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In vitro neurite guidance effects induced by polylysine pinstripe micropatterns with polylysine background

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Abstract: Engineered culture substrates with chemical neurite guidance cues have been used for studying the mechanism of axon pathfinding at cellular level. In this study, we designed a novel poly-L-lysine (PLL) micropattern ("pinstripe micropattern") to investigate how the same biomolecules with slightly different surface concentration can affect *in vitro* neuronal growth. The pinstripe micropattern was fabricated by stamping PLL on a PLL-coated glass coverslip, which resulted in denser PLL lines and a less-dense PLL background. There were two effects of the substrate on cultured primary hippocampal neuron: neurite initiation and growth cone turning. Although the whole surface was permissive for neurite outgrowth, we observed that the growth direction of neurites had a strong tendency to follow the stamped PLL line patterns with PLL background. However, the micropattern did not affect the spreading of cell body on the substrate. According to these investigations, we concluded that the PLL pinstripe pattern with PLL background, which had the step difference of polylysine concentrations, would be very useful for designing novel cell assays for the investigation of neurite guidance mechanisms, and suggested it as a new design method for controlling the direction of neurite growth on *in vitro* neural network. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2015.

Key Words: microcontact printing, primary neuron, axon guidance, polylysine

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INTRODUCTION

During the development of nervous system, axons find directions to form neural circuits in response to various guidance signals present in the extracellular matrix. These extracellular signaling cues include extracellular matrix proteins (e.g., laminin and fibronectin), extracellular signaling proteins (e.g., semaphorin, slit, ephrin, and netrin), cell adhesion molecules (e.g., cadherin and L1-family), and neurotrophic factors (e.g., NGF, BDNF, and NT).[1–3] In the process of axon pathfinding, growth cones sense the signaling cues and navigate toward targets.[4,5] Especially, the relative steepness of gradient of the signaling cues are believed to be one of the important factors that determine the sensing and pathfinding behaviors of the growth cone.[6–8]

For the past decades, microfabrication technology has been exploited to build cell culture substrates that had surface micropatterns of chemical cues for the study of neuritogenesis and axon guidance at subcellular levels. Microfluidic channels were used to generate the surface-bound gradient of signaling molecules such as slit, netrin, laminin, chondroitin sulphate proteoglycan, and ephrin-A5.[9–16] Microcontact printing (μCP) technique was used to generate the micropatterns of signaling molecules (e.g., laminin, L1-Fc, and Ncad-Fc), and such micropatterns were effective in a selective guidance of *in vitro* axonal growth.[17–21] In particular, Von Philipsborn et al.[22] developed the substrate patterned ephrin-A5 dot array, which has a gradually increased spacing using μCP and showed that the growth cone of neurons cultured on the substrate recognized and integrated the discontinuous local concentrations of ephrin-A5. Meanwhile, synthetic polymers, which are not found in the native microenvironment of neurons, have been also used to fabricate micropatterned culture substrates. Discontinuous dot array of surface-bound polylysine was fabricated to demonstrate the polarity control of neurons[23] or to show branch formation of neurites.[24,25] In addition, discontinuous periodic lines with different spacing were used to control the direction of axonal growth.[26] Previous studies showed that various uses of polylysine as surface-bound axon guidance cue *in vitro*. However, there was no study about the effect of concentration difference of polylysine for axon pathfinding remains. Using microfabrication technologies, it is necessary to generate guidance cue composed of polylysine

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concentration difference and research into growth of axons on the guidance cue.

In this study, we designed a surface-bound pinstripe pattern that is composed of a denser poly-L-lysine (PLL) line and a less-dense PLL background. We intended to study the interaction between the axon growth and the step change of PLL. Using the pinstripe pattern with PLL background, we tested the effect of PLL concentration on the neurite initiation and outgrowth. The pinstripe pattern with PLL background was fabricated by stamping PLL thin stripes on a PLL-coated surface. We found two different neurite guidance effects in pinstripe pattern with PLL background, and the effects also depended upon the line width. Finally, we used pharmacological manipulation to show that filopodial activity is involved in the positive-charge–induced neurite guidance effects.

MATERIALS AND METHODS

Microcontact printing

Polydimethoxysiloxane (PDMS) stamps were manufactured by soft-lithography. Briefly, an SU8 mold was fabricated by standard photolithography process using negative photoresist SU8 2002 (negative photoresist, Microchem, Newton, MA).[24] The thickness of the mold was 2 μm. To cast a stamp, the mixture of PDMS prepolymer and curing agent (10:1 (w/w), Sylgard 184 silicon elastomer kit, Dow Corning, Midland, MI) was poured on the SU8 mold and cured for 5 h at 60°C in a convection oven. Then, cured PDMS was gently peeled off from the mold and cut into small pieces (1 cm × 1 cm). The PDMS stamp was sonicated with acetone, isopropyl alcohol, and deionized (DI) water for 5 min in each solution. The cleaned PDMS stamp was immersed in a 10% sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, MO) solution for 5 min and 10 min with and without sonication, respectively. Next, the stamp was blow-dried with compressed air and coated with a PLL–fluorescein isothiocyanate (FITC) solution (0.1 mg mL^{-1} in DI water). After 30 min, the stamp was rinsed with DI water and gently blow-dried with compressed air. The coated stamp was immediately used for the μCP.

