Neural Stem Cell Differentiation Using Microfluidic Device-Generated Growth Factor Gradient

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INTRODUCTION

Stem cells hold great promise as biopharmaceuticals and cell-based therapies because they can be expanded in culture, genetically modified using ectopic gene delivery systems, and differentiated into multiple cell types (Clarke et al., 2000; Muller et al., 2006; Capowski et al., 2007; Kim and Jin, 2012). Like other stem cells, neural stem cells (NSCs) are capable of self-renew while maintaining their capability to differentiate neurons, astrocytes and oligodendrocytes (Gage, 2000; Abe-matsu et al., 2006; Germain et al., 2010; Alenzi and Bahkali, 2011). To obtain enriched populations of the desired cell types for clinical applications, optimizing the stem cell microenvironment is crucial in controlling the differentiation of cell types in vitro (Jessell, 2000; Gurdon and Bourillot, 2001; Rallu et al., 2002; Hebert and Fishell, 2008).

Growth factors or biological molecules play significant roles in biological processes of NSCs, regulating cell growth, migration, differentiation and apoptosis in a temporal and spatial manner (Altmann and Brivanlou, 2001; Lee and Pfaff, 2001; Panchision and McKay, 2002; Rogulja and Irvine, 2005). Therefore, efforts have been made to understand the influences of concentration gradients of signaling molecules to find the optimal concentration that regulate NSC fate commitment (Lee et al., 1997; Tropepe et al., 1999; Dorsky et al., 2000; Xu et al., 2000; Megason and McMahon, 2002). However, traditional cell culture methods are unable to control stem cell behaviors precisely (Bavister, 1995; Beebe et al., 2002; Sia and Whitesides, 2003). The traditional approaches of screening for optimal conditions with multiple growth factors require substantial effort and time. For example, conventional cell culture technologies require multiple independent cultures exposed to fixed growth factor concentrations and thus consume large volumes of media, expensive reagents and enormous amounts of cells.

Microfluidic gradient systems have been recently developed to overcome the limitations of conventional systems. These systems have attempted to create microenvironments with greater relevance to normal physiological processes as well as high-throughput platforms for determining cell behavior-biological factor interactions (Beebe et al., 2002; Ng et al., 2002; Park and Shuler, 2003; Sia and Whitesides, 2003; Walker et al., 2004). The microfluidic platform enables the manipulation of fluid flow, the generation of stable concentration gradients of signaling molecules to find the optimal concentration that regulate NSC fate commitment (Lee et al., 1997; Tropepe et al., 1999; Dorsky et al., 2000; Xu et al., 2000; Megason and McMahon, 2002). However, traditional cell culture methods are unable to control stem cell behaviors precisely (Bavister, 1995; Beebe et al., 2002; Sia and Whitesides, 2003). The traditional approaches of screening for optimal conditions with multiple growth factors require substantial effort and time. For example, conventional cell culture technologies require multiple independent cultures exposed to fixed growth factor concentrations and thus consume large volumes of media, expensive reagents and enormous amounts of cells.

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GRADIENTS ON SURVIVAL, PROLIFERATION AND DIFFERENTIATION OF ECTOPTIC GENE-EXPRESSING RAT NEURAL STEM CELLS IN MICROFLUIDIC CHIP

Given these notable characteristics, microfluidic devices have attracted widespread interest as powerful tools for various biological applications. In this study, we described a microfluidic-based gradient platform to control NSC behaviors. Using this microfluidic device, we examined the effects of growth factor concentration gradients on survival, proliferation and differentiation of ectopic gene, ASCL1-delivered NSCs as well as unmodified NSCs.

