Enhanced Differentiation of Human Neural Stem Cells into Neurons on Graphene

*Sung Young Park, Jaesung Park, Sung Hyun Sim, Moon Gyu Sung, Kwang S. Kim, Byung Hee Hong, and Seunghun Hong**

To use human neural stem cells (hNSCs) for brain repair and neural regeneration, it is critical to induce hNSC differentiation that is directed more towards neurons than glial cells.[1–5] However, most previous studies report that hNSCs, without biochemical motifs or co-culturing, differentiated more towards glial cells than neurons.[6–8] On the other hand, although graphene has attracted much interest for biological applications due to its exotic properties such as biocompatibility, electric conductivity, and transparency,[9–12] it has not been explored for neural stem cell behavior, yet. Herein, we report a graphene substrate that enhanced the differentiation of hNSCs into neurons. Microarray studies were performed to explore a plausible explanation for this effect. Furthermore, we demonstrated an electrical stimulation on the cells differentiated from hNSCs using graphene as a transparent electrode. Our findings suggest that graphene has a unique surface property that can promote the differentiation of hNSCs toward neurons rather than glia, which should open up tremendous opportunities in stem cell research, neuroscience, and regenerative medicine.

Our experimental procedure is summarized in **Figure 1**. Graphene was synthesized on a large scale and transferred onto a glass substrate, following a previously reported method (see also Figure S1 and S2 in the Supporting Information).[10,11] The graphene film on glass was then placed into a laminin solution (20 μg mL^{-1} in culture media for 4 h) so that laminin molecules adhered to both the graphene and the glass and helped hNSC attachment. The hNSCs were seeded on the substrate and proliferated in the culture media with basic fibroblast growth factor (bFGF, 10 ng mL^{-1}) and epidermal growth factor (EGF, 10 ng mL^{-1}). Afterwards, the hNSCs were differentiated in the culture media without bFGF and EGF for a long-term period (≈one month). Immunocytochemistry and microarray experiments were performed to characterize their neural differentiation on graphene.

First, we observed the adhesion of hNSCs on graphene (**Figure 2**). 10 h after cell seeding, more hNSCs were found on graphene than on glass, indicating a rapid hNSC attachment onto graphene (Figure 2a). However, when the hNSCs were proliferated in the culture media with the growth factors for five days, the entire surface area, including graphene and glass regions, was fully occupied with hNSCs, and we could not see any difference in cell number between the graphene and the glass regions (left of Figure 2b). The immunofluorescence image shows that the hNSCs were immunopositive for nestin (green), a NSC marker, on both graphene and glass regions, indicating that hNSCs grown on the both regions proliferated very well and exhibited the NSC properties (right of Figure 2b). We also performed a cell viability assay to evaluate the biocompatibility of graphene (Figure S3 in the Supporting Information). The result shows that the hNSCs grew very well on both the graphene and glass regions, which is somewhat consistent with a report that graphene is less toxic than single-walled carbon nanotubes (SWCNTs).[12] Although the biocompatibility is a very complicated issue requiring more thorough investigation in the future, we could not find any more-harmful effect of graphene on hNSCs, in comparison to other cell-growth substrates, such as glass.

The differentiation of hNSCs was initiated simply by exchanging the culture media with media without the growth factors EGF and bFGF (**Figure 3**).[13] After three days of differentiation, we could not find any difference in the hNSC growth between graphene and glass regions (left of Figure 3a). However, after two weeks, the bare region of glass was partially exposed due to the detachment or retraction of hNSCs during the differentiation process (middle of Figure 3a). After three weeks of differentiation, we observed a clear difference in morphology between the cells on graphene region and those on glass region (right of Figure 3a). Note that the graphene region was fully occupied with the differentiated hNSCs with neurite-outgrowths, while many hNSCs were detached from the glass region during the differentiation process.

