Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B

Supporting Information

Graphene-Incorporated Chitosan Substrata for Adhesion and Differentiation of Human Mesenchymal Stem Cells

Jangho Kim,‡[a] Yang-Rae Kim,‡[b] Yeonju Kim,[c] Ki Taek Lim,[a] Hoon Seonwoo,[a] Subeom Park,[b] Sung-Pyo Cho,[b] Byung Hee Hong,[b] Pill-Hoon Choung,[d] Taek Dong Chung*[b] Yun-Hoon Choung,*[c] and Jong Hoon Chung*[ae]

[a] *Department of Biosystems & Biomaterials Science and Engineering, Seoul National University, Seoul 151-742, Republic of Korea. E-mail: jchung@snu.ac.kr*

[b] *Department of Chemistry, Seoul National University, Seoul 151-747, Republic of Korea. E-mail: tdchung@snu.ac.kr*

[c] *Department of Otolaryngology, Ajou University School of Medicine, Suwon 443-721, Republic of Korea. E-mail: yhc@ajou.ac.kr*

[d] *Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Republic of Korea*

‡ These authors contributed equally to this work.

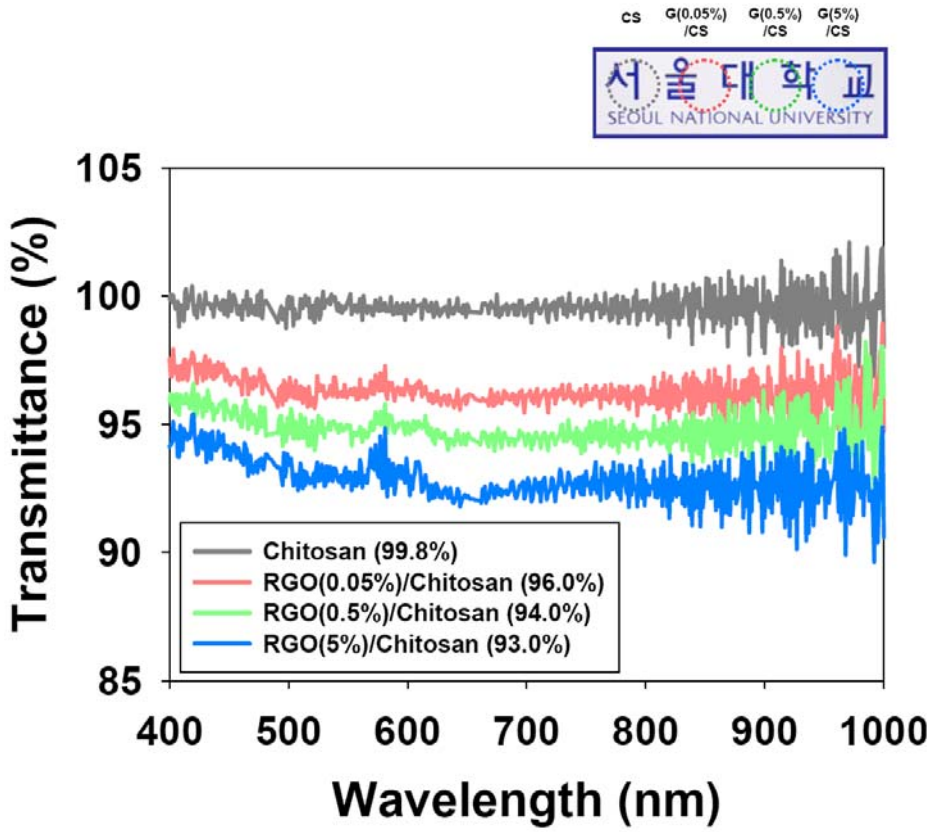

Fig. S1. UV–vis spectra of transferred chitosan and RGO/chitosan substrata.