MATERIALS AND METHODS

Microfluidic system design
Microfluidic chips and osmotic pumps were kindly provided by Dr. Sang-Hoon Lee (Korea University, Seoul, Korea). Microfluidic system is based on a concentration gradient generated by an osmotic pump (Park et al., 2009). Briefly, the system is composed of microchannel, inlet reservoir made of tips, osmotic pump, and coiled tube outlet reservoir (Fig. 1). Two solutions of different concentrations of growth factors were introduced into the main channel and a concentration gradient is generated at the interface of the two solutions. Two micropipette tips (AxyGen, NY, USA) were filled with the same amounts of medium and connected to each inlet, serving as inlet reservoirs. The osmosis occurred by concentration difference between water and 0.082 M polyethylene glycol (PEG, Sigma-Aldrich, MO, USA) solution was used as driving force of the microfluidic system (Park et al., 2009). To increase the liquid capacity, we wound flexible polyethylene tube (Natumseisakusho, Tokyo, Japan) into a compact size and used as outlet reservoir.

Rat neural stem cell cultures
The cortices of the embryonic stage 14.5 (E14.5) Sprague Dawley (SD) rat (Orient bio, Seongnam, Korea) brain were isolated and cultured as previously reported (Kong et al., 2017). Briefly, NSCs were expanded as neurospheres in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Invitrogen, CA, USA) containing 1% (v/v) antibiotic-antimycotic (Gibco, CA, USA), 2% (v/v) B27 (Invitrogen), 20 ng/ml epidermal growth factor (EGF, Merk, Darmstadt, Germany) and 20 ng/ml basic fibroblast growth factor (bFGF, Merk). The cells were maintained at 37°C, 5% CO2 incubator (Sanyo, Osaka, Japan) and the medium was conditionally changed every other day by removing half the media and adding fresh media containing 2% B27 and 20 ng/ml EGF and bFGF. After 1 week, neurospheres were dissociated with accutase (Merk) and used for microfluidic culture system and traditional culture system.

Retrovirus preparation and transduction
Retroviral GFP vector and retroviral ASCL1 vector were used to generate retroviruses, pMX vector containing cDNA of ASCL1, internal ribosome entry site (IRES), and cDNA of GFP was used for the overexpression for ASCL1 (Kim et al., 2009). pMX-IRES-GFP (Addgene, MA, USA) was used for the control. PLAT-E cells transfected with gag-pol and env were used for the packaging of viruses. Briefly, PLAT-E cells were transfected with retroviral GFP vector or retroviral ASCL1 vector using X-tremeGENE 9 (Roche, Basel, Switzerland) following manufacturer’s instruction. Supernatant containing viral particles was collected 2 days after incubation and centrifuged at 12500 g for 12 h to concentrate retroviruses. To determine the titer of viruses, 6.0×104 dissociated rat NSCs were plated onto the poly-D-lysine (Sigma-Aldrich) and laminin (Invitrogen) coated microscope cover glasses (Superior, Lauda-Königshofen, Germany) in 24 well plates (ThermoFisher Scientific, MA, USA). NSCs were cultured for 1 day to 50-60% confluency, and then exposed to retroviruses encoding green fluorescence protein (GFP) for 6 h in the presence of polybrene (4 µg/ml, Sigma-Aldrich). After washing twice with DMEM/F12, cells were cultured in the presence of EGF and bFGF for 2 days. Then cells were fixed for immunocytochemistry.

Microfluidic culture of ectopic gene-expressing neural stem cells
After 1 week of propagation, 6.0×105 dissociated rat NSCs were plated at 12 well plates (ThermoFisher Scientific) and exposed to retroviruses for 6 h in the presence of polybrene (4 µg/ml). After 6 h incubation, ectopic gene-expressing NSCs (200 µl of a suspension of 7.5×105 cells/ml) were loaded into a microchannel coated with poly-D-lysine/laminin via outlet port using a micropipette and allowed to adhere for 90 min in a 37°C, 5% CO2 incubator. Adherent cells were cultured for 1 day in medium containing 2% B27 and 20 ng/ml EGF and bFGF. After that, ectopic gene-expressing NSCs were exposed to growth media (containing 20 ng/ml EGF and bFGF) via inlet 1 port or differentiation media (without EGF and bFGF) via inlet 2 port for 3 days. Then, cells were fixed for immunocytochemistry. To compare the graded effects of growth factors in a microfluidic device with that in a traditional culture system, dissociated rat NSCs were also plated onto the poly-D-lysine/ laminin-coated microscope cover glasses in 24 well plates at a density of 6×103/500 µl, transduced with retroviruses for 6 h in the presence of polybrene (4 µg/ml), washed twice with DMEM/F12 and then cultured in the same medium for 2 days.