After one month differentiation, the cells on both of graphene and glass regions exhibited elongated cell shapes with neurite outgrowths, leading to neural network formations, which was typical after neuronal differentiation of hNSCs

Prof. S. Hong
Department of Physics and Astronomy Department of Biophysics and Chemical Biology Interdisciplinary Program in Nano-Science and Technology Seoul National University Seoul
151-747, Korea
E-mail: seunghun@snu.ac.kr
Dr. S. Y. Park
Interdisciplinary Program in Nano-Science and Technology Department of Physics and Astronomy Seoul National University Seoul
151-747, Korea
J. Park, Prof. K. S. Kim
Department of Chemistry Pohang University of Science and Technology
Pohang, 790-784, Korea
S. H. Sim, Prof. B. H. Hong
Department of Chemistry and SKKU Advanced Institute of Nanotechnology Sungkyunkwan University Suwon
440-746, Korea
M. G. Sung
Department of Physics and Astronomy Seoul National University Seoul
151-747, Korea

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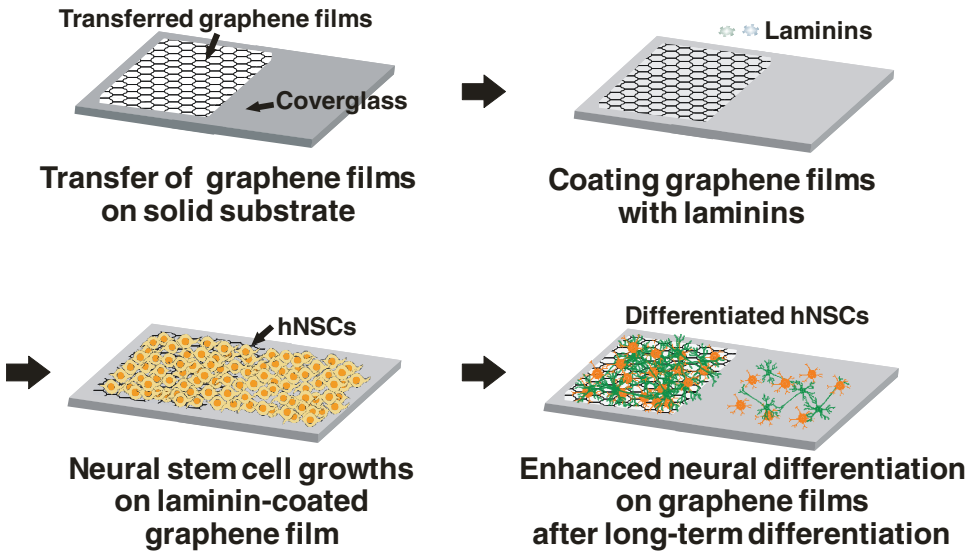

Figure 1. Schematic diagram depicting the growth and differentiation of hNSCs on graphene. Graphene was synthesized on a large scale and transferred onto a cover glass, and laminin was coated on both the graphene and glass regions to help the hNSC attachment. After hNSC proliferation for several days, they were subsequently differentiated by withdrawing the growth factors for a long-term period (≈one month).

(Figure 3b). During the differentiation process, some of hNSCs differentiate to neurons, while others differentiate to glia, which support the activity of neurons.[2–5] We utilized glial fibrillary acidic protein (GFAP, red) and neuron-specific class III beta-tubulin (TUJ1, green) to mark glia and neurons, respectively (bottom of Figure 3b). The immunofluorescence images show the glia and the neurons differentiated from the hNSCs on both of graphene and glass regions. Here, it should be noted that the results of the hNSC differentiation on graphene and glass regions were quite different (Figure S4 in Supporting Information and Figure 3b). During the long-term differentiation process, the cells on the glass region aggregated and often detached from the substrate, resulting in a much reduced number of the differentiated cells (left column of Figure 3b). Meanwhile, the majority of the differentiated cells on graphene remained and adhered quite stably, even after the long-term differentiation process (right column of Figure 3b). For quantitative analysis, we measured the number of cells per unit area by counting the cell nuclei stained by DAPI marker over a surface area of 0.64 mm^2 on graphene and glass regions (Figure 3c and Figure S4 in the Supporting Information). We found that the number of differentiated cells (225 ± 13 cells per area) on the graphene region was larger than that 114 ± 17 cells per area on the glass region ($n = 5$, $p < 0.001$). The result indicates that the graphene provided more favourable microenvironments for hNSC differentiation and promoted cell adhesion and neurite outgrowths than conventional substrates such as glass.