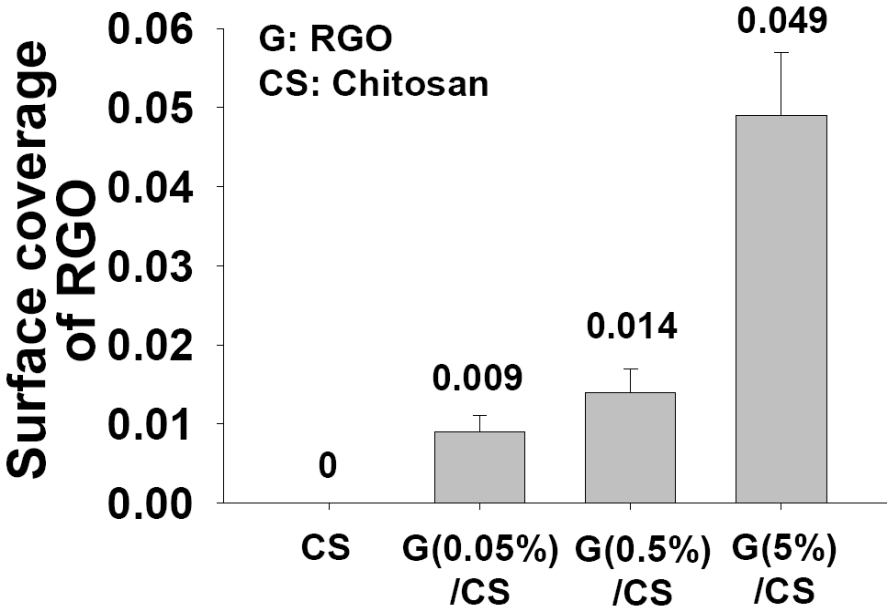

Fig. S2. Surface of coverage of RGO on chitosan and RGO/chitosan substrata.

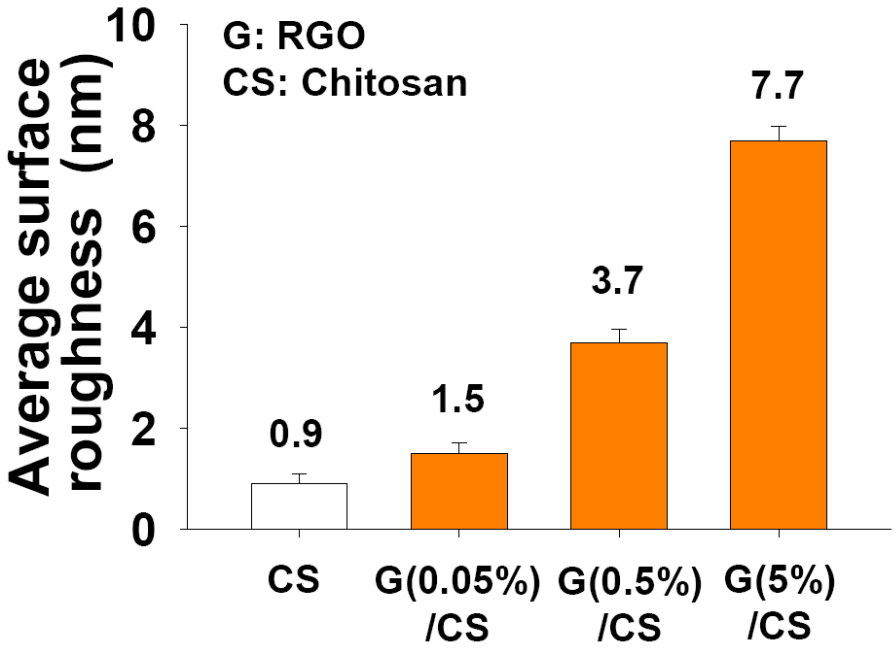

Fig. S3 Average surface roughness of chitosan and RGO/chitosan substrata.