Fig. 1. Microfluidic chip device experimental device scheme. (A) The size of microchip channel is 4 mm in width, 10 mm in length. (B) For inlet reservoirs, 1,000-µl micropipette tips were used. (C) The main driving force for fluid flow is generated by the osmotic pump. (D) The flexible tube was used as an outlet reservoir.

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Immunocytochemistry and cell counting

We did immunocytochemistry as previously reported (Kong et al., 2015; Lee et al., 2016). NSCs were fixed with 4% (v/v) paraformaldehyde (Affymetrix, CA, USA) for 30 min and rinsed with phosphate-buffered saline (PBS). Fixed cells were blocked for 30 min in PBS containing 5% (v/v) normal goat serum (Millipore, MO, USA) and 0.2% (v/v) triton X-100 (Amresco, PA, USA) and probed with primary antibodies to GFP (anti-GFP, mouse IgG2a isotype, monoclonal, 1:500; Molecular Probes, OR, USA) or β-tubulin type III (TuJ1, mouse IgG2b isotype, monoclonal, 1:1000; Sigma-Aldrich). After 1 h incubation with primary antibodies, the cells were rinsed with PBS and probed with Alexa Fluor 488-conjugated secondary antibody (goat anti-mouse IgG 2a, 1:1000; Molecular Probes) or Alexa Fluor 546-conjugated secondary antibody (goat anti-mouse IgG2b, 1:1000; Molecular Probes) for 40 min. Following rinsing with PBS, cell nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI, 1:10,000 in PBS; Sigma-Aldrich) to count the total cell number. The images were obtained using an inverse fluorescence microscopy (DMIL; Leica, Wetzlar, Germany). Quantification of cells for traditional culture system was performed by counting the number of specifically immunostained cells in 3 randomly chosen microscopic fields. For microfluidic chip, the phase contrast images were captured at 6 different fields (10x magnification) and the fluorescent images were captured at 9 different fields (20x magnification) of microchannel. Quantification of cells for microfluidic culture system was performed by counting the number of specifically immunostained cells in each selected fields of over three independent experiments.

Statistical analysis

The ratio of GFP-positive cells to total cells was calculated to draw transduction efficiency. The number of TuJ1/GFP-double positive cells was divided by GFP-positive cells to get the percentage of ectopic gene-expressing neurons. The percentage value of ASCL1 retrovirus-transduced group was divided by that of control retrovirus-transduced group to get the fold increase. Quantitative data were expressed as the mean ± SD. For the determination of statistical significance, differences between groups were tested by the Student’s t-test and considered to be statistically significant if p<0.05.
RESULTS

Differential NSC proliferation and differentiation induced by EGF/bFGF concentrations generated in the microfluidic chip

We first observed whether rat NSCs could be successfully cultured in the microfluidic device under continuous flow, and examined the effects of a concentration gradient of extracellular biological molecules on survival, proliferation and differentiation. Microfluidic device previously reported were kindly provided by Prof. Sang-Hoon Lee (Korea University) (Park et al., 2009; Fig. 1). One week after dissection and expansion, NSCs were dissociated into single cells, loaded into the poly-D-lysine and laminin coated microchannel of chip device at a density of 1.5×10^6 cells/200 µl, and then cultured for 24 h in the presence of growth factors. After 1 day, adherent cells were exposed to media with EGF and bFGF in one side and without EGF and bFGF in the other side for 3 days and then fixed to perform immunocytochemistry (Fig. 2A). We used 20 ng/ml EGF and bFGF because it is the condition we and others generally use for NSC proliferation (Nelson and Svendsen, 2006; Lee et al., 2016; Cha et al., 2017). NSCs proliferate in the presence of growth factor and differentiate into neurons, and glia in the absence of growth factors (Kong et al., 2015; Lee et al., 2016). To observe the effect of the growth factor gradient on rat NSCs, we took phase contrast images from 6 different fields of the microchannel at 10x magnification (Fig. 2B-2H). Images taken immediately after fixation showed that the cells directly exposed to media with growth factors were elongated and showed a normal proliferation (Fig. 2G). The cells directly exposed to media lacking growth factors exhibited extensive outgrowth of processes and formed a cellular network, but showed little proliferation (Fig. 2H). The cells cultured near the outlet port, where diffusive mixing of media both with and without growth factors occurs, appeared to not only proliferate but also differentiate and were evenly distributed across the microchannel (Fig. 2C, 2D). This result shows that the growth factor gradient was successfully generated across the entire microfluidic device and NSCs can be cultured in the device.

