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Enhanced neuronal differentiation of pheochromocytoma 12 cells on polydopamine-modified surface

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ABSTRACT

Since pheochromocytoma 12 (PC12) cells have the ability of neuronal differentiation upon nerve growth factor (NGF) treatment, they are a good model for studying the neuronal differentiation. Establishing a strong adhesion of PC12 cells to the culture substrate may increase neuronal differentiation, and the use of L-3,4-dihydroxyphenylalanine (L-DOPA), which is responsible for the adhesive property of mussel adhesive proteins (MAPs), is a feasible strategy for such strong adhesion. We hypothesized that a polydopamine-modified surface can promote PC12 cell adhesion and subsequent neuronal differentiation. We examined whether polydopamine-modified surface promotes PC12 cell adhesion, and further evaluated the neuronal differentiation of these cells. The polydopamine modification enhanced the cell adhesion and viability, and also promoted the neuronal differentiation of NGF-stimulated PC12 cells, as evidenced by the elongation of neurites and expression of neuronal differentiation markers, by increasing the activation of NGF/Trk-Rho GTPase signal pathway. Our findings will help develop an improved strategy for functionalizing biomaterial substrates for less-adhesive cells including neural cells.

1. Introduction

Pheochromocytoma 12 (PC12) cells, which are derived from a rat pheochromocytoma, have been used for studying neuronal differentiation mechanisms since the discovery of their neuronal differentiation ability upon nerve growth factor (NGF) treatment [1]. In serum-free differentiation medium supplemented with NGF, PC12 cells stop proliferating, sprout neurites, and acquire the properties of sympathetic neurons [2]. The neurite outgrowth, a characteristic of neuronal differentiation, has been suggested to be linked to enhanced cell adhesion [3–5], suggesting cell adhesion to the culture substrate may play an important role in neuronal differentiation of PC12 cells [6,7]. Consequently, establishing a strong adhesion of PC12 cells to the culture substrate may result in the increase of neuronal differentiation.

One of the possible strategies to facilitate the strong adhesion of PC12 cells to the culture substrate is the use of L-3,4-dihydroxyphenylalanine (L-DOPA). The mussel adhesive proteins (MAPs) [8] enable the mussels to adhere firmly to wet marine surfaces. The adhesive property of MAPs is attributed to the presence of DOPA [9]. L-DOPA is known to have cytotoxic effects on neurons [10]. However, a low dose of L-DOPA has been reported to protect PC12 cells from serum depletion-induced cell death [11], and a more recent study demonstrated that the polydopamine surface modification attenuated cytotoxicity of underlying materials [12]. Thus, we hypothesized that polydopamine surface modification can promote PC12 cell adhesion and subsequent neuronal differentiation without any significant cytotoxicity.

We examined whether surface modification with polydopamine promotes the adhesion of PC12 cells in a conventional serum-containing growth medium and also assessed PC12 cell adhesion in a serum-free medium to clarify the effect of polydopamine modification on PC12 cell adhesion without interference from the cell adhesion molecules contained in the serum. Additionally, the neuronal differentiation of these cells were induced by subsequently culturing in an NGF-supplemented, serum-free N2 differentiation medium to evaluate the effect of the polydopamine surface modification on the neural differentiation of the PC12 cells. The spreading, viability, and apoptosis of the PC12 cells cultured on surfaces with different coatings were also examined. The neuronal differentiation of the PC12 cells, which was evaluated by measuring the neurite length and determining the expression levels of neuronal differentiation markers, on the polydopamine-modified surface

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was compared to that of the PC12 cells cultured on the gelatin-coated surface [13], which served as a control.

2. Materials and methods

2.1. Gelatin coating and polydopamine modification on culture surface

Culture dishes were coated with gelatin by incubating with 0.1% (v/v in distilled water) gelatin solution overnight at room temperature, followed by washing with sterile phosphate-buffered saline (PBS, Sigma–Aldrich, St. Louis, MO, USA). For the polydopamine modification, L-DOPA solution was prepared by dissolving 2 mg of L-DOPA (D9628, Sigma–Aldrich) in 1 ml of 10 mM Tris buffer base (pH 8.5, Sigma–Aldrich). Under this basic condition, L-DOPA undergoes oxidative polymerization resulting in formation of polydopamine layer on the substrate. The culture dishes were treated with the L-DOPA solution overnight at room temperature, followed by washing with sterile PBS.

