell differentiation in a concentration gradient of signaling molecules are discussed below. (a) Isolation: the system should be isolated. Cells must be maintained under sterile conditions; hence any peripheral systems such as pumps or air pressure conduits, which usually connect an incubator with the nonsterile environment, are unacceptable. (b) Long-term use: a long-term culture should be possible under a stable concentration gradient by minimizing the consumption of culture media or signaling molecules. Particularly in stem cell research, the required cell culture period often exceeds multiple weeks. Thus, long-term maintenance of a stable growth factor concentration gradient with minimum consumption of costly reagents is required. (c) Ease of use: the use and maintenance of the system should be simple and straightforward; delicate components that are vulnerable to temperature, light, and operator (lack of) skill should be eliminated, and the operational scheme should be simple. (d) Biological safety: side effects such as nonphysiologic shear stresses on cells should be avoided as much as possible during culture because long-term exposure to such side effects may have undesirable outcomes. (e) Portability: the entire setup should be small. Although current microfluidic systems are very simple and small, peripheral devices are often complex and large. The experimental setup should be portable so that the microfluidic system can be a component of other biological experimental systems. From this point of view, passive device operation seems highly desirable. (f) Accessibility: cell observation and functional assays should be convenient. Real-time observation should be possible, and the cellular assays in the microfluidic network should be operated easily and efficiently.

Among the various specialized cells differentiating from ESCs, neural stem cells (NSCs) have attracted much attention [10, 11]. During development of the vertebrate nervous system, distinct classes of neurons appear in precise locations along the dorso-ventral (D-V) and anterior-posterior (A-P) axes of the neural tube. Sonic hedgehog (Shh) and bone morphogenetic protein (BMP) were shown to influence cell fate along the D-V axis, [12] whereas fibroblast growth factor (FGF)-2, FGF8, retinoic acid, and Wnt1 can change cell fate along the A-P axis (Fig. 1) [13]. Despite strong evidence for existence of these positional cues, little is known about how the various factors influence proliferation and differentiation of neural progenitor cells. Earlier, human NSCs were cultured in a microfluidic device with a growth factor gradient [14]. A microfluidic device in which postnatal primary hippocampal neurons were cultured at low densities to analyze local cell-to-cell neuronal signals and to investigate substrate biocompatibility has been described [15]. However, although these systems fulfilled research purposes, they lacked one or more of the desirable features outlined above. For example, high shear stress was applied on cells, and this may have biased the results; bulky peripheral devices prevented isolation of the system; or neuronal growth was tested in the absence of biomolecular gradients. Thus, more versatile microfluidic devices are required to provide more in vivo-like realistic environments, including maintenance of concentration gradients of signaling molecules.

In this study, we examine the effect of a concentration gradient of extracellular signaling molecules on survival, proliferation, and differentiation of human ESC-derived neural progenitor cells using a microfluidic gradient chip satisfying most of requirements described above. To this end, we developed an improved microfluidic system enabling long-term (more than 1 week) culture of stem cells in a stable concentration gradient with a very slow and controlled liquid flow generated by an osmotic pump [16]. No peripheral devices were required. An interstitial flow speed of only a few micrometers per second was achieved, giving a Péclet number (*Pe*) close to unity, so that unwanted effect of shear stress was avoided. Other innovations will be described below.

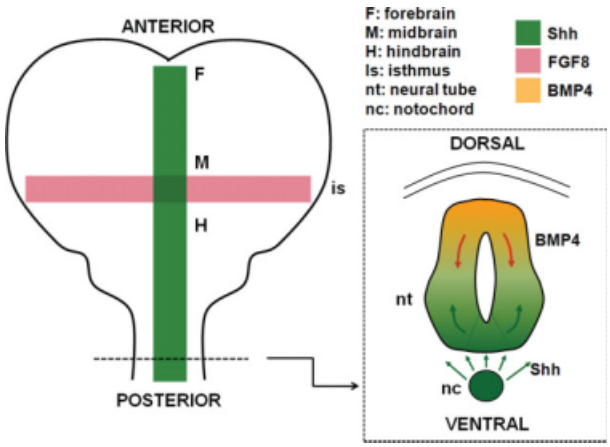

Figure 1. The interplay between Shh, FGF8, and BMP4 that specifies neuronal identities along the dorso-ventral and anterior-posterior axes during the early development of the vertebrate nervous system. On the left is a ventral view of the neural tube. Inductive signaling molecules known to control neuronal identities originate from the neural plate at the mid-hindbrain junction (FGF8, red) and floor plate (Shh, green). A transverse section of the neural tube is shown on the right. A gradient of Shh (green) specifies ventral neurons, whereas a BMP gradient (orange) specifies dorsal neurons. Abbreviations: BMP4, bone morphogenetic protein 4; FGF8, fibroblast growth factor 8; Shh, sonic hedgehog.

Materials and Methods

Microfluidic System Design

The improved platform and operational scheme are shown in Figure 2. As summarized in our previous work [16], the system is based on an osmotic pump and a gradient generated by two laminar flows (Fig. 2A); solution A and solution B of different concentrations are introduced into the main channel by an osmotic pump (the osmosis occurs between water and 0.082 M polyethylene glycol [PEG] solution that drives very steady and slow flow). At the interface of the two solutions, diffusion generates a concentration gradient due to such slow speed. The main microchip channel was 4 mm in width, 10 mm in length, and 250 μm in height (supporting information Fig. 1).