Figure 1(A) shows the fabrication process of PLL pinstripe pattern with or without PLL background. A glass coverslip (18 mm in diameter, Marienfield, Lauda-Königshofen, Germany) was sonicated in acetone, isopropyl alcohol, and DI water for 5 min in each solution. The coverslip was then treated with an air-plasma (30 W, 0.7 Torr, Femto Science, Korea) for 1 min, and a drop of PLL-FITC solution (0.1 mg mL^{-1} in DI water, MW 30,000–70,000, Sigma-Aldrich) was applied on the coverslip for 2 h at room temperature. Then, the coverslip was rinsed with DI water for several times and dried with compressed air. To print micropatterns on coverslip, a PLL-FITC–coated PDMS stamp was brought in contact with the bare coverslip and coated coverslip for 1 min at a constant pressure (20 g). After removing the stamp, the stamped coverslip was sterilized with 70% ethanol (1 mL) for 1 min in the laminar flow hood. Sterilized coverslip were dried and left in the hood until cell plating.

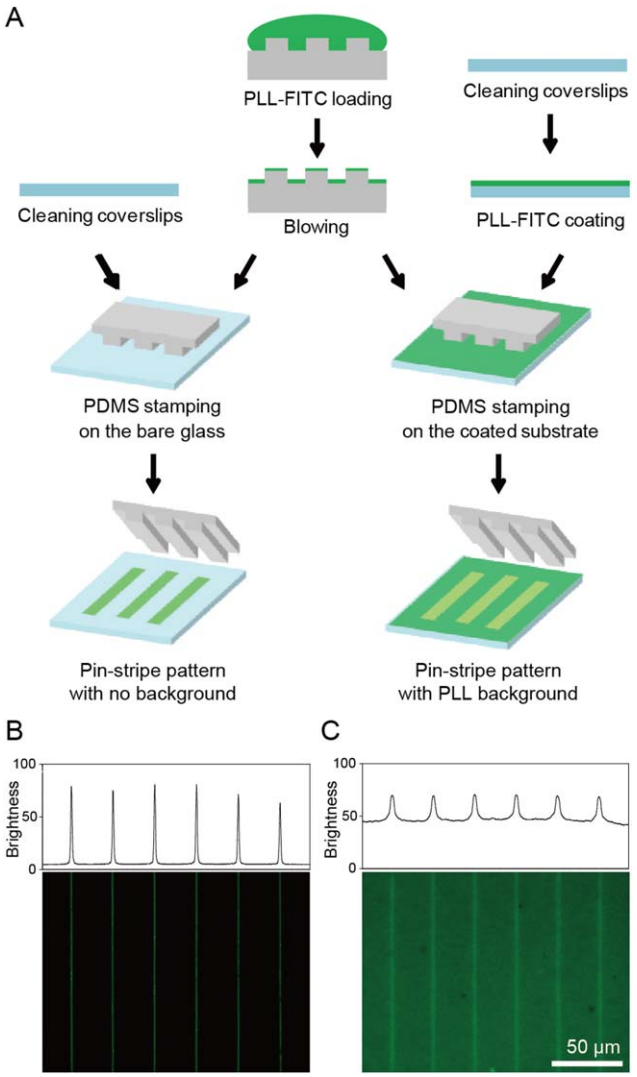

FIGURE 1. Rat hippocampal neurons on pinstripe pattern with PLL background. A: Schematic diagram for the fabrication process of pinstripe pattern without background (left) and with PLL background (right). Contrast profiles and fluorescent images for pinstripe pattern (3 μm in width and 30 μm in spacing) without background (B) and with PLL background (C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

We used micropatterns with the following specifications: 3, 5, 10, and 15 μm in width and 30 μm in spacing.

Cell culture

Fresh hippocampi were dissected from E18 Sprague-Dawley rat (Koatech, Korea), and they were rinsed in a Hank's buffer salt solution (WelGENE, Korea). The tissues were mechanically dissociated with pipette into the homogeneous solution. The supernatant was removed by passing through centrifugal process, and the remaining cells were mixed with plating medium which consisted of Neurobasal medium (Gibco, USA), 10 mL of B-27 supplement (×50, Gibco), 2 m*M* of GlutaMAX (Gibco), 12.5 μ*M* of L-glutamate (Sigma-Aldrich), and 1% penicillin–streptomycin (Gibco). The cell suspension was filtered with a cell strainer (BD

Falcon, Bedford, MA) to remove cell clusters. Cells were plated on patterned substrates at the density of 50 cells/mm. Cultures were maintained in a humidified incubator under 5% CO_2 and 37°C condition. After 2 days *in vitro* (DIV), the plating medium was replaced with maintenance medium (Neurobasal medium, B27 supplement, 2 m*M* of GlutaMAX, and 1% penicillin–streptomycin). All procedures were done according to approved animal use protocols of the KAIST Institutional Animal Care and Use Committee.