2.2. PC12 cell culture

PC12 cells (Paragon Biotech, Baltimore, MD, USA) were maintained in Roswell Park Memorial Institute 1640 growth medium (RPMI 1640, Gibco-BRL, Gaithersburg, MD, USA) supplemented with 7.5% (v/v) fetal bovine serum, 7.5% (v/v) horse serum, and 1% (w/v) penicillin/streptomycin. The cells were cultured at 37 °C in a humidified incubator with 5% CO_2 in air, and cells within 10 passages were used for the experiments. The medium was changed every 2 days. For neuronal differentiation, the medium was replaced by the chemically defined N2-supplemented serum-free medium, which was a 2:1 mixture of Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) and PBS with 5 mg/ml insulin (Sigma–Aldrich), 100 mg/ml transferrin (Sigma–Aldrich), 20 nM putrescine dihydrochloride (Sigma–Aldrich), and 30 nM sodium selenite (Sigma–Aldrich) [14]. For the induction of neuronal differentiation, nerve growth factor (NGF, 100 ng/ml, Invitrogen, Carlsbad, CA, USA) was added daily for 3 days.

2.3. Contact angle measurements

All of the samples were rinsed 3 times in distilled water, soaked for 30 min to ensure the removal of the residues from the surface, and dried in a vacuum drier. The contact angles were measured using a contact angle analyzer (DSA 100, KRÜSS, Hamburg, Germany).

2.4. Scanning electron microscopy (SEM) imaging

The PC12 cells were cultured on coverslips coated with either gelatin or L-DOPA for 2 h. The cells were fixed in 1% (v/v) glutaraldehyde in distilled water for 1 h. After dehydration in a series of graded ethanol, the coverslips were dried and coated with platinum particles for SEM imaging using the JSM-6701F device (JEOL, Tokyo, Japan).

2.5. Neutral Red assay

The cell viability was evaluated by a rapid colorimetric test based on the uptake of a cationic supravital dye, Neutral Red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride), into viable cells. Briefly, the cells were rinsed with PBS, a medium containing 50 μg/ml of Neutral Red was replenished, and the cells were incubated for an additional 3 h. After this incubation, the solution was rapidly removed and 0.2 ml of acetic acid (1%,v/v) and ethanol (50%,v/v) was added to each well to extract the dye. After 5 min at room temperature, the absorbance at 540 nm was measured. The intensity of the red color was directly proportional to the number of viable cells.

2.6. Mitochondrial metabolic activity assay

The mitochondrial metabolic activity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT solution (2 mg/ml in PBS, Sigma–Aldrich) was added to the culture plates and incubated for 4 h at 37 °C and then replaced with dimethyl sulfoxide (DMSO, 100 ml/well) to dissolve the formazan crystals. The absorbance at 570 nm was measured.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was performed using 5 μg of pure total RNA and SuperScript™ II reverse transcriptase (Invitrogen), followed by PCR amplification of the synthesised cDNA. The PCR product were electrophoresed on a 2% (w/v) agarose gel, visualized using ethidium bromide staining, and analyzed using a gel documentation system (Gel Doc 1000, Bio-Rad, Hercules, CA, USA). β-actin served as an internal control. The results of the RT-PCR were quantified using an Imaging Densitometer (Bio-Rad).

2.8. Western blot

Equal amounts of protein from each sample were mixed with sample loading buffer, loaded, and separated by electrophoresis. The proteins separated by SDS–PAGE were transferred to Immobilon-P membranes (Millipore Corp., Billerica, MA), and the membranes were incubated with primary antibodies overnight at 4 °C and then washed and incubated with secondary antibodies conjugated to horseradish peroxidase (Sigma–Aldrich) for 50 min at room temperature. The blots were developed using an enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ, USA). The luminescence was recorded on X-ray film (Fuji super RX, Fujifilm Medical Systems, Tokyo, Japan), and the bands were imaged and quantified using an Imaging Densitometer (Bio-Rad).

2.9. Immunocytochemistry

The cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed in PBS. Primary antibodies against β-tubulin (Sigma–Aldrich), caspase-3 (Abcam, Cambridge, MA, USA), and fascin (ECM biosciences, Versailles, KY, USA) were used. The slides were then incubated in PBS containing rhodamine- or FITC-conjugated secondary antibodies (Jackson-Immunoresearch, West Grove, PA, USA) for 1 h at room temperature. For the cell adhesion area measurement, the PC12 cells were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). After washing twice with PBS, the DiI-labeled cells were fixed with 4% paraformaldehyde. The Alexa Fluor 488-labeled phalloidin staining kit (Invitrogen) was used to stain F-actin according to the manufacturer's instructions. The color of F-actin staining was changed from green to red, as a pseudocolor, for clear comparison to FITC-labeled fascin. All of the slides were mounted with mounting solution containing 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) to stain the nuclei and were photographed using a fluorescent microscope (Olympus, Tokyo, Japan).