We used 1,000-μL micropipette tips (T-1000; AxyGen Inc., Union City, CA, http://www.axygen.com) as inlet reservoirs (supporting information Fig. 2). Inlet reservoirs need to be carefully chosen because even slight flow instability between two inlets may cause a large gradient distortion profile in the main channel. We therefore analyzed the hydrodynamic pressure balance and the capillary force of the liquid surface of the inlet reservoir (Fig. 2B). The main driving force for fluid flow was generated by the osmotic pump (Fig. 2C); note that flow is not caused by the hydraulic pressure of the inlet reservoir because this pressure is negligible compared with osmotic pressure applied at the membrane of the osmotic pump. Osmosis was achieved by concentration difference between pure water and a high level of PEG (0.082 M) in the PEG reservoir, separated from water by a cellulose membrane. In addition, we increased the capacity of the outlet

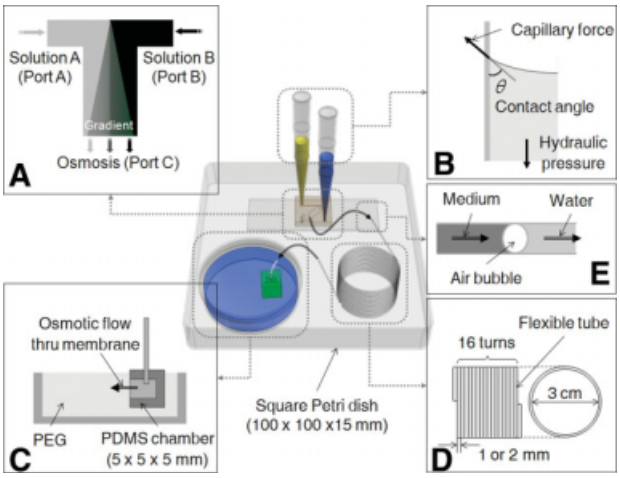

Figure 2. Experimental setup. The system consists of four different components **(A–D). (A):** A concentration gradient in a 4 mm-wide microchannel is achieved by an osmotic pump. **(B):** Micropipette tips were used as inlet reservoirs. **(C):** The osmotic pump was dipped into the PEG solution reservoir to generate the main driving power of the system; and **(D)** the coiled tube has a large liquid capacity and was used as an outlet reservoir. **(E):** The spent medium flow from the outlet was separated from the distilled water flowing to the osmotic pump unit. Abbreviations: PDMS, poly(dimethylsiloxane); PEG, polyethylene glycol.

reservoir by winding the flexible tube to increase length while maintaining compact size (Fig. 2D).

Fabrication of Chip

We fabricated the microchannel using a conventional soft lithographic process using poly(dimethylsiloxane) (PDMS) [17, 18]. For the osmotic pump, PDMS cubic chambers (1 × 1 × 1 cm) with a window of cellulose membrane (5 × 5 cm) were fabricated (Fig. 2C); bonding of cellulose membrane to the PDMS chamber was performed using PDMS solution as adhesive. The osmotic flow rate was 0.15 μL/hour when 0.082 M PEG solution was used [16]. Two micropipette tips were filled with the same amounts of medium and plugged into each inlet, serving as inlet reservoirs. For the outlet reservoir we used a wound, flexible polyurethane tube (Polyethylene Tube; Natume Seisakusho, Tokyo, http://www.nazme.co.jp/index.html; tube diameter: 1 mm or 2 mm) with a coil diameter of 3 cm (Fig. 2D). One end of the flexible tube (filled with distilled water before connection) was connected to port C, and the other end to the osmotic pump. The PEG reservoir was a 60-mm Petri dish containing approximately 10 ml of 0.082 M PEG solution.

Visualization of Protein Gradient Generated in the Microfluidic Chip

To investigate the protein gradient in the main channel of the chip, inlet ports A and B were respectively loaded with cholera toxin subunit B conjugated with the fluorescent dye Alexa Fluor 488 or 594 (1 mg/ml; Molecular Probes, Eugene, OR, http://probes.invitrogen.com). Due to the difficulty of directly measuring the concentration gradient of cytokines used in our study, we used cholera toxin subunit B as a substitute. After stabilizing the gradient for 30 minutes, the fluorescence of the protein conjugates was visualized using a fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany, http://www.leica.com).

Diffusion flux (J), governed by the Fick's law, is expressed as the following formula: $J = -D(dc/dx)$, where c is the concentration, x is the distance, and D is the diffusivity. Here, D is equivalent to $kT/(6\pi\mu r)$ by the Einstein relation for the diffusion of particles, where k is Boltzmann's constant, T is the absolute temperature, μ is the fluid viscosity, and r is the particle radius. Thus, D is inversely proportional to the diffusing molecule's radius, r. Accordingly, we measured the fluorescent intensity of cholera toxin subunit B (57 kDa) and extrapolated the gradient profile to get the profile of the cytokines (~20 kDa for Shh, BMP4, and FGF8).