Immunostaining

A mouse monoclonal anti-beta-III tubulin antibody (Sigma-Aldrich) was used to label whole structure of the neuron. To fix the samples, samples were washed three times with 1 × PBS (phosphate-buffered saline, pH 7.4, Gibco), and they were treated with 4% paraformaldehyde (Sigma-Aldrich) for 15 min. After rinsing with 1 × PBS three times, samples were treated with 1% Triton X-100 (Sigma-Aldrich) for 5 min. After the permeabilization and rinsing, samples were treated with 6% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min, and samples were reacted for 1 h at 37°C with a mouse monoclonal anti-beta-III tubulin antibody which was diluted by 1: 500 in 1.5% BSA as a primary antibody. After the primary antibody reaction, samples were rinsed three times in PBS and incubated for 1 h at 37°C with a secondary antibody. We used Alexa Fluor 594 mouse anti-rabbit antibody with 1: 200 (v/v) dilution factor in 1.5% BSA. The stained samples were mounted on a slide glass with Faramount (Dako, Denmark).

Imaging and data analysis

Fluorescent images and phase-contrast images were taken by an inverted microscope (IX71, Olympus, Japan) using 20× objective lens. The length of neurites and the number of processes were measured using MetaMorph (Molecular Devices, Silicon Valley, CA), and the eccentricity of cell body was measured using ImageJ (NIH image). Data were represented by mean ± standard deviation. Statistical significance was evaluated by two-tailed ($\alpha = 0.05$) one-way analysis of variance with Bonferroni's multiple comparison and an unpaired Student's two-tailed *t*-test (significant for $p < 0.001$).

To measure the eccentricity of a soma, the boundary of somata was manually extracted from phase-contrast image using Photoshop (Adobe). Then, the encircled cells were filled with black color and saved as a separate binary image. The binary image was loaded from ImageJ, and the major axis and minor axis of each cell were calculated by the "Fit ellipse" function. Finally, eccentricity of somata was calculated by $(1 - b^2/a^2)^{1/2}$ (a: major axis and b: minor axis).

RESULTS

Fabrication and characterization of pinstripe pattern

In order to confirm that the pinstripe pattern with PLL background had two different surface concentrations of PLL, we investigated the fluorescence intensity profile of PLL-FITC patterns with and without PLL background. Figure 1(B) shows the pinstripe pattern on bare glass surface. In this substrate, the fluorescence intensities in cell-adhesive area (PLL pinstripe) and cell-repellent area (glass coverslip) showed a large contrast. Figure 1(C) shows the pinstripe pattern on PLL-coated glass surface. Because of the background PLL-FITC, the contrast was low, but the intensity profile clearly indicated that the brighter pinstripe pattern that was printed by μCP had higher concentration of PLL than the background.

Neurite outgrowth on pinstripe patterns with PLL background

To confirm whether patterning effect was due to the difference in concentration of PLL between the background and the printed PLL stripes, we tested neurite patterning effect with or without PLL stripes. First group (PLL-PLL) had PLL background with thin PLL stripes. Second group (PLL-BSA) served as a negative control for PLL; it had PLL background with thin BSA stripes. For the sake of visualizing the BSA pinstripe pattern, BSA solution (0.1 mg mL^{-1} in DI water) was mixed with Rhodamine (Sigma-Aldrich) solution (0.1 mg mL^{-1} in DI water). Figure 2(A,B) shows representative images from two groups at 3 DIV. Among the two groups, neurite patterning effect was observed only in the PLL-PLL group [Fig. 2(A)]. On this substrate, cell bodies adhered everywhere because of the PLL background coating, whereas many neurites had the tendency to follow the thin PLL stripe that had the higher concentration of PLL. In contrast, there were no patterning or consistent neurite alignment effect on PLL-BSA groups, which led us to conclude that neurite patterning effect occurred because of the difference in PLL concentration formed by PLL pinstripe patterns.

Effect of line width on the neurite guidance

As the growth direction of neurites was strongly influenced by the pinstripe pattern, the effect of the line width on the neurite guidance was investigated. Figure 3(A) shows the fluorescence image of beta-III tubulin-stained hippocampal neurons on pinstripe pattern with PLL background in variable widths (3, 5, 10, and 15 μm and uniformly-coated substrate as a control), 2 days after the cultivation. When 3-μm lines were used, the guidance effect was observed, and the substrate with the widths of 5 μm and 10 μm also had the guidance effect. In case of 15-μm lines, however, there was no effect, and the growth pattern was similar to that of controls. Figure 3(B) indicates that neurites were highly aligned to the pattern in the cases of 3, 5, and 10 μm, whereas not in 15 μm. If neurites localized equally on the line and off the line, the percentage of on line and off line would be same. Figure 3(C) shows the average length of the longest neurite on each substrate. The longest neurite on 3-μm pinstripe pattern was much longer than that in the control group. Even though the neurites on the 5-μm and 10-μm pinstripe pattern were guided by the lines, their lengths were smaller than the neurites on the control group. However, there was no difference in the average length of the longest neurites between 15-μm pinstripe pattern and control. Figure 3(D) clearly shows the differences in distribution of number of neurites per neuron. The neurons on 3-,