To assess the degree of neuronal differentiation with the growth factor concentration gradient, immunocytochemistry was performed with antibody against β-Tubulin type III (TuJ1) and fluorescence images from 9 different fields of the microchannel were captured at 20x magnification (Fig. 3A). Representative images are shown in Fig. 3B, and quantitative analyses are shown in Fig. 3C. Cells in the low growth factor compartment showed increased neuronal differentiation (1.96% for R-inlet) compared to cells in the high growth factor compartment (0.89% for L-inlet) (Fig. 3C, lower panel). Cells in the intermediate growth factor compartment, where growth and differentiation media (without growth factors) are mixed, exhibited the highest degree of neuronal differentiation (4.15% for R-outlet, 3.11% for M-outlet and 2.21% for M-middle) (Fig. 3C, upper and middle panel). These results suggest that rat NSCs remained healthy throughout the entire culture period under fluidic flow, and importantly, proliferated and differentiated in response to growth factor concentration gradient. In addition, the presence of low amount of growth factors near the outlets, generated by mixing of supplemented media and differentiation media (media without growth factors occurs, appeared to not only proliferate but also differentiate and were evenly distributed across the microchannel (Fig. 2C, 2D). This result shows that the growth factor gradient was successfully generated across the entire microfluidic device and NSCs can be cultured in the device.

![Fig. 3. NSCs differentiated better with low concentration of GF than without GF condition. (A) Nine spots were chosen to take microscopic photos. M-O, middle-outlet; M-M, middle-middle; M-I, middle-inlet. (B) Immunocytochemistry data for neuron (TuJ1, green). DAPI was used to obtain the total number of cells. Scale bar=50 µm. (C) Quantification of the immunostaining data. Cells positive to TuJ1 were counted and the ratio of TuJ1-positive cells to total cells was calculated and presented. Quantitative data are expressed as the mean ± SD (n=3).](image-url)
gests that NSCs generate more differentiated neurons in the presence of low growth factor.

Differentiation of ASCL1-overexpressing NSCs into neurons in a traditional culture system

To explore whether ectopically delivered genes function in NSCs, and if transduced cells also respond to a growth factor concentration gradient under continuous fluidic flow, we next overexpressed ASCL1, a basic helix-loop-helix protein that is known to induce neurogenesis, using a retroviral delivery system (Kim et al., 2007). Before applying transduced NSCs to the microfluidic device, we examined cell fate and behavior of control GFP or ASCL1 retrovirus-transduced NSCs in the presence or absence of growth factors using a traditional culture system. The titer of retroviruses for ectopic gene delivery was first determined using NSCs (Supplementary Fig. 1A-1C).