Directed Differentiation of Neural Progenitor Cells from Human ESCs

To obtain neural progenitor cells, human ESCs (HSF6) were differentiated as previously described [19]. Briefly, human ESC colonies were harvested by treatment with collagenase type IV (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), gently triturated, and cultured for 8 days in suspension culture dishes (Corning Costar, Corning, NY, http://www.corning.com/index.aspx) containing human ESC culture medium without human basic fibroblast growth factor (bFGF), to form embryoid bodies (EBs). The EBs were then plated onto tissue culture dishes in insulin/transferrin/selenium/fibronectin medium [19]. Over a time span of 8 days, cells exhibiting the morphology of neuroepithelial cells were observed and rosette formation was visualized in differentiating EBs. To obtain a large number of highly purified neural progenitor cells, differentiating cells from EBs were dissociated with 0.05% (wt/vol) trypsin (Invitrogen) and transferred to plates coated with poly-L-ornithine (PLO; 50 μg/ml; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and laminin (5 μg/ml; Roche, Mannheim, Germany, http://www.roche.com) at a density of 3×10^5 cells/cm^2 in N2 medium containing bFGF (20 ng/ml) [19]. All experimental procedures involving human ESCs were approved by the Minister for Health, Welfare, and Family Affairs, Korea, and the Korean Stem Cell Research Center (Institutional Review Board permit number 24).

Flow Cytometry

The purity of hESC-derived neural progenitor cells was quantitatively analyzed by flow cytometry. Briefly, cells were fixed and permeabilized with BD Cytofix/Cytoperm buffer (BD Biosciences, San Diego, http://www.bdbiosciences.com) for 20 minutes at 4°C, and washed twice with BD Perm/Wash buffer (BD Biosciences). Cells were next sequentially incubated with primary antibody to Nestin (R&D Systems, Minneapolis, http://www.rndsystems.com) and immunofluorescent secondary antibody, and finally analyzed using a FACSCalibur and CellQuest software (BD Biosciences).

Microfluidic Culture of Human ESC-Derived Neural Progenitor Cells

After 4 days of propagation, human ESC-derived neural progenitor cells were detached from culture dishes using 0.05% (wt/vol) trypsin, washed, and resuspended in N2 medium containing bFGF (20 ng/ml). Cells (100 μL of a suspension of 5×10^6 cells/ml) were loaded into a microchannel coated with PLO (50 μg/ml) and laminin (5 μg/ml) via outlet port C (Fig. 2A), using a micropipette. Adherent neural progenitor cells were exposed to media containing Shh (500 ng/ml), FGF8 (100 ng/ml), or BMP4 (10 ng/ml) for 4 days (all growth factors were from R&D Systems), and the cells were then terminally differentiated in N2 medium without bFGF or other cytokines for an additional 4 days. To compare the effect of cytokines in a microfluidic device with that in a traditional culture system, the detached neural progenitor cells were also plated on PLO/laminin-coated culture dishes and grown in the same culture medium.

Immunocytochemistry

Before and after culturing of human ESC-derived neural progenitor cells inside microfluidic devices, cells were fixed in cold 4%

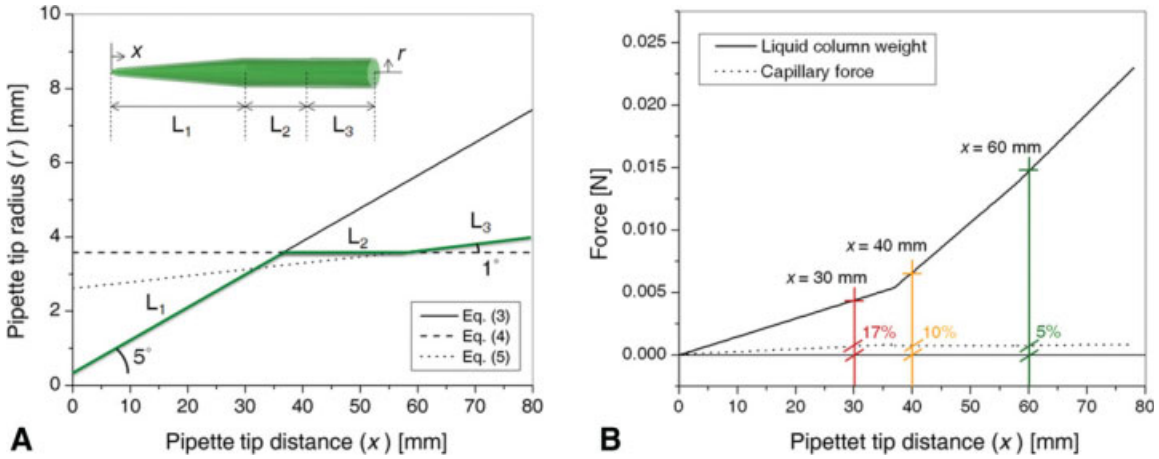

Figure 3. Analysis of the micropipette tip. **(A):** The inner radius of the tip is measured at three sections: L_1, L_2, and L_3. **(B):** Hydraulic pressure is linearly dependent on the height of the liquid column. As the liquid height falls, capillary action (a surface tension force) becomes dominant. Abbreviation: Eq., equation.

(vol/vol) paraformaldehyde for 20 minutes at 4°C. After blocking and permeabilization with 0.3% (vol/vol) Triton X-100 and 10% (vol/vol) goat serum in 0.1% (wt/vol) bovine serum albumin/phosphate-buffered saline, cells were probed with primary antibodies to Nestin (R&D Systems) or class III β-tubulin (TuJ1; Covance, Denver, PA, http://www.covance.com). After incubation with primary antibodies, cells were washed and probed with appropriate Alexa Fluor secondary antibodies (Molecular Probes) for 90 minutes in the dark at room temperature. Finally, fluorescent images were captured using a fluorescence microscope (Axiocam; Carl Zeiss, Thornwood, NY, http://www.zeiss.com) after counterstaining with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), and the image was analyzed using ImageJ software (NIH, Bethesda, MD).