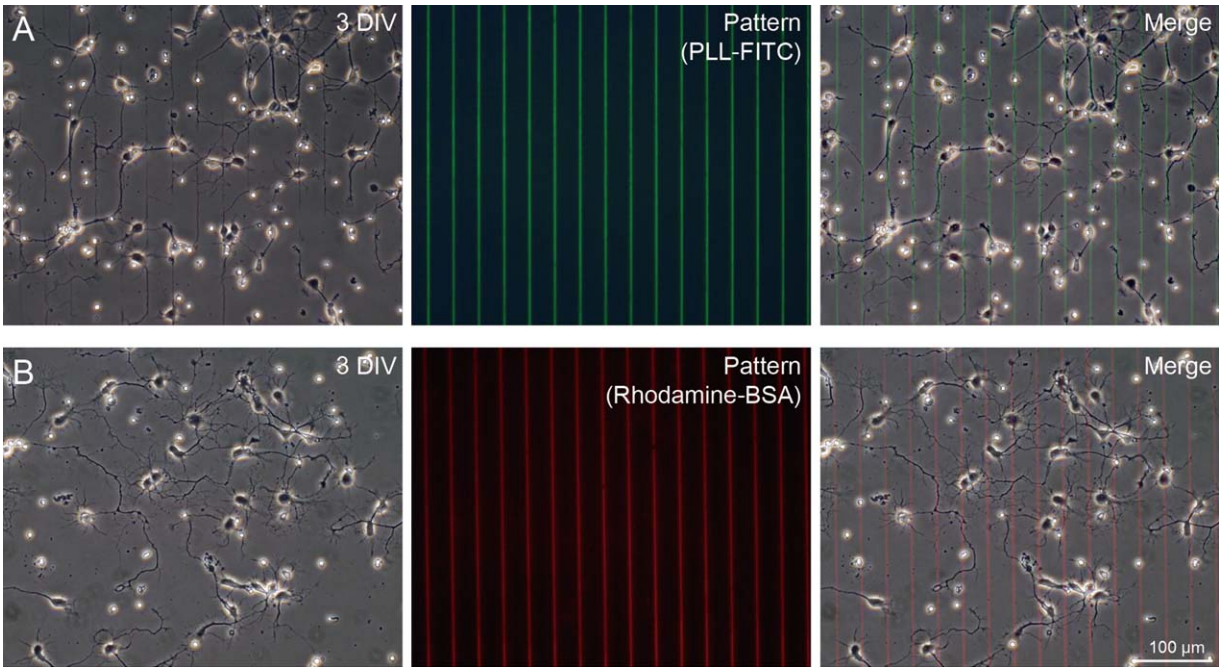

FIGURE 2. Patterning effect on different substrates. A: Phase-contrast image (left) of neurons on PLL pinstripe pattern with PLL background (3 μm in width and 30 μm in spacing) at 3 DIV, fluorescent image (middle) of pattern and mixed image (right). B: Phase-contrast image (left) of neurons on BSA (mixed with Rhodamine) pinstripe pattern with PLL background (3 μm in width and 30 μm in spacing) at 3 DIV, fluorescent image (middle) of pattern and mixed image (right). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

5-, and 10-μm pinstripe pattern had less neurites than that in the control group, but there was no difference of distribution between neurons on 15-μm pinstripe pattern and control group. According to the results, the width of pinstripe pattern must be under 10 μm to pattern the neurites of neurons on the substrates. In case of under 10 μm, the width of line should be considered for the length of neurites on the pinstripe pattern with PLL background.

Morphology of somata on a pinstripe pattern with PLL background

Next, we investigated the effect of pinstripe pattern with PLL background on the morphology of soma (cell body). Figure 4(A) shows neurons cultured in three different substrates: pinstripe pattern without PLL background (3 μm in width and 30 μm in spacing), pinstripe pattern with PLL background (3 μm in width and 30 μm in spacing), and PLL-coated substrate without patterns as a control. To compare the shape of soma, the eccentricity of the soma was measured as an index of cell shape [Fig. 4(B)]. Figure 4(C) shows the eccentricity of cells on each substrate. Eccentricity of cells on a pinstripe pattern without background was 0.82 ± 0.068 (mean $\pm$ SD, $n = 29$), which was significantly different from that of the control groups (mean $\pm$ SD $= 0.66 \pm 0.14$, $n = 43$). This difference indicated that cells on a pinstripe pattern without PLL background had oval shape compared with those in the control group. On the other hand, the eccentricity of cells on a pinstripe pattern with PLL background was 0.69 ± 0.15 (mean $\pm$ SD, $n = 37$), which was similar to the case of the control samples. Neurons on a pinstripe pattern with and without PLL background had patterned neurites through the lines. The pinstripe pattern without PLL background put a constraint on adhesion of somata as an oval shape. On the contrary, somata on the pinstripe pattern with PLL background had a similar morphology in the case of control substrate because of sufficient area of PLL background.