The neural lineages of the differentiated cells were analyzed by immunostaining the differentiated cells and counting the number of cells immunopositive for GFAP (glial cell marker) and TUJ1 (neural cell marker) (Figure S5 in the Supporting Information). Figure 3d shows the percentage of glia (GFAP-positivie

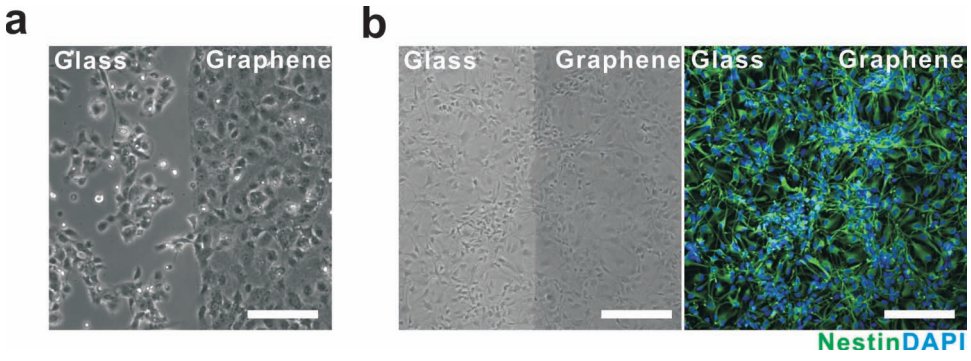

Figure 2. Growth of hNSCs on graphene. The graphene regions appeared slightly darker in all images. All scale bars represent 200 µm: a) Bright-field image of hNSCs on the boundary area between glass (left) and graphene (right) 10 h after cell seeding. Note that more hNSCs were attached on graphene region than on glass region at this early period of cell adhesion. b) Bright-field (left) and fluorescence (right) images of hNSCs proliferated for five days. Immunostaining markers were nestin (green) for neural stem cells and DAPI (blue) for nuclei. There was not a difference in cell numbers between the graphene and glass regions. Note that all the cells were immunopositive for the nestin marker indicating they exhibited the property of NSCs.