2.10. Statistical analysis

All of the quantitative data are expressed as the mean ± standard deviation (SD) of at least 5 independent experiments. For

the two-group comparisons, two-sided *t*-tests were used, whereas one-way ANOVA tests with Bonferroni corrections were performed for the comparison of more than 3 groups (OriginPro 8 SR4 software, ver. 8.0951, OriginLab Corporation Northampton, MA, USA). $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Contact angle of the culture surfaces

The contact angle of the non-coated polystyrene surface was 66.4 ± 1.7°, and this was significantly changed by modifying the

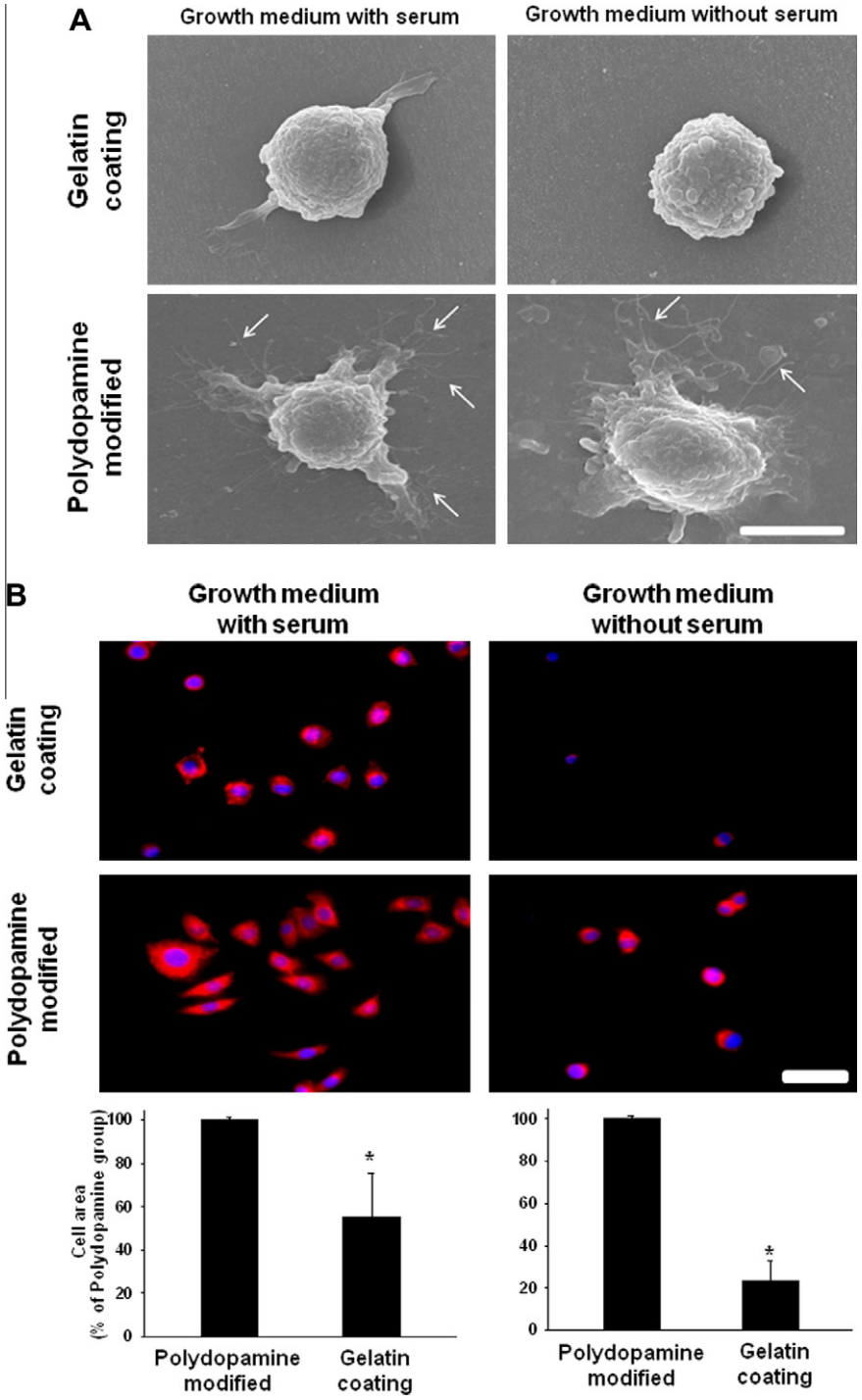

Fig. 1. PC12 cell adhesion on either polydopamine-modified or gelatin-coated surfaces in growth medium with or without serum. (A) The SEM images were taken 2 h after cell seeding. The arrows indicate the filopodia. The scale bar indicates 10 μm. All of the photomicrographs were obtained at the same magnification. (B) Enhanced PC12 cell spreading by culturing on polydopamine-modified surfaces, as determined by DiI staining (red). The cells were cultured on either polydopamine-modified or gelatin-coated surfaces for 24 h in growth medium with or without serum. The nuclei were stained with DAPI (blue). $^{*}p < 0.05$. The scale bar indicates 25 μm. All of the photomicrographs were obtained at the same magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surface (Supp. 1). The gelatin coating increased the contact angle (75.8 ± 0.8°) compared to the non-coated polystyrene surface, whereas the modification with polydopamine decreased the contact angle (40.2 ± 3.8°).