Next, we tested the degree of cell clustering on pinstripe patterns with or without PLL background. On a pinstripe pattern with PLL background, cell clustering was found to be suppressed. We cultured the neurons on pinstripe pattern (3 μm in width and 30 μm in spacing) without PLL background and with PLL background and compared the number of clusters and cells alone between the two substrates. Figure 4(D,E) shows the phase-contrast image of the neurons on both substrates at 1 and 3 DIV. On a pinstripe pattern without PLL background, the average number of clusters was 8.3 ± 4.0 (mean $\pm$ SD, $n = 16$) at 1 DIV, whereas the number of clusters significantly increased to 14.3 ± 6.7 ($n = 16$) at 3 DIV. This indicated that 3-μm line was not adequate to grow a patterned neuronal network for 3 DIV. As a result, the average number of cells alone decreased greatly from 24.5 ± 8.6 ($n = 16$) to 6.8 ± 5.4 ($n = 16$) for 2 days [Fig. 4(F)]. However, on a pinstripe pattern with PLL background, the number of clusters slightly increase from 5.6 ± 3.9 ($n = 16$) to 10.0 ± 5.1 ($n = 16$), but there was no change in average number of cells alone for 2 days [Fig. 4(G)]. After considering the results, sufficient adhesive area of a pinstripe pattern with PLL background facilitated in maintaining patterned cell shape for a long