Statistical Analysis

For quantitative data, the values are expressed as mean ± SD. For the determination of statistical significance, results were analyzed by a one-way analysis of variance followed by the Tukey's multiple comparisons test with the appropriate control. p values less than .05 were judged to be statistically significant.

Results

Performance of System Components

A steady and stable microchannel flow is very important for maintenance of the concentration gradient. To this end, the flows from the two inlet ports A and B (Fig. 2A) must be accurately balanced; the easiest strategy is to use the hydrodynamic pressure of the liquid columns to this end. Micropipette tips are very convenient in this regard because the user can control the amounts of sample solution, up to a maximum of 2000 μL. However, we observed in preliminary experiments that the gradient pattern was not well maintained, especially in the later stages of culture (supporting information Video 1). This was attributable to so-called liquid molecular events. These included surface tension mediated by cohesive forces between liquid molecules, and capillary action occurring when adhesive interaction forces between liquid molecules and walls were stronger than cohesive forces. As micropipette tips do not have ideal surface roughness, uncontrolled capillary action may occur. The micropipette has a cone-shaped geometry (T-1000 model: diameter 7.8 mm at the top and 0.7 mm at the bottom; length about 78 mm); it was essential to determine the height of the liquid column that would allow capillary action to be neglected. The hydraulic pressure (P) of a liquid column is presented simply as

$$P = \rho g h \tag{1}$$

where ρ is the liquid density (1000 kg/m^3 for water), g is the gravitational acceleration (9.81 m/s^2), and h is the height of the liquid column (m). However, because of capillary action, the upward force acting around the circular circumference (F) is

$$F = \gamma \cos\theta 2\pi r \tag{2}$$

where γ is the surface tension (0.07 N/m by water at 35°), θ is the angle of contact (Fig. 2B), and r is the inner diameter of the micropipette tip (m). We experimentally measured the contact angle of distilled water on the pipette tip wall; the receding contact angle of the micropipette (62.5°) was much smaller than the advancing contact angle (97°) (supporting information Fig. 3), and this should be considered in calculation of the capillary force. The height of the liquid column lifted by the capillary force is derived from the difference between the liquid column weight (W) and F. For derivation of W and F, the geometry of the tip must be considered. From the manufacturer's design drawings, we constructed three first-order linear functions for r as follows:

$$R = 0.0003188 + 0.08868x \text{ at } L_1 \tag{3}$$

$$R = 0.0036 \text{ at } L_2 \tag{4}$$

$$R = 0.002098 + 0.0259x \text{ at } L_3 \tag{5}$$

where x is the distance from the pipette tip, and L_1, L_2, and L_3 are sections of 0–37 mm, 37–58 mm, and 58–78 mm, respectively, as indicated in Figure 3A. Here, r and x are in meters. By applying Eqs 3–5 to Eq 2, we obtained F; and, using simple calculus and mathematical formulae, we could derive W for each of the sections L_1, L_2, and L_3 (see supporting information data for detailed derivation):