To investigate the response of control GFP or ASCL1 introduced NSCs to the growth factors, 6.0×10^4 dissociated cells were plated onto microscope cover glasses, grown for 1 day, and transduced with retroviruses encoding either GFP alone or ASCL1 with GFP. Ectopic gene-transduced cells were then cultured for additional 2 days in media with or without growth factors followed by fixation (Supplementary Fig. 1D). Immunocytochemistry analysis with anti-GFP antibody and anti-β-Tubulin type III antibody revealed that ectopic gene expression of ASCL1 increased neuronal differentiation in both growth media (media with EGF/bFGF) and differentiation media (media without EGF and bFGF) (Fig. 4). When the cells were cultured in growth media containing EGF and bFGF, none of the control GFP retrovirus-transduced cells were positive for anti-β-Tubulin type III, whereas 1.70 ± 0.11% of the ASCL1 retrovirus-transduced cells were positive for anti-β-Tubulin type III, a marker for neurons (Fig. 4A-4F, 4M). In the case of NSCs cultured in differentiation media without EGF and bFGF, the number of anti-β-Tubulin type III positive cells in the ASCL1 retrovirus-transduced group was 1.54 fold higher than the control GFP retrovirus-transduced group (Fig. 4G-4L, 4N). These data indicate that ectopic expression of ASCL1 induced differentiation of NSCs into neurons and the effect of ASCL1 on neuronal differentiation was dramatic when the cells were cultured in differentiation media (increase about 15%) compared with cells cultured in growth media (increase about 1.5%).

ASCL1-expressing NSC differentiation in a microfluidic chip system revealed that low concentrations of EGF/bFGF increases neurogenesis

To identify the optimum conditions for NSC differentiation
using a microfluidic chip, we cultured control GFP or ASCL1 retrovirus-transduced rat NSCs in the microfluidic gradient device and assessed the degree of neurogenesis by calculating the numbers of TuJ1-positive neurons. NSCs were dissociated and 6.0×10⁵ cells were plated onto 12-well plates and the numbers of TuJ1-positive neurons. NSCs were dissociated and assessed the degree of neurogenesis by calculating retrovirus transduced NSCs in the microfluidic gradient device of both the control GFP and ASCL1 retrovirus-transduced groups (data not shown). Representative fluorescence images for the control GFP and ASCL1 retrovirus-transduced groups are shown in Fig. 5A, and 6A, and 6B, respectively. When NSCs were transduced with the control GFP retrovirus, the cells expressed GFP, as shown by immunocytochemistry (Fig. 5A). However, TuJ1 positive neurons were rare in the control GFP retrovirus-transduced cells. Only a few cells cultured in the intermediate growth factor compartment were positive for both GFP and β-Tubulin type III (1.35 ± 1.22% for R-outlet and 1.12 ± 1.53% for M-outlet). In contrast, a significant number of ASCL1 retrovirus-transduced cells developed into anti-β-Tubulin type III positive neurons.

![Fig. 5.](image1) Response of control GFP retrovirus-transduced rat NSCs in a growth factor concentration gradient using microfluidic culture system. (A) Immunocytochemistry for control GFP retrovirus-transduced cell (anti-GFP, green) and neuron (TuJ1, red) of culture in a growth factor concentration gradient. Scale bar=50 μm. (B) Graphical quantification of the immunostaining data. Cells positive to TuJ1 and GFP were counted, and the ratio of TuJ1/GFP-double positive cells to GFP-positive cells was calculated and presented. Quantitative data are expressed as the mean ± SD (n=3).

![Fig. 6.](image2) ASCL1 retrovirus-transduced rat NSCs differentiate more into neurons when GF concentration is low or absent. (A) Immunocytochemistry for ASCL1 retrovirus-transduced cells (anti-GFP, green) and neurons (TuJ1, red) of culture in a microfluidic chip generated growth factor concentration gradient. Arrows indicate TuJ1⁩-GFP⁩ cells. Scale bar=50 μm. (B) Graphical quantification of the immunostaining data. Cells positive to TuJ1 and GFP were counted, and the ratio of TuJ1/GFP-double positive cells to GFP-positive cells was calculated and presented. Quantitative data are expressed as the mean ± SD (n=3). *p<0.05; **p<0.01 vs. control GFP retrovirus-transduced cell shown in Fig. 4. Abbreviation: L, left; M, middle; R, right; GF, growth factor; GFP, green fluorescence protein.
Direct exposure to media lacking growth factors (R-inlet) resulted in the highest number of cells positive for both GFP and the neuronal marker TuJ1 (26.74 ± 0.47%), followed by the cells in the intermediate growth factor area, such as R-middle (15.02 ± 1.20%), R-outlet (11.79 ± 4.08%) and M-outlet (12.38 ± 8.21%) (Fig. 6B). The cells directly exposed to media with growth factors (L-inlet) also expressed GFP. However, few GFP-positive cells expressed TuJ1 (3.14 ± 1.56%) compared with cells exposed to the low growth factor compartment or intermediate growth factor compartment (Fig. 6B). Thus, neuronal differentiation of ASCL1 retrovirus-transduced cells was inversely proportional to growth factor concentration in the gradient microchannel.