3.2. The polydopamine-modified surface improved the adhesion, spreading, and cytoskeletal organization of PC12 cell

The SEM images clearly indicated that the filopodia formation of the PC12 cells was enhanced on the polydopamine-modified surfaces compared to the gelatin-coated surfaces (Fig. 1(A)). DiI staining indicated that the spreading of the PC12 cells was significantly greater on the polydopamine-modified surfaces compared to the gelatin-coated surfaces, regardless of the presence of serum (Fig. 1(B)). Immunofluorescent staining for F-actin and fascin, key cytoskeletal proteins, demonstrated that the expression levels of these key cytoskeletal proteins increased in the PC12 cells cultured on the polydopamine-modified surfaces compared to the cells cultured on the gelatin-coated surfaces (Supp. 2). Serum in the medium increased both the F-actin and fascin staining intensity in both groups.

3.3. The polydopamine-modified surface increased PC12 cell viability and proliferation

Both the cell viability (Fig. 2(A) and (B)) and mitochondrial metabolic activity (Fig. 2(C) and (D)), which were assessed by Neutral Red and MTT assays, respectively, were significantly enhanced when the PC12 cells were cultured on the polydopamine-modified surfaces compared to the gelatin-coated surfaces. Such increase of cell viability and mitochondrial metabolic activity was observed even without serum supplementation. The Western blot analysis indicated that the polydopamine-modification enhanced the activation of Akt and Erk, important signaling molecules for the cell viability and proliferation of PC12 cells in the absence of NGF stimulation (Fig. 2(E)) [15].

The proliferation of PC12 cells was significantly increased when they were cultured on the polydopamine-modified surfaces (Supp. 3). On day 1, the number of cells cultured on the polydopamine-modified surface in the growth medium supplemented with serum was significantly higher compared to both the gelatin-coated groups (with and without serum supplementation). In addition, the number of cells cultured on the polydopamine-modified surface in the growth medium without serum was significantly higher compared to the gelatin coating group without serum supplementation. On days 2 and 3, the number of cells significantly differed among the groups, except that there was no significant difference between the cells cultured on the polydopamine-modified surface in the growth medium with serum and the cells cultured on the polydopamine-modified surface in the growth medium without serum.

3.4. Viability of the PC12 cells cultured on the polydopamine-modified surface during differentiation

Neutral Red (Supp. 4(A) and (B)) and MTT assays (Supp. 4(C) and (D)) were performed after the PC12 cells were cultured in