$$F = 0.000127 + 0.03535X \text{ and } W = 0.146x \text{ at } L_1 \tag{6}$$

$$F = 0.001371 \text{ and } W = -0.009378 + 0.3994x \text{ at } L_2 \tag{7}$$

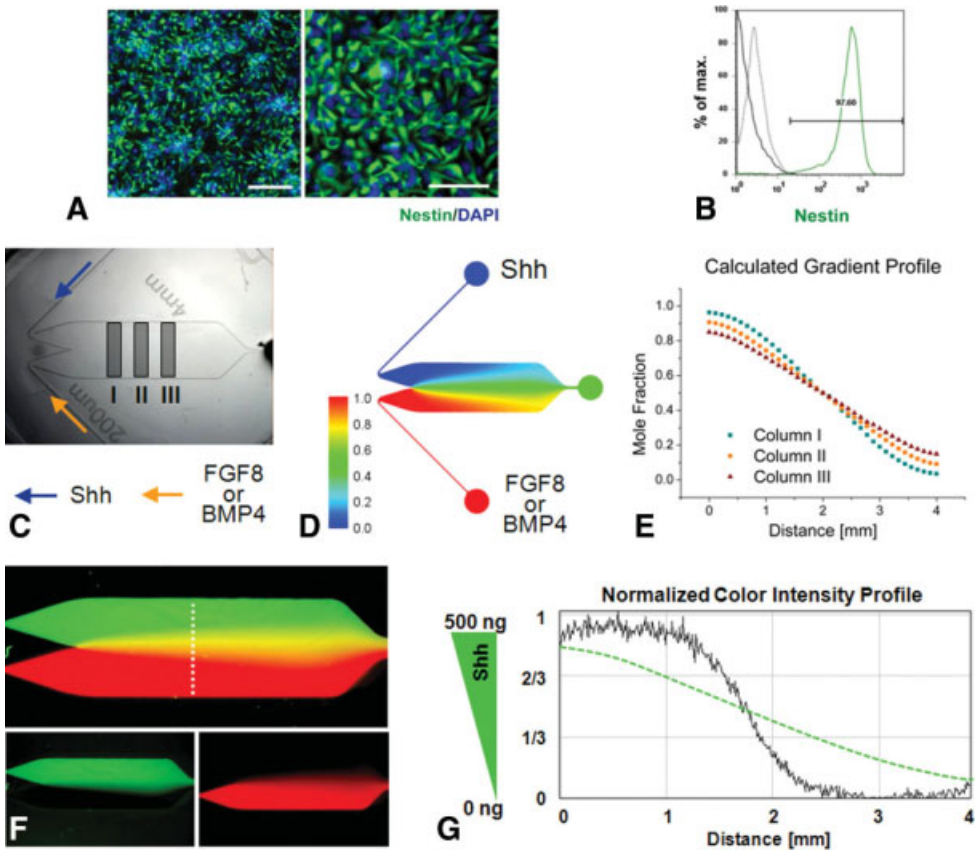

Figure 4. Differentiation of human ESC-derived neural progenitor cells in the microfluidic gradient chip. The purity of the input cell population was confirmed by immunostaining **(A)** and flow cytometry **(B)** using antibody against Nestin. Scale bars = 100 μm (**A**, left) and 50 μm (**A**, right). A flow cytometric plot **(B)** shows the no-stain control profile (black solid line), the isotype control IgG-staining profile (black dotted line), and the Nestin staining profile (green solid line). After seeding Nestin-positive neural progenitor cells into the gradient chip, two pairs of cytokines were added to the culture medium: Shh and FGF8; Shh and BMP4. **(C):** Two different cytokine flows (Shh vs. FGF8 or BMP4) in the main channel in which three observation window columns (I, II, III) were located. **(D–E):** Gradient profile estimated by the computational simulation. Almost linear profiles were developed in the three columns, and the error ranges were below 5%. **(F):** Visualization of the concentration gradient of fluorescent-labeled proteins (cholera toxin subunit B conjugated with Alexa Fluor 488 and 594). **(G):** Normalized color intensity (green fluorescence) shows that the gradient profile covers about one third of the main channel, implying the cytokine gradient profiles (e.g., Shh) will follow the green dotted line. Abbreviation: BMP4, bone morphogenetic protein 4; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; FGF8, fibroblast growth factor 8; max., maximum; Shh, sonic hedgehog.

$$F = 0.0008071 + 0.009963x \text{ and}$$
$$W = -0.004887 + 0.4596x \text{ at } L_3 \quad (8)$$

Plots of Eqs 6–8 clearly show the interaction of hydraulic pressure (derived from the weight of the liquid column) and capillary force (Fig. 3B). As x approaches 0, surface tension is dominant. Our experience suggested that the liquid column (x) should be kept higher than 40 mm for prevention of flow fluctuation caused by capillary action, which means that the magnitude of F in Eq 2 is recommended to be 10% of hydraulic pressure force during the entire period of cell culture. A margin of 5% prevents even minor fluctuations in gradient profile. A margin of 17% may cause spontaneous fluctuation. Thus, the liquid height should be no less than 30 mm and the reservoirs should be refilled during culture.

Although it is easy in practice to replace the osmotic pump at any time, a few simple precautions eliminate the need for this extra handling. The reservoir for spent reagents should be of adequate volume. To retain a compact size while enlarging volume capacity, we wound the flexible tube 16 times around a cylinder of diameter 3 cm (Fig. 2D). We thus achieved approximately 1,200 μL of reservoir capacity. When it is considered that the typical osmotic pumping flow rate is 15 μL/hour, we could maintain pumping for 80 hours ($\sim$3 days) with 1,200 μL of outflow capacity. With flexible tubing of diameter 2 mm (capacity $\sim$4,700 μL when wound 16 times around a 3-cm diameter cylinder), we could maintain operation over 10 days (240 hours) without any extra handling or use of peripheral devices. However, as we used an osmotic pump, the flexible tube was filled with distilled water prior to operation, and spent reagents must not directly contact the water in the tube over the experimental period. We used a small air bubble (Fig. 2E) to successfully prevent mixing; the bubble was stable throughout the experiment.

On-Chip Differentiation of Human ESC-Derived Neural Progenitor Cells

We tested whether human ESC-derived neural progenitor cells could be successfully cultured in the microfluidic device under continuous fluid flow, and whether their survival, proliferation, and/or differentiation reflected gradient concentrations of added extracellular signaling molecules. The human ESCs were differentiated into neural progenitors as described in Materials and Methods. Almost all cells derived from human ESCs expressed Nestin, a marker of primitive neural progenitors, and the purity of input population was quantitatively