By culturing ectopic gene-expressing cells as well as unmodified NSCs under a continuous gradient of growth factors, we showed that these cells proliferated and differentiated in a graded fashion and the cell properties reflected the concentrations of growth factors. Furthermore, a higher percentage of ASCL1 retrovirus-transduced NSCs differentiated into β-Tubulin type III positive neurons whereas control GFP retrovirus-transduced cells produced few neurons. Unlike non-transfected NSCs, ASCL1 expressing NSCs differentiated more into neurons where growth factors are absent. These results show that the optimum conditions for NSC proliferation or differentiation can be identified by using the microfluidic chip.

DISCUSSION

In the past few years, on-chip-based microfluidic systems have provided tremendous promise for many biological applications such as controlling cell-extracellular microenvironment interactions and optimizing conditions for stem cell cultures (Park et al., 2009). In this study, we also used a gradient-generating microfluidic platform and investigated the optimal conditions for NSC microenvironment.

NSCs cultured in a continuous concentration gradient of growth factors were well adhered, remained healthy, and proliferated in response to growth factors. Interestingly, the cells in the intermediate growth factor compartment showed a higher percentage of neuronal differentiation than cells in other fields of the microchannel. Consistent with our observations, it has been reported that low concentrations of bFGF have been shown to promote significant neurogenesis in rodent progenitor cells (Qian et al., 1997). Similarly, low levels of bFGF also increased the number of neurons generated from human neural progenitor cells (hNPCs) (Nelson and Svendsen, 2006). Another study using adult rat cortical cells showed that low-level stimulation with bFGF induced the activation of a neurogenic progenitor that normally remains inactive in the adult cortex (Palmer et al., 1999). A reasonable explanation for this phenomenon of low concentrations of bFGF increasing neurogenesis is that it may drive the progenitor to a neuronal fate. Enhancement of the survival of newly formed neurons could also address the neurogenic effect of bFGF. It is also notable that NSCs are sensitive and responsive to the environment. Thus, fluid flow, shear stress in the microfluidic chips can affect the NSC biology. It is known that fluid mechanical stress resulted in differential gene expression compared to static control (Nishii et al., 2018). Thus, it is possible that NSCs in the microfluidic chamber may show different feature compared to the static culture.