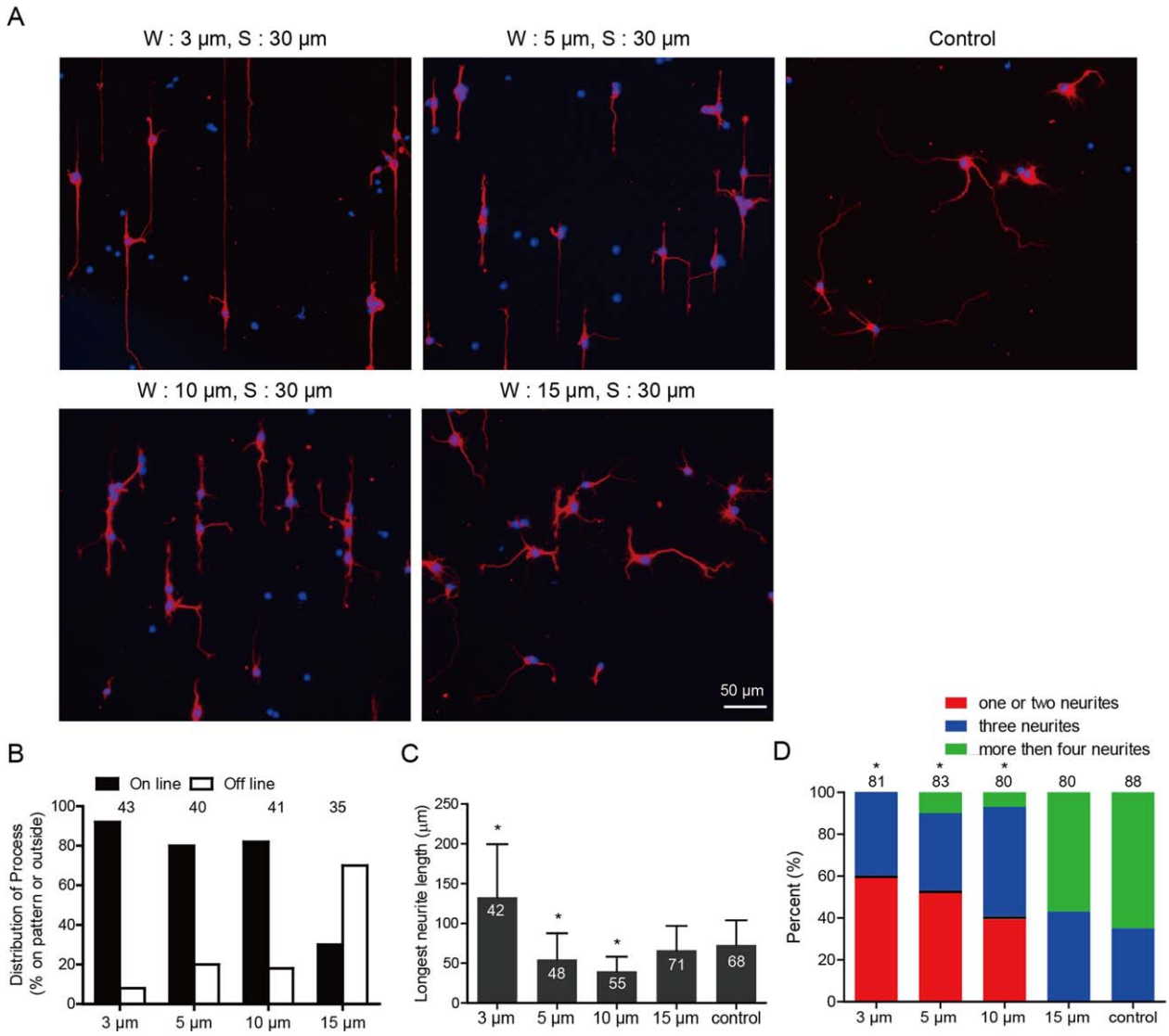

FIGURE 3. Effect of line width on the neurite guidance. A: Beta-III tubulin stained neuron at 2 DIV on the variable widths of pinstripe pattern with PLL background. B: Distribution of processes on line pattern. C: One-way analysis of variance statistical analysis was used, and "*" indicates that results are significantly different from control (mean ± SD, $p < 0.001$). D: Percentages of neurons in each stage on each substrate at 2 DIV. There was significant difference from control group by the chi-square test ($*p < 0.0001$). The numbers indicate the data points for statistics. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

time on a fine line with few microns without cell clumping. This is a distinguishable characteristic to improve neuronal network formation in comparison with conventional PLL μCP.

Filopodia senses the surface concentration difference of polylysine

To examine the importance of filopodial activities in sensing differences in polylysine surface concentration, we treated the neurons on a pinstripe pattern with PLL background (3 μm in width and 30 μm in spacing) with cytochalasin D, an inhibitor of the formation and function of F-actin.[27] We added cytochalasin D (1 μ*M*) to the culture media immediately after seeding, and cultured neurons for 1 day [Fig. 5(A)]. Figure 5(B) shows that 80.0% ($n = 50$) of neurites on the substrate were on line pattern without cytochalasin D but the probability that neurites were on pattern decreased greatly to 10.5% ($n = 51$) with cytochalasin D. This result implied that filopodia in growth cone were involved in the guidance effect from a pinstripe pattern with PLL background.

DISCUSSION

In this work, we generated a step gradient of polylysine, which is a cell adhesive synthetic polymer, by using μCP on a glass coverslip coated with the same molecule. Neurites showed strong tendency following this pinstripe pattern that had high concentration than outside. Notably, we found that line width of pinstripe is less than 15 μm for controlling direction of axon growth.

According to our results, the pinstripe pattern with PLL background could affect the neuritogenesis and axon guidance. When a neuron adhered on a denser PLL line of pinstripe pattern, sprouting neurites were aligned to the line [Fig. 6(A)]. When a growth cone encountered the denser polylysine line, it turned its direction to follow the line [Fig. 6(B)]. However, both effects were completely abolished by the treatment with cytochalasin D, which strongly indicated that F-actin polymerization process was involved in the two effects. Considering the fact that neurite initiation and growth cone turning processes are triggered by the localized perturbations in actin dynamics,[28–30] it is possible that denser polylysine line of pinstripe pattern with PLL background induced more adhesion sites and subsequently drove active actin polymerization and microtubule synthesis in a specific direction. The positively charged surface might stimulate the localized actin polymerization.[31] Furthermore, our results about pattern width specific guidance effect meant that under 10 μm line was needed to induce the localized contrast of actin polymerization. We also proposed a unique method to fabricate two different concentrations using same materials by creating a PLL pinstripe pattern with PLL background. A conventional technique to produce stripe pattern is to use microfluidic channel.[32] Compared with the microfluidic method, μCP method can produce not only pinstripe patterns, but also more complex patterns (e.g., circles and grids) that are difficult to be produced with microfluidic channels. Another noticeable point in our experiment was that we printed positive-charged polymers on a positively-charged surface. This might be counter-intuitive, as electrostatic repulsion may result in no deposition of the second layer. However, according to the fluorescence intensity measurement, we found out that printing PLL on PLL layer was a reproducible technique and it formed an adlayer that resulted in a higher intensity profile in the stamped area.