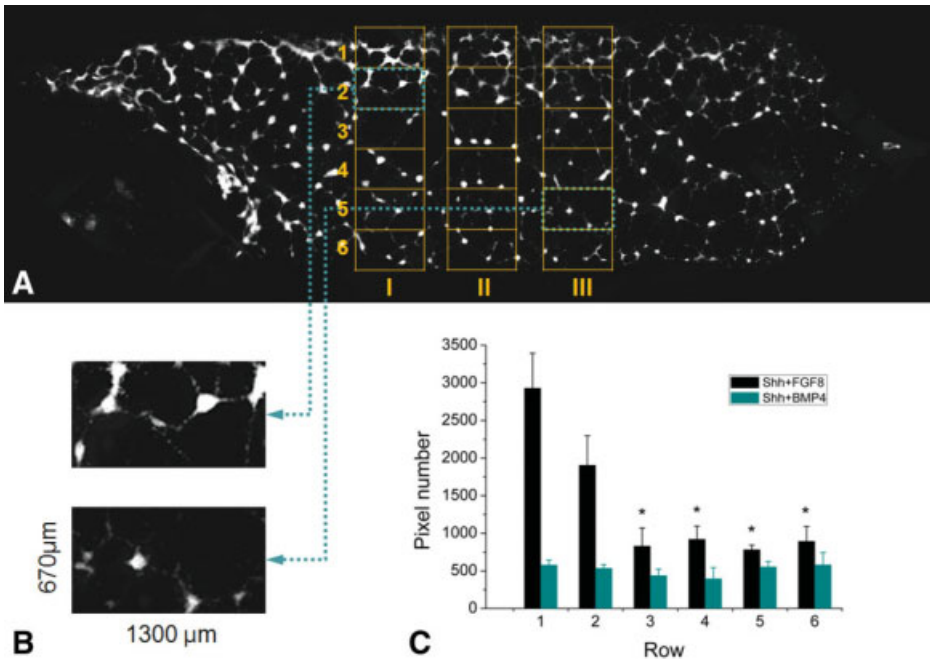

Figure 5. Quantification of total cell numbers upon culture in gradient chambers. **(A):** Three columns (I, II, and III) of a rectangular mesh (1300 × 670 μm; with rows numbered 1–6) were used for quantitative analysis of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI)-stained cells in the gradient chip. **(B):** Magnified image of DAPI-stained nuclei in the selected rows of the rectangular mesh. **(C):** Cell numbers were estimated by pixel counts of DAPI-stained images. Each bar represents the average pixel number in each row (1–6). Values are expressed as mean ± SD. $*p < .05$ versus row 1. Abbreviations: BMP4, bone morphogenetic protein 4; FGF8, fibroblast growth factor 8; Shh, sonic hedgehog.

analyzed by flow cytometry before applying the microfluidic device (purity was >97%; Fig. 4A, 4B). To observe the effect of cytokine gradients on cells, three imaginary window columns were placed in the main channel where the diffusion mixing of two different cytokine solutions (Shh vs. FGF8 or BMP4) occurs (Fig. 4C). Due to its cost/time effectiveness, a three-dimensional fluid dynamics model was implemented to predict the gradient profiles in our microchannel (Fig. 4D) (see the supporting information data for the computational method); the resulting profiles were found to be almost linear across the width of the main channel (4 mm) (Fig. 4E). We wanted to then compare the computational results with the actual protein gradients in the microchannel; however, due to the difficulty of directly measuring the concentration gradient of cytokines, fluorescent-labeled proteins (cholera toxin subunit B conjugated with Alexa Fluor 488 or 594) were used as a substitute to visualize the cytokine gradient generated in the microfluidic chip. The developed protein gradients, after stabilizing for 30 minutes, were as shown in Figure 4F. For quantitative analysis we measured the color intensity of the green fluorescence of Figure 4F and the result was as shown in Figure 4G. The gradient-developed region was about one third of the channel width, however, it is important to note that the fluorescent-labeled cholera toxin subunit B is larger than the original cytokines (Shh, BMP4, FGF8), resulting in a lower diffusion flux. At neutral pH, the cholera toxin subunit B exists as a 57-kDa pentamer, which is approximately three times larger than size of the cytokines used in the present study (Shh: 20 kDa, BMP4: 22 kDa, FGF8: 23.6 kDa). Since cholera toxin subunit B is approximately three times larger than cytokines (e.g., Shh), the actual span of the cytokine gradients can be predicted (green dotted line) by expanding the graph of cholera toxin subunit B (black line) in Figure 4G three times; the expanded graph for cytokines was also found to be almost linear from 90%--10% in the *y*-axis, as is its corresponding simulation result, the column II graph (orange dotted line) in Figure 4E. Therefore, our computational model was proven useful for estimation of actual concentration profiles of cytokines Shh, BMP4, and FGF8 in the microfluidic chip.

Dissociated human ESC-derived neural progenitor cells were then plated into the microfluidic chamber and exposed to concentration gradients of Shh, FGF8, or BMP4, for 4 days in the presence of bFGF, a well-known mitogen of neuroepithelial cells. After an additional 4 days of neural differentiation without bFGF (thus, a total of 8 days of culture in the microfluidic chamber: 4 days of proliferation followed by 4 days of differentiation), cells displayed an elongated bipolar shape and had fine extended processes similar to axons and dendrites. In multiple independent experiments ($n = 3$), similar patterns of cell distribution across the cytokine gradient were observed, although minor variations were found due to initial cell seeding density and cell survival/proliferation rate in the chip, and the results of a representative experiment are presented in Figures 5 and 6. Differentiation into a mature neural cell type was analyzed by immunostaining using antibody against TuJ1, a neuronal marker (supporting information Fig. 4). Quantification of cell numbers in the gradient chamber was performed by counting DAPI-positive image pixels in a rectangular mesh (1300 × 670 μm; six rows numbered 1–6 and three columns numbered I to III) imposed on a digital image of cells distributed in the main channel (Fig. 5A, 5B). In the chamber with a continuous gradient of Shh and FGF8, cell numbers were much greater in areas of higher Shh and lower FGF8 concentrations. In contrast, the numbers of cells in the chamber with a gradient of Shh and BMP4 did not significantly differ with cytokine concentrations (Fig. 5C).