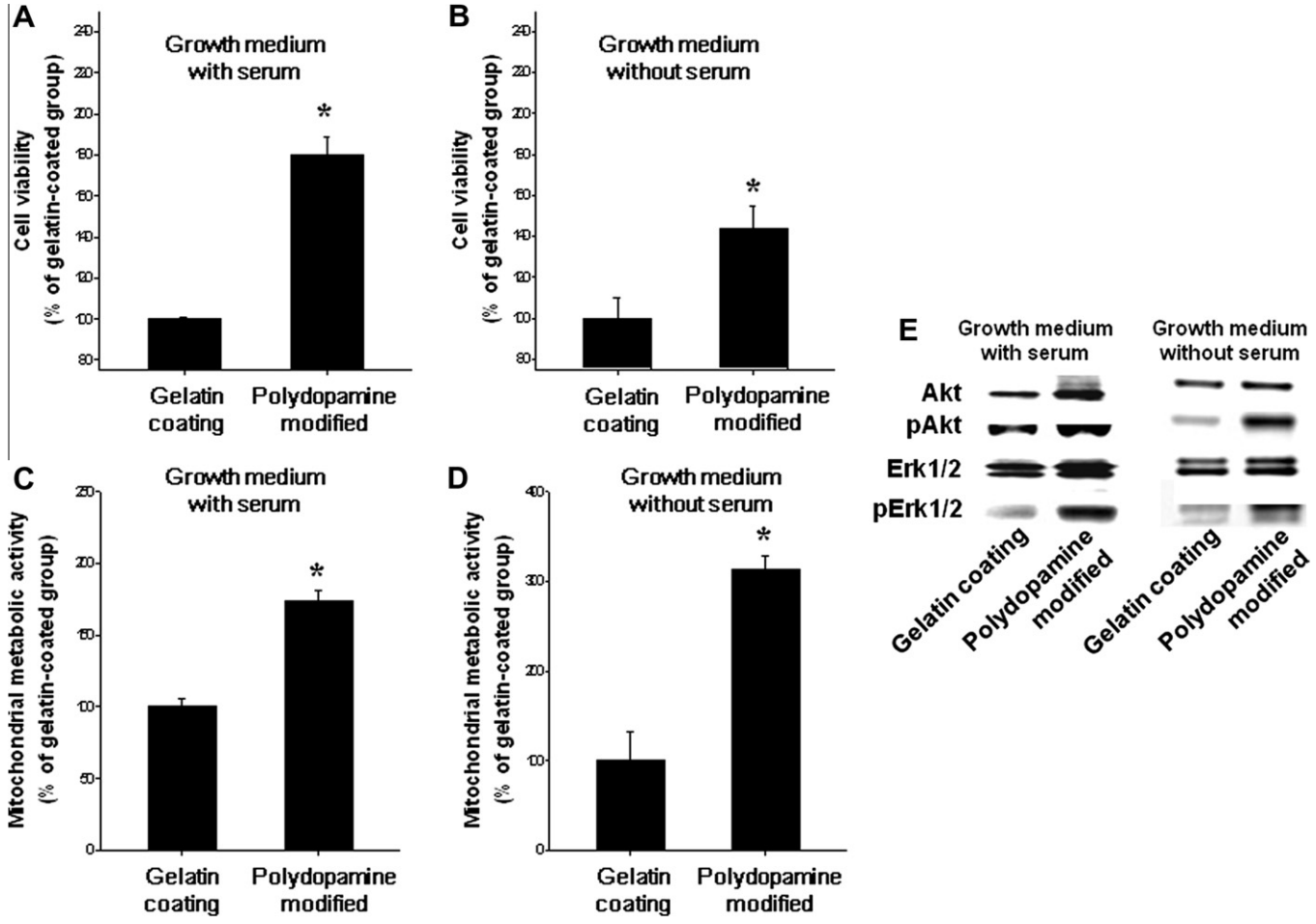

Fig. 2. Enhanced viability and mitochondrial metabolic activity of PC12 cells by culturing on polydopamine-modified surfaces. PC12 cells were cultured either polydopamine-modified or gelatin-coated surface in growth medium with (A) and (C) or without serum (B) and (D) for 24 h. The cell viability was evaluated by the Neutral Red assay, and the mitochondrial metabolic activity was evaluated by the MTT assay. $^{*}p < 0.05$. (E) The expression levels of Akt, pAkt, Erk, and pErk in the PC12 cells cultured on either polydopamine-modified or gelatin-coated surfaces for 3 days, as evaluated by Western blot analysis.

growth medium with or without serum supplementation for 1 day and were subsequently cultured in neuronal differentiation medium (N2-supplemented medium with NGF) for 3 days. The PC12 cells differentiated on the polydopamine-modified surfaces showed higher cell viabilities and mitochondrial metabolic activities compared to the PC12 cells cultured on the gelatin-coated surfaces.

3.5. Apoptotic activity of PC12 cells during differentiation

The ratio of caspase-3-positive cells to DAPI-stained cells was significantly decreased for the PC12 cells cultured on the polydopamine-modified surfaces compared to that of the cells cultured on the gelatin-coated surfaces (Fig. 3(A)). The apoptotic activity of the PC12 cells cultured on the polydopamine-modified surface was significantly decreased compared to that of the PC12 cells cultured on the gelatin-coated surface, even without serum supplementation before the induction of differentiation. Similarly, the polydopamine-modified surfaces resulted in decreased expression levels of pro-apoptotic genes (BAX and p53) while increasing anti-apoptotic gene (Bcl-XL) expression in the PC12 cells, regardless of the presence of serum supplementation before the induction of differentiation (Fig. 3(B)). The Western blot analysis indicated that activation of Akt, a neuroprotective signal [16], increased in the cells grown on the polydopamine-modified surfaces, whereas caspase-3 expression decreased (Fig. 3(C)).

3.6. The polydopamine-modified surface promoted the neuronal differentiation of PC12 cells

The PC12 cells cultured on the gelatin- or polydopamine-modified surfaces were stained positively for β-tubulin, a neuronal marker (Fig. 4(A)). The PC12 cells cultured on the polydopamine-modified surfaces displayed elongated-morphological characteristics of neurites. The differentiation of the PC12 cells was enhanced by culturing on the polydopamine-modified surfaces, as determined quantitatively by measuring the length of the extended neurites (Fig. 4(B)). Furthermore, the PC12 cells cultured on the polydopamine-modified surfaces showed enhanced expression of neuronal differentiation marker proteins, such as growth-associated protein 43 (GAP43) [17] and postsynaptic density protein 95 (PSD95) [18], compared to the cells cultured on the gelatin-coated surfaces. The polydopamine surface modifiecation also increased the expression of cdc42, a member of the Rho family GTPases, and activated Trk (pTrk), a high-affinity NGF receptor (Fig. 4(C)).