Surface patterning techniques have been applied for making patterned neuronal network *in vitro*. In 1988, Kleinfeld et al.[33] introduced neuronal pattering method to pattern cell adhesive organosilane molecule among cell repellent alkyl-trichlorosilanes-coated surface. After this work, various culture substrates that had both cell repellent and adhesive area were introduced to create patterned neuronal network *in vitro*. Also, microfabrication technologies, such as μCP,[34–36] photoresist patterning for lift-off,[33,37] micro-molding,[36,38] and laser patterning[39,40] were applied because it was essential to pattern cell repellent and adhesive area in a subcellular scale. To form a cell adhesive area, nonbioactive cell adhesive molecules, such as positively charged synthetic polymer[34–36] and positively charged organosilane molecule,[33,41] have been used. To form a cell repellent area, ethylene glycol groups,[35–37] serine,[36] and hydrophobic organosilane[34,41] were used. More recently, agarose hydrogel was patterned on polylysine-coated substrate as a cell-repulsive mask.[38] In contrast to the conventional neuronal patterning principle, which uses the difference in cell adhesion on predefined areas, we showed that neuronal networks could be patterned only using cell

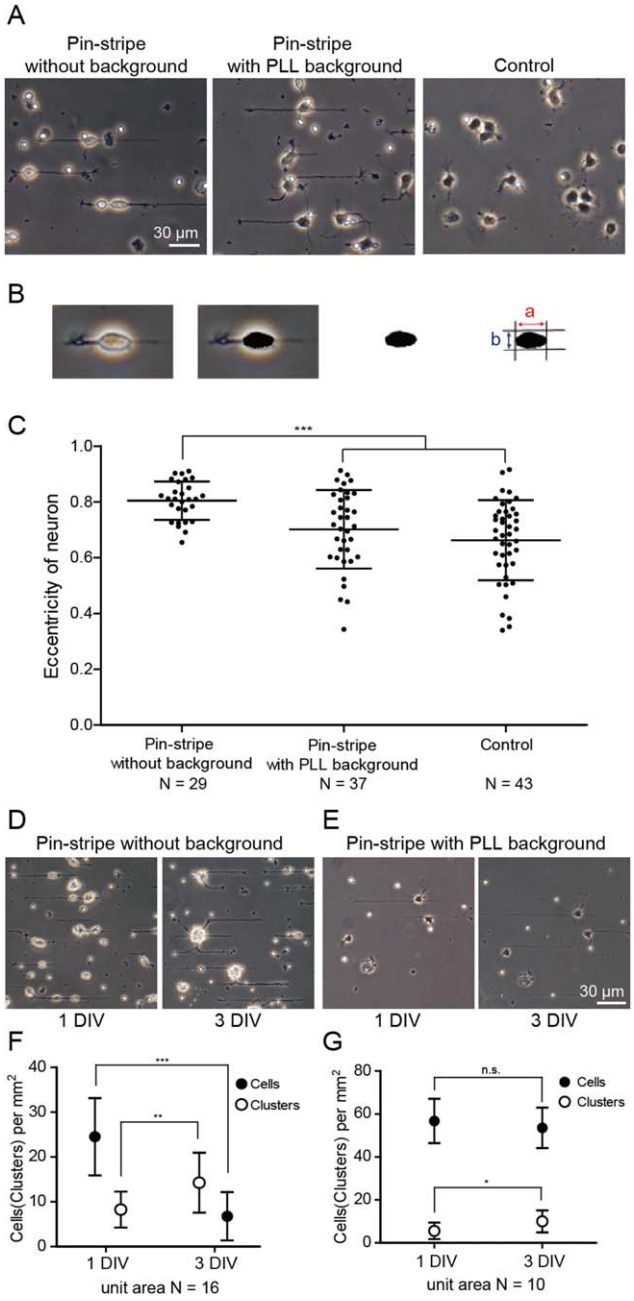

FIGURE 4. Morphology of somata on a pinstripe pattern with PLL background. A: Neurons cultured on pinstripe pattern without background and pinstripe pattern with PLL background (3 μm in width and 30 μm in spacing both) and PLL uniformly coated substrates at 1 DIV. The scale bar is 30 μm. B: Schematic illustration of measuring process of eccentricity of neuronal cell body. C: Scatter plot of eccentricity of neuron on each substrate (mean ± SD, one-way analysis of variance, $***p < 0.0001$). D: Neurons cultured on pinstripe pattern without background (3 μm in width and 30 μm in spacing) at 1 DIV and 3 DIV (top). E: Neurons cultured on a pinstripe pattern with PLL background (3 μm in width and 30 μm in spacing) at 1 DIV and 3 DIV. F: A quantitative analyses of the cells and clusters/mm^2 at 1 DIV and 3 DIV on pinstripe pattern without background (mean ± SD, unpaired *t*-test, $***p < 0.0001$ and $**p < 0.01$). G: A quantitative analyses of the cells and clusters/mm^2 at 1 DIV and 3 DIV on a pinstripe pattern with PLL background (mean ± SD, unpaired *t*-test, $*p < 0.1$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