Fluorescence micrography after immunostaining for TuJ1 showed that extensive outgrowths of differentiated neurons formed a complex cellular network with a highly branched structure across the gradient chamber (Fig. 6). Interestingly, the number of TuJ1-positive neurons was higher and the branched structure was more prominent in the chamber with gradients of Shh and FGF8 than the chamber with Shh and BMP4 gradients (Fig. 6A, 6C). Moreover, many TuJ1-negative cells were detected at elevated BMP4 concentrations (Fig. 6C). We quantitatively analyzed neural differentiation by counting TuJ1-positive neuronal cell body clusters and neurite bundles in imaginary observation windows of three different areas across the concentration gradient (Fig. 6B). The average numbers of both neuronal cell body clusters and neurite bundles were shown to be directly proportional to Shh

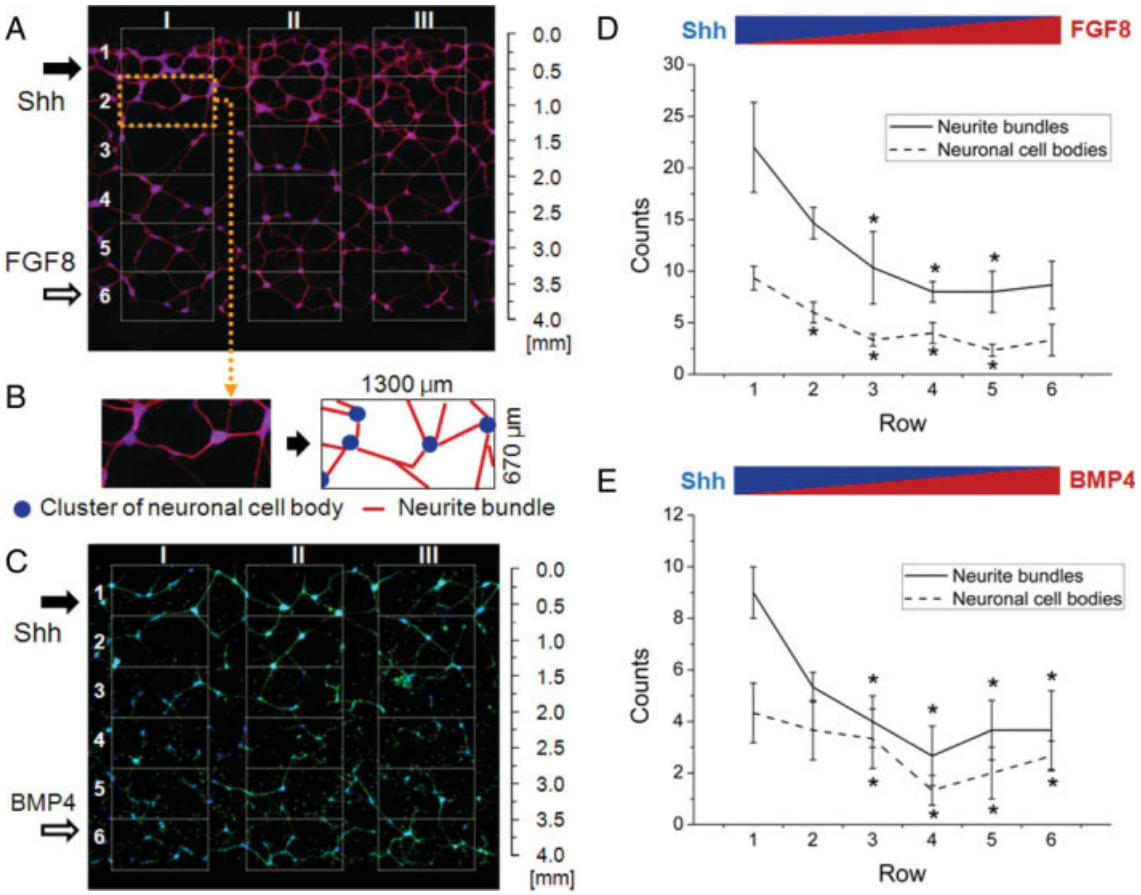

Figure 6. Quantification of neuronal cell body clusters and neurite bundles in the neuronal network generated with Shh-FGF8 and Shh-BMP4 gradients. **(A):** Cells were immunostained using antibody against TuJ1, a neuronal marker. Three columns (I, II, and III) of a rectangular mesh (1300 × 670 μm; with rows numbered 1–6) were used for quantitative analysis of neurite bundles and clusters of neuronal cell bodies in the neuronal network generated by Shh-FGF8 gradients. **(B):** Clusters of neuronal cell bodies and neurite bundles were marked by circles and lines, respectively, and manually counted. **(C):** An analysis as in **(A)** of cells cultured in the Shh-BMP4 gradient. **(D–E):** The average counted numbers of neuronal cell body clusters and neurite bundles in each row. Values are expressed as mean ± SD. *$p < .05$ versus row 1. Abbreviations: BMP4, bone morphogenetic protein 4; FGF8, fibroblast growth factor 8; Shh, sonic hedgehog.

concentration in chambers with gradients of either Shh and FGF8 or Shh and BMP4 (Fig. 6D, 6E). The cytokine effects on neural progenitor cells were also tested using a traditional culture system. Significant increases in TuJ1-positive cells were observed only in the group treated with both Shh and FGF8, but not in the group treated with either Shh or FGF8 alone (supporting information Fig. 5). Importantly, the significantly different level of neurogenic effect between Shh and FGF8 observed in the microfluidic system was not found in the traditional culture system, although Shh seemed to increase TuJ1-positive cells slightly more than FGF8. It is likely that paracrine molecules released by cells were accumulated during the culture period and possibly masked the effect of exogenous cytokines. Thus we speculate that continuous flow of the cytokine gradient in our system washed out the soluble factors and might allow for cells to respond solely to Shh and/or FGF8, producing the more clear difference in the cytokine effects.