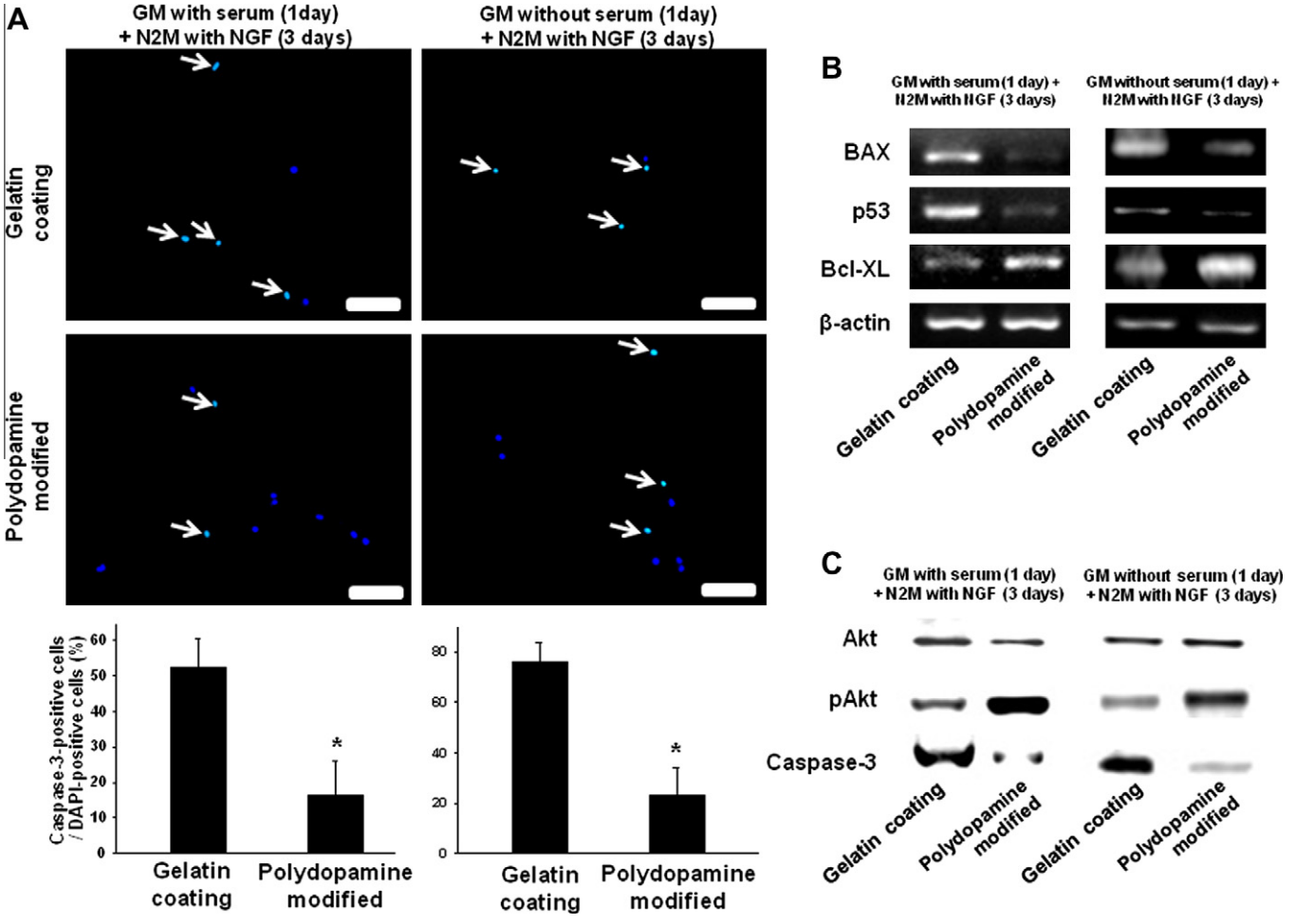

Fig. 3. Reduced apoptosis of PC12 cells cultured on polydopamine-modified surfaces in neural differentiation medium (N2-supplemented medium with daily doses of 100 ng/ml NGF for 3 days). Before the induction of the neuronal differentiation, the cells were cultured in growth medium with serum or without serum for 1 day. (A) Immunofluorescent staining for caspase-3 (green) and quantification of caspase-3-positive cells on day 3. The nuclei were stained with DAPI (blue). The arrows indicate the DAPI/caspase-3-double-positive cells (light blue: green and blue merged). The scale bars indicate 100 μm. $^{*}p < 0.05$. (B) RT-PCR analysis for pro-apoptotic (BAX and p53) and anti-apoptotic (Bcl-XL) factors on day 3. (C) Western blot analysis for Akt and caspase-3. GM: growth medium, N2 M: N2-supplemented medium. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In the present study, the polydopamine surface modification significantly enhanced the adhesion and spreading of PC12 cells compared to the control gelatin coating, and there can be a few possible explanations. First, under serum-supplemented conditions, polydopamine can act as a surface modifier that minimizes the denaturation of surface-deposited serum-contained adhesion molecules by adjusting the surface energy, making them well-preserved in their native confirmation so that they are available for the cell adhesion process [19].

Second, the amine functional group of polydopamine might be also responsible for the enhanced PC12 cell adhesion and spreading under serum-free conditions. Since the amine functional groups of polydopamine layer can be positively charged and the glycoproteins of cell membrane are negatively charged under physiological conditions, polydopamine can promote cell adhesion due to the electrostatic interactions with the glycoproteins of the cell membrane [20,21]. Such amine group-mediated cell attachment and cell spreading has been well reported [22,23].