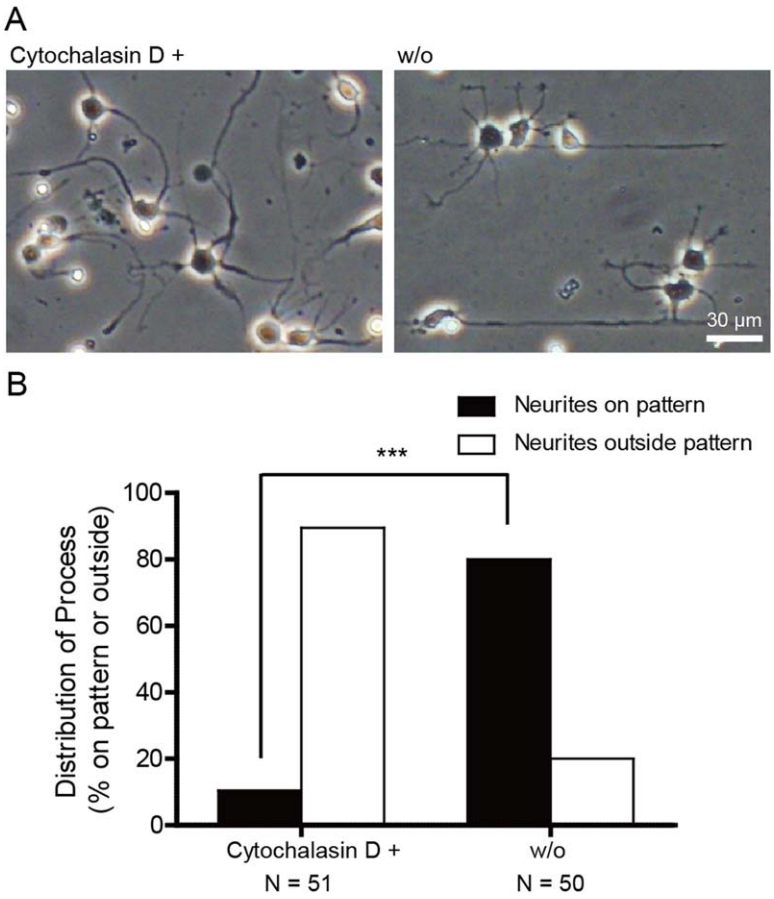

FIGURE 5. Pharmacological manipulation of neurite growth on pinstripe pattern with PLL background. A: Phase-contrast image of hippocampal neurons cultured on pinstripe pattern with PLL background with or without the treatment of cytochalasin D at 1 DIV. The scale bar is 30 μm. B: Distribution of processes for cells on the substrate. There was significant difference between the two conditions by chi-square test (***$p < 0.0001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

adhesive material with different surface concentrations. This new approach in neuronal patterning could give us some advantages over conventional patterning techniques. First, we can think of designing a "neurite guiding area" using the guidance effect 2 [Fig. 6(B)], which could make a site-specific axon guidance assays for constructing unidirectional neuronal networks *in vitro*.[42–45] Second, it can be beneficial for creating a patterned neuronal network with subcellular line patterns. As the whole surface is positively charged, cell bodies tend to spread out more uniformly and firmly on the substrate, and they were not influenced by the size of the testing patterns. This was reflected in the observation that there were significantly less cell clustering or clumping after a few days of cultivation (Fig. 4). In contrast, when neurons were forced to grow on a narrow adhesive area against the nonadhesive background, they tend to form clusters even after a few days under the low-density cell culture conditions. The reduction in cell clustering using pinstripe pattern with PLL background is important from the viewpoint of maintaining a patterned neuronal cultures for some extended period of time.[36,46]

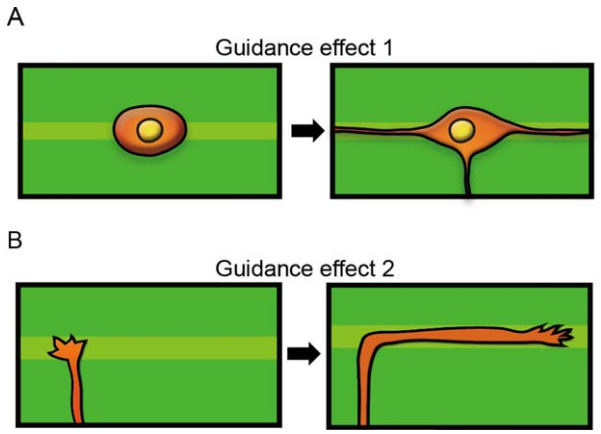

FIGURE 6. Illustration of two different neurite guidance effects in pinstripe pattern with PLL background. A: When neuron adheres right on the line pattern, neurites sprout from the each side of line pattern and grow along the line. B: When the tip of the neurite touched the line pattern, the growth cone turned its direction to follow the line. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

Most of the neuronal patterning techniques focus on patterning the surfaces into two regime: cell-adhesive versus cell-repulsive. This principle works well for growing neuronal networks in certain shapes. In this work, we suggested that

cell-adhesive versus cell-adhesive can also be a patterning principle for controlling neurite outgrowth. What we showed as a novel principle was that two different levels of positively charged surfaces can be adopted in the design of axon guidance assays. It showed some interesting effects on neuritogenesis and axon guidance. This new surface micropatterning method will be a useful experimental tool for elucidating axon guidance and the development of neural circuits in neurobiology. We further expect that the newly found axon guidance principle could be applied to biomaterial in regenerative medicine.

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