Several earlier studies report that ectopic expression of bHLH genes like Neurogenin 2 can induce neurogenesis from different types of cells (Farah et al., 2000; Kim et al., 2007; Ribes et al., 2008; Kim et al., 2009; Thoma et al., 2012). In our current study, we observed enhanced neuronal differentiation in NSCs that are retrovirally transduced with ASCL1 (achaete-scute family bHLH transcription factor 1), a member of the bHLH protein family, which plays an important role in the generation of neurons during embryonic development (Kim, 2011). Notably, with the microfluidic gradient chip, we found that the number of neurons-derived from ASCL1 retrovirus-transduced NSCs was inversely proportional to the growth factor concentrations, with the highest percentage of neurons in the low growth factor compartment (Fig. 6B). We have previously reported that ASCL1 expressing progenitor cells continued to divide in the presence of growth factors, and subsequently differentiated into neurons after growth factor withdrawal (Kim et al., 2007). However, the concentration of growth factors may be important to determine the level of proliferation and differentiation of ASCL1 expressing NSCs. NSCs introduced with ASCL1 probably proliferated to increase the numbers and then differentiated to neurons in the high growth factor section. When growth factors are low or intermediate, some may be still proliferating but not as much as NSCs in the section where growth factors are high, and differentiated before increasing the ASCL1 expressing cell numbers. Therefore, we see more GFP+ neurons in high growth factor area. In the postnatal subventricular zone, ASCL1 expression was conserved in progenitors for both the neuronal and oligodendrocyte lineages (Parras et al., 2004). Thus, we speculate that ASCL1 may be involved in maintaining cells as neural progenitors while retaining the neurogenic potential. In contrast to ASCL1 retrovirus-transduced NSCs, there were few control GFP retrovirus-transduced NSCs that had differentiated into neurons. The cells that were transduced with the control GFP retrovirus had differentiated the most in the intermediate growth factor compartment as seen with the unmodified NSCs (Fig. 5B). This could be explained by the protective effects of growth factors onto neurons (Chadi et al., 1993; Nakata et al., 1993; Qian et al., 1997; Nelson and Svendsen, 2006). Importantly, the increased number of neurons induced by ASCL1-overexpression was also observed in the traditional culture system (Fig. 4). The number of GFP-expressing cells positive for β-III Tubulin antibody in the microfluidic devices under continuous flow was lower than that in the conventional culture plates lacking fluid flow (data not shown). This could be because of the removal of autocrine and paracrine factors in our microfluidic system, which would not occur in the traditional culture system.

To create a biomolecular gradient in a well-defined microenvironment, flow-based and diffusion-based microfluidic gradient generators have been previously developed (Chung et al., 2005; Abhyankar and Beebe, 2007; Chung et al., 2007; Park et al., 2009). We used a microfluidic chips previously reported to provide a stable growth factor concentration gradient without an external power source (Park et al., 2009). It has been reported that with the same type of microfluidic chip, concentration gradients of sonic hedgehog (Shh)/fibroblast growth factor 8 or Shh/bone morphogenic protein were generated and NPCs differentiated into neurons, generating a complex neural network that was proportional to the Shh concentration (Park et al., 2009). Similarly, in the current study, we
were also able to generate and maintain EGF/bFGF gradients through an osmotic pump by adjusting the capillary action and hydraulic pressure in the reservoirs. NSCs proliferate in the presence of mitogens and differentiate into neurons or glia when the growth factors are removed. Thus, media without the addition of any growth factors are generally used for the induction of differentiation. However, in the current study, using the microfluidic chip, we found that to induce neurogenesis, low concentrations of growth factors provide a better environment than the condition without growth factor condition.

In conclusion, we showed that foreign gene-expressing NSCs as well as unmodified NSCs could be successfully cultured under a continuous fluid flow which generated a growth factor concentration gradient. These advantages of the microfluidic system will provide a powerful tool to explore the optimal microenvironments for various types of stem cells prior to use in cell therapy and transplantation.

CONFLICT OF INTEREST
All authors declare no conflict of interest.

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lowing cerebral ischemia in the gerbil. Brain Res. 605, 354-356.
Supplementary Figure 1. About 20% of rat NSCs are transduced using the retrovirus-mediated ectopic gene delivery method

(A-B) Immunocytochemistry for control GFP retrovirus (A) or ASCL1 retrovirus (B) transduced cell (anti-GFP, green) and nuclei (DAPI, blue). Scale bar = 50μm. (C) Graphical quantification of the immunostaining data. Cells positive to GFP were counted, and the ratio of GFP-positive cells to total cells was calculated and presented. Quantitative data are expressed as the mean ± S.D. (n=3).
Supplementary Figure 2. Quantification of cell processes in L-inlet and R-inlet

(A-F). Cells shown in the Fig 1G were enlarged to measure the cell processes. (G-L) Cells shown in Fig. 1H were enlarged to measure the cell processes. The length of cell processes was measured and quantified (M). Data are expressed as the mean ± S.D. (n=15).