Another factor involved in the polydopamine-mediated enhancement of cell adhesion and spreading may be the hydrophilicity. It has been reported that PC12 cell adhesion was enhanced in the specific contact angle of 38°–56° [24], and our contact angle measurement results demonstrated that the polydopamine surface modification created a surface with the hydrophilicity within such range (contact angle of 40.2°) (Suppl. 1). In fact, the amine functional groups of L-DOPA have been reported to increase the surface hydrophilicity promoting both cell adhesion and spreading [25].

We believe such polydopamine-mediated increase of PC12 cell adhesion and spreading subsequently increased the viability and proliferation of the PC12 cells on the polydopamine-modified surface (Fig. 2 and Suppl. 3) by preventing anoikis, a type of apoptosis caused by insufficient survival signals from cell-ECM interactions [26], as well as the neuronal differentiation of the PC12 cells based on the previous studies demonstrated that cell adhesion and spreading are prerequisites for neurite outgrowth and that the attachment of either filopodia or lamellipodia to the substratum regulates the rate of neurite elongation [3,27].

Regarding the potential use of polydopamine surface modification in manipulating neural cells, a recent study reported that surface immobilization of NGF and adhesion peptides using polydopamine modification enhanced neural stem cell proliferation and differentiation [28], suggesting a possible use of polydopamine surface modification in developing bio-functional substrate for neural stem cells. However, the experimental design and focus of that particular study were different from those of the present study in that they utilized polydopamine to facilitate the immobi-