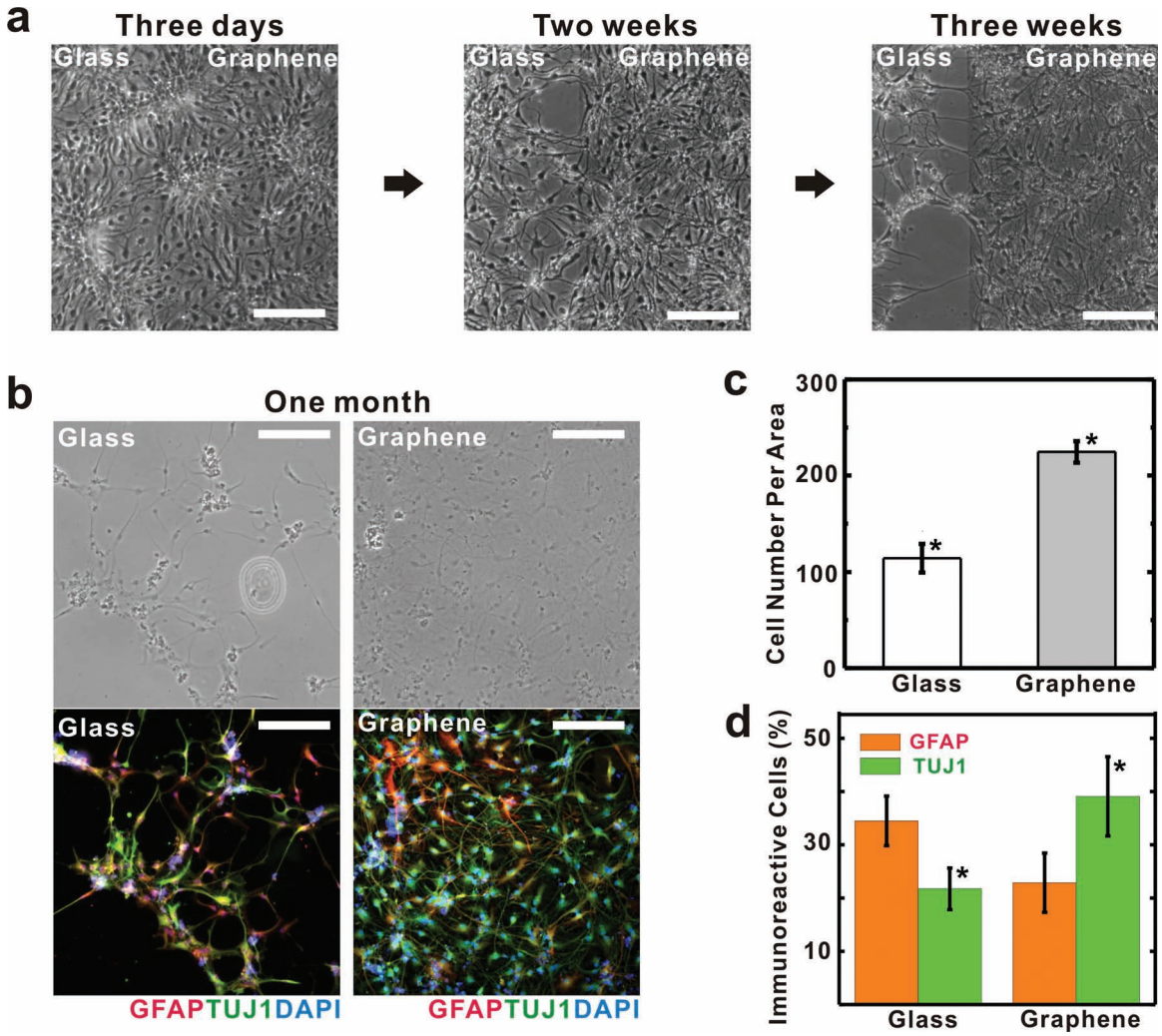

Figure 3. Enhanced neural-differentiation of hNSCs on graphene films. All scale bars represent 200 μm: a) Bright-field images of the hNSCs differentiated for three days (left), two weeks (middle), and three weeks (right). Note that the hNSCs on glass were gradually retracted and detached after two weeks, while those on graphene remained stable even after three weeks of differentiation. b) Bright-field (top row) and fluorescence (bottom row) images of hNSCs differentiated on glass (left) and graphene (right) after one month differentiation. The differentiated hNSCs were immunostained with GFAP (red) for astroglial cells, TUJ1 (green) for neural cells, and DAPI (blue) for nuclei. Note that more hNSCs were adhered to graphene than to glass. c) Cell counting per area (0.64 mm^2) on graphene and glass regions after one-month differentiation. Note that much more cells were observed on graphene in comparison to the glass regions ($n = 5$, $p < 0.001$). d) Percentage of immunoreactive cells for GFAP (red) and TUJ1 (green) on glass and graphene. Note that glass regions show more GFAP-positive cells (glia) than TUJ1-positive ones (neurons), while graphene regions have more TUJ1-positive ones (neurons) than GFAP-positive ones (glia) ($n = 5$, $p < 0.05$).

cells) and neurons (TUJ1-positive cells) on graphene and glass regions. Significantly, we found more neurons (39.1 ± 7.4%) than glial cells (22.9 ± 5.6%) on graphene surface, while there were more glial cells (34.5 ± 4.7%) than neurons (21.7 ± 3.9%) on glass surface. In addition, the percentage of neurons on graphene was larger than that on glass with statistical significance ($n = 5$, $p < 0.05$). The result shows that the graphene substrate enhanced the probability for the differentiation of hNSCs to neurons compared with glass substrates.