Discussion

In the past few years, on-chip-based microfluidic systems have been under the spotlight and increasingly widespread for many biomedical applications. High-throughput bioassay microscale systems offer many advantages over conventional cellular analysis methods. One great advantage is the ability to generate gradients of biomolecules. We have previously shown that the osmosis-driven system was useful for cell culture in a biomolecular concentration gradient over several hours, with no requirement for extra handling [16]. However, the use of a capillary glass tube (diameter: 1 mm, height: 75 mm) and flexible tubing (inner diameter: 1 mm, typical length in use: 20 cm) prevented long-term (days to weeks) system operation. We modified these two components to allow the system to be used for stem cell differentiation, which requires a much longer cell culture time. We used 1000 μL pipette tips as inlet reservoirs, and showed that the tips substituted effectively for capillary glass tubes as inlet reservoirs. The glass tube held 58 μL; the tip normally holds a 17-fold greater volume, rising to a maximum 34-fold larger capacity when filled to the brim (2000 μL).

In microfluidic systems, convective (or convection-dominant) flow provides a continuous fresh nutrient supply but often imposes non-physiologic shear stress on cells [20]. On the other hand, diffusive flow is free of such unwanted effects, but cannot guarantee long-term stability (even over several hours) [21, 22]. These limitations were overcome in our system, by achieving a flow of Pe $\sim$ 1; this served to eliminate

the drawbacks of convection and diffusion.16 With such a low interstitial-level flow rate, cells could be exposed to very stable, long-term (weeks; pumps only need to be replaced every 10 days) cytokine concentration gradients.

We demonstrated that our microfluidic system is more efficient than traditional culture systems to eliminate cell-derived soluble factors and evaluate the effect of exogenous cytokines more directly during neural differentiation of hESCs. We showed that neural progenitors derived from hESCs successfully differentiated into neurons in the microfluidic chamber under continuous gradients of developmentally relevant signaling molecules. The average numbers of both neuronal cell body clusters and neurite bundles were shown to be directly proportional to Shh concentration in chambers with gradients of either Shh and FGF8 or Shh and BMP4. This correlation with Shh concentration may be because of increased proliferation and survival of human ESC-derived neural progenitors in response to Shh. This view is supported by previous studies showing that Shh treatment in the presence of bFGF increased neural progenitor cell proliferation [23] and that Shh acted as a mitogen as well as a survival factor during neurogenesis of ESC [24].

However, the mitogenic effect of Shh was not prominent in the result of quantification of total cell number (pixel count of DAPI) in Shh-BMP4 gradients of the microfluidic system. In addition, we observed a significant decrease in TuJ1-positive cells at high BMP4 concentrations in the microfluidic device. These results are in good agreement with the results from our traditional culture system (Fig. S5 in the Supplementary Information) and many other in vitro studies using conventional culture, showing opposing actions of Shh and BMPs on cellular proliferation and differentiation. BMPs have been shown to antagonize the Shh-mediated proliferation of neural progenitor cells [23, 25], to inhibit neurogenesis, and to concomitantly promote astroglial lineage commitment [23, 26, 27].

Together, our results show that human ESC-derived neural progenitor cells could be cultured for up to 8 days under a continuous fluid flow which provide stable concentration gradients of growth factors. Cells proliferated and differentiated in a controlled fashion and cell properties clearly reflected concentrations of extracellular signaling molecules. The traditional approach to such work requires multiple independent cultures exposed to fixed concentrations of the signaling molecules. Our gradient-generating chip allowed concentration-dependent cellular responses to be induced and quantified within a single micro culture chamber.

Conclusion

The optimal concentrations or gradients of a single or multiple cytokine(s) are critical for the commitment of stem cells, as well as region-specific patterning of organs and embryos. In this paper, we emphasize that long-term culture in a microfluidic system generating stable cytokine gradients is very useful in stem cell research. Several technical modifications to our gradient generation chip were quantitatively analyzed and found useful to permit cell culture for up to 10 days without the need for any special handling. We demonstrated that our microfluidic system is more efficient than traditional culture systems to eliminate cell-derived soluble factors and evaluate the effect of exogenous cytokines more directly. We also show that opposing effects of agonist (Shh) and antagonist (BMP4) on the proliferation and differentiation of human ESC-derived neurons could be successfully recapitulated according to the concentration gradients in our microfluidic system. Osmotic pump replacement (which is simple) and replenishment of reagents will allow the culture period to be extended at will. A combination of useful features, such as slow flow ($Pe \sim 1$), accurate controllability, freedom from unwanted convective flow, the passive mechanism, small size, and easy visualization of concentration-dependent cellular responses, make the microfluidic chip a very powerful tool for research on stem cell differentiation or other biological phenomena requiring long-term maintenance of a stable concentration gradient. Although further studies are needed for the effect of microchip-generated cytokine gradients on stem cells, our microsystem provides a basic platform for the development of efficient cellular microenvironment that may allow a robust generation of clinically relevant cell types from stem cells.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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