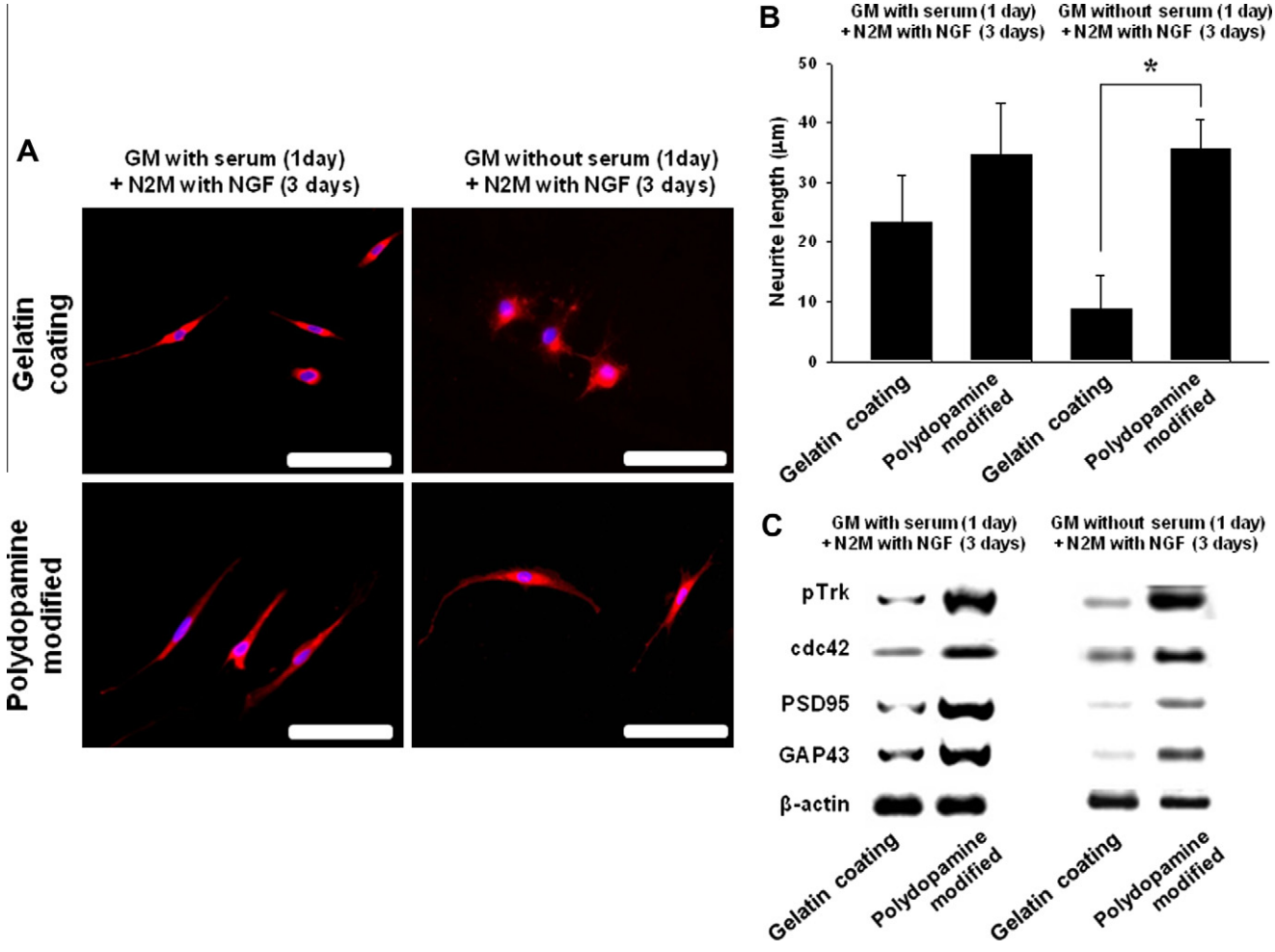

Fig. 4. Enhanced neuronal differentiation of PC12 cells in neural differentiation medium (N2-supplemented medium with daily doses of 100 ng/ml NGF for 3 days) by culturing on polydopamine-modified surfaces. Before the induction of neuronal differentiation, the cells were cultured in growth medium with serum or without serum for 1 day. (A) The PC12 cells were stained for β-tubulin (red) at day 3. The nuclei were stained with DAPI (blue). The scale bars indicate 100 μm. (B) The neuronal differentiation of the PC12 cells was quantified by measuring the neurite length of the PC12 cells. $^{*}p < 0.05$. GM: growth medium, N2 M: N2-supplemented medium. (C) The expression of neuronal differentiation-related factors (pTrk, cdc42, PSD-95, and GAP 43) in PC12 cells, as evaluated by Western blot analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lization of growth factors/adhesion peptides rather than directly attaching cells and also they did not examine underlying molecular mechanisms, as we tried in the present study.

In addition to the neurite elongation and expression of neuronal differentiation makers (PSD95 and GAP43), we examined the expression of pTrk and cdc42 to elucidate the underlying mechanism of enhanced neuronal differentiation of PC12 cells on polydopamine (Fig. 4(C)). Since Trk is a high-affinity NGF receptor and it is phosphorylated upon NGF binding [29,30], the increased phosphorylation of Trk suggested that more NGFs bound to Trk on the polydopamine-modified surface. Since polydopamine immobilizes bioactive molecules by simple dipping [28], it is possible that polydopamine attracted NGFs from the culture medium and retained them in the vicinity of cells increasing the possibility of NGFs contacting the Trk.

Furthermore, increased expression of cdc42, a member of Rho GTPase and signaling molecule downstream of Trk [31], also indirectly proves that polydopamine surface modification increased NGF binding to Trk. Since activated Rho GTPase promotes neurite outgrowth by inducing the formation of lamellipodia and filopodia [32,33], polydopamine-mediated increase of the activation of NGF-Trk-Rho-GTPase signal pathway is believed to be one of the major mechanisms of increased neurite outgrowth on the polydopamine-modified surface.

In summary, polydopamine surface modification can improve PC12 cell adhesion and viability compared to the conventional gelatin coating by providing a favorable microenvironment for PC12 cells during routine maintenance culture. Furthermore, with NGF stimulation, culturing on the polydopamine-modified surface can promote the neuronal differentiation of PC12 cells through NGF/Trk-mediated Rho GTPase activation. Our findings will help develop an improved strategy for functionalizing biomaterial substrates for less-adhesive cells including neural cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.123.

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