To explore plausible explanations for the enhanced neuronal differentiation of hNSCs on graphene, we investigated the gene expressions of the differentiated cells on graphene via microarray experiments, where the cells differentiated on glass were used as a control group. The detailed procedure for the microarray experiment can be found in the Supporting Information. The results show that the laminin-related receptors of the extracellular matrix (ECM) in hNSCs on graphene were significantly upregulated compared with the control group (Figure S6 in the Supporting Information). This indicates the adhesion of hNSCs on graphene was enhanced, which is also consistent with the larger cell number per area on graphene than that on glass (Figure 3c). A previous report showed that hNSCs could have an enhanced differentiation into neurons when they were surrounded by a larger number of glial cells.[14] A plausible explanation for the enhanced neuronal differentiation on graphene could be that this larger cell number was retained on graphene during the differentiation process in comparison with the glass substrate. Specifically, during the

differentiation process of hNSCs, the upregulated laminin receptors of the hNSCs on graphene supported the better adhesion of hNSCs on graphene. Thus, a while after the hNSCs began the differentiation process, a larger number of cells, including glial cells and undifferentiated hNSCs, could be expected to remain on graphene than on glass. At this moment, the undifferentiated hNSCs on graphene were probably surrounded by a larger number of glial cells than those on glass, which could have induced the enhanced differentiation of hNSCs into neurons on graphene. Thus, after a long time period, we can expect a higher ratio of neurons to glial cells on graphene than on glass.

It is also worth noting that genes related to the calcium signaling pathway, such as NCX (Na/Ca exchangers) and GPCR (G protein-coupled receptors), were significantly upregulated when hNSCs were differentiated on graphene (Figure S7 in the Supporting Information). This implies that the differentiated neurons on graphene had key functional components such as ion channels or neurotransmitter-related proteins, which cast a very promising opportunity of using graphene as novel and functional scaffold materials for stem-cell based applications.

Previous reports about the exotic electrical properties of graphene allow us to envision various therapeutic applications such as neural stimulating electrodes for brain disease treatments.[15–17] Here, the electrochemical property of graphene electrode was characterized by cyclic voltammetry using a potentiostat with two-electrode system (Figure S8 in the Supporting Information). The setup consists of graphene as a working electrode (surface area = 0.2826 cm^2) and a Ag/AgCl coil as the common reference and counter electrode in the electrolyte (phosphate-buffered saline, PBS, pH = 7.4). The voltammogram was featureless within the operational potential window from −0.5 V to 1.0 V (vs. Ag/AgCl), except for a small peak around −0.5 V, probably due to any adsorbed oxygen on graphene surface, as reported previously.[18] This result indicates that the graphene films can deliver electric currents to cells via capacitive charge injection without much chemical reaction, which is thought to be ideal for neural stimulation.[17,18]

We observed the electrical neural activity of the differentiated cells using underlying graphene films as a stimulating electrode to confirm their neuronal differentiation (**Figure 4**). The neural differentiation of hNSCs can be confirmed by measuring their neural activity using the graphene films as a stimulating electrode. Here, hNSCs were first seeded and differentiated on a single-layer graphene film on the glass substrate for at least two weeks. The cells were stained with a Fluo-4-AM dye to monitor the change of intracellular calcium ion concentrations (Figure 4a). Previous reports showed that the voltage pulse stimuli on a neuron from an electrode opened calcium ion channels and increased the calcium ion concentration in the neuron, resulting in the enhanced fluorescence intensity of Fluo-4-AM dye in the neuron.[18–20] A series of voltage pulses (usually, 1 ≈ 10 of 500 mV monophasic/cathodic voltage pulses with 1 ≈ 100 ms duration in a second) were applied to the differentiated cells via the graphene electrodes, while monitoring the fluorescence intensity from the cells using a fluorescence microscope. The left and right fluorescence images in Figure 4a show several differentiated cells before and after electrical stimuli, respectively. Note that the cell in the dotted circle clearly exhibited the

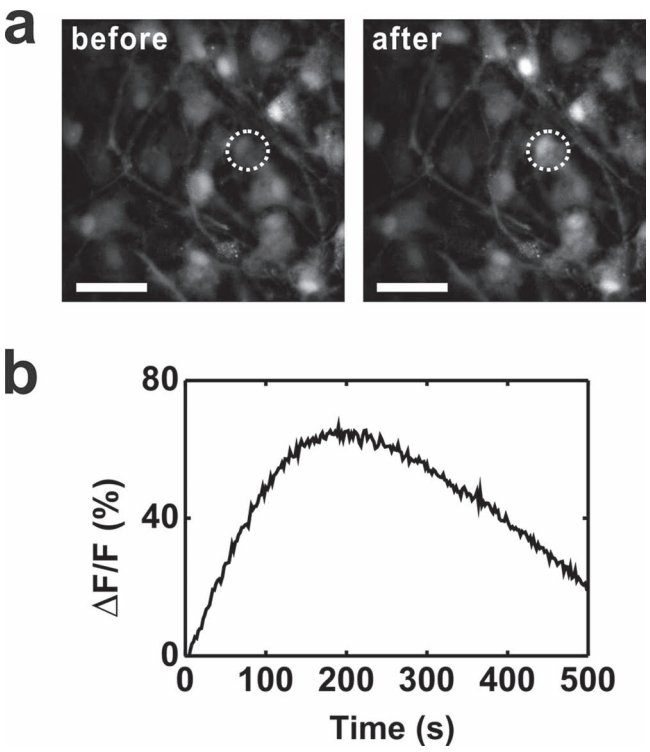

Figure 4. Electrical stimulation of the cells differentiated from hNSCs on graphene electrodes. All scale bars represent 50 μm: a) Calcium imaging of the differentiated cells on transparent graphene electrode before (left) and after (right) electrical stimulation (cathodic/monophasic voltage-pulses, 500 mV, 100 ms, 10 pulse trains in a second). Increased fluorescence intensity of Fluo-4 AM dye in a cell (marked by a dotted circle) was observed after stimulation. b) Plot of the relative fluorescence intensity change $\Delta F/F$ of the cell (marked by the dotted circle in (a)) versus the stimulation time period. The data show the increased calcium ion level after repeated cell stimulation by the graphene electrode.

increased fluorescence intensity after stimulation, indicating an increased calcium level inside the cell. The amount of fluorescence intensity change by the electrical stimuli varied from cell to cell. Presumably, it is because individual cells had different coupling with the graphene electrode surface and some cells were glia, which did not respond to electrical stimuli.

The relative change $\Delta F/F$ of its fluorescence intensity was plotted versus the stimulation time period (Figure 4b). The cell exhibited over 60–70% fluorescence intensity increase by electrical stimuli. We also achieved a similar result using a multiple-layer graphene electrode (Figure S9 in the Supporting Information). This result clearly showed that the differentiated cells from hNSCs on graphene films were functioning as a neuron, and the graphene films can be utilized as a neural-stimulation electrode. Moreover, its excellent optical transparency can be another advantage for further applications such as live-cell imaging compatible with conventional inverted microscopy.

In summary, we discovered the enhanced neuronal differentiation of hNSCs on graphene. The graphene worked as an excellent cell-adhesion layer during the long-term differentiation process and induced the differentiation of hNSCs more toward neurons than glial cells. We also found that graphene had a good electrical coupling with the differentiated neurons

for electrical stimulation. The neural activity of the differentiated cells was confirmed by electrical stimulation using the graphene electrode. Our results suggest that graphene can be used as an excellent nanostructured scaffolds for promoting NSC adhesion and differentiation for long-term periods as well as possible neural prosthetics, which may give rise to various applications in neural regenerative medicine.

Experimental Section

Preparation of Graphene: Graphene was synthesized and transferred onto a cover glass, according to a method reported previously.[10,11] Briefly, SiO_2/SiO wafers coated with Ni (300 nm) or Cu (700 nm) were placed in a tubular quartz tube and heated (≈1000 °C for 5 min) in a flow of CH_4, H_2, Ar/He gas mixtures (CH_4:H_2:Ar = 250:325:1000 sccm for Ni and CH_4:H_2:He = 50:15:1000 sccm for Cu). Subsequently, the sample was rapidly cooled to room temperature. After etching of the metal (Ni or Cu), graphene films were transferred to glass substrates.

Culturing Stem Cells: Immortalized hNSCs (ReNcell VM, Millipore, Temecula, CA, USA) were maintained according to the manufacturer's protocol. Graphene on glass substrates were incubated in laminin solution (20 μg mL^{-1}, Sigma, MO, USA) for 4 h and then washed with PBS by several times. Subsequently, the suspensions of hNSCs were seeded at a cell density of ≈10^5 per mL. All hNSC experiments were carried out between passages of 3 and 10. Neural differentiation could be initiated by removing the growth factors (bFGF and EGF) from the culture media, and the cells were allowed to differentiate for two weeks to one month.

Immunocytochemistry: In brief, hNSCs were first fixed for 15 min in paraformaldehyde (4% by volume) in PBS and permeabilised with Triton X-100 (0.1% by volume) in PBS for 15 min, followed by overnight incubation at 4 °C in the following primary antibodies: anti-nestin (1:200; clone 10C2, Millipore, Temecula, CA, USA), TUJ1 (1:500; clone SDL.3D10, Sigma, MO, USA), and GFAP (1:1000; Dako, Glostrup, Denmark). The cells were washed with PBS, incubated with either goat anti-mouse FITC (1:200; Sigma, MO, USA) or goat anti-rabbit TRITC (1:500; Sigma, MO, USA) and subsequently mounted using a mounting reagent with 4′,6-diamidino-2-phenylindole (DAPI) (ProLong Gold antifade reagent with DAPI, Invitrogen, CA, USA). The mounted samples were imaged using an inverted fluorescence microscopy (TE2000, Nikon, Tokyo, Japan) with an EMCCD monochrome digital camera (DQC-FS, Nikon, Tokyo, Japan). ImageJ software[21] was used for subsequent image processing of the fluorescence images.

Statistical Analysis: Data are expressed as mean ± standard deviation and analyzed by a one-tailed Student's t-test. Statistical significance was determined with *P* values less than 0.001 or 0.05, which was specified each time.

Electrical Stimulation & Calcium Imaging: A cloning cylinder (O.D. 10 mm & height 10 mm, Pyrex, Corning, NY, USA) was mounted on transferred graphene films on cover glass with uncured PDMS solution, and then it was cured at 60 °C for 8 h. The hNSCs were cultured for three days on the graphene films and were differentiated for more than two weeks. For electrical stimulation experiment, culture media were exchanged to extracellular media (pH 7.4, 136 mmol NaCl, 2.5 mmol KCl, 2 mmol $CaCl_2$, 1.3 mmol $MgCl_2$, 1.3 mmol $MgCl_2$, 10 mmol HEPES, 10 mmol Glucose). After washing the cells by PBS, the cells were loaded with Fluo-4 AM dye (2.5 μmol, Invitrogen, CA, USA) for 30 min at room temperature followed by washing for 30 min at room temperature. Time-lapse calcium level in live NSCs was imaged using the inverted fluorescence microscopy with a 40× oil immersion lens (N.A. 1.30, W.D. 0.22 mm, CFI S Fluor 40× oil, Nikon, Tokyo, Japan) and the EMCCD monochrome digital camera. ImageJ software[21] was used for the quantitative analysis of fluorescence intensity.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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