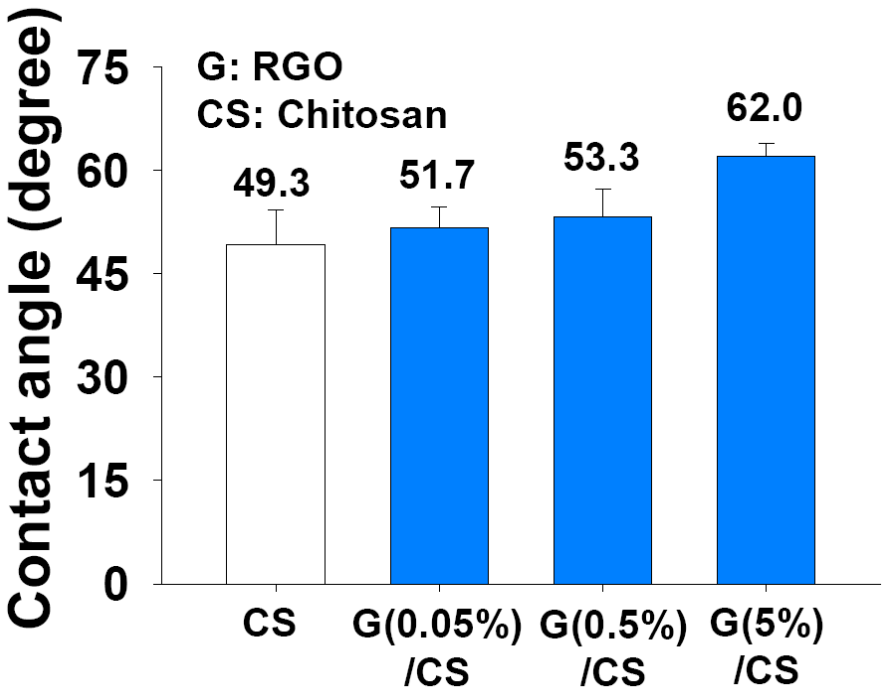

Fig. S4 Contact angle of chitosan and RGO/chitosan substrata.

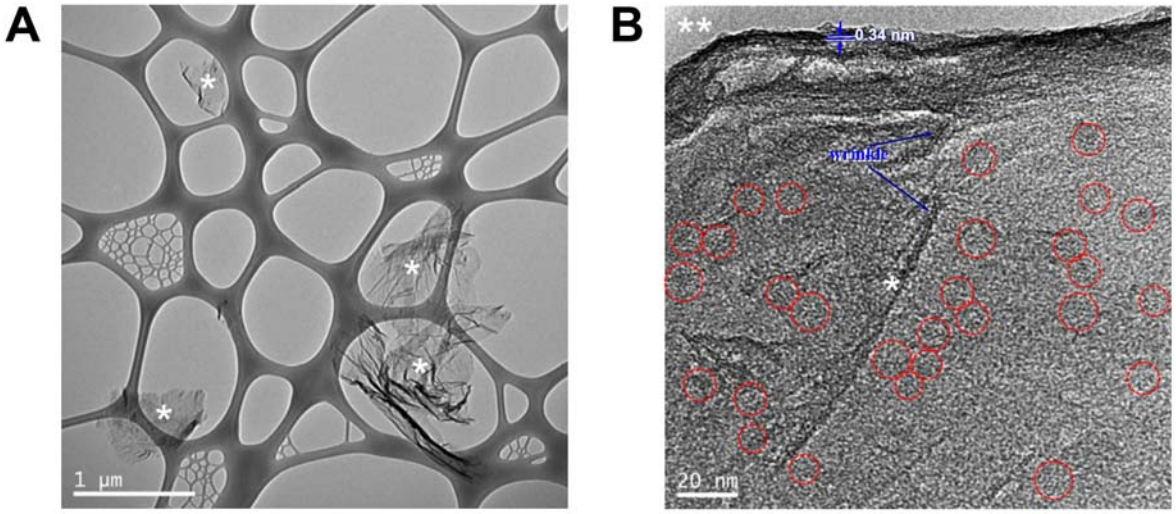

Fig. S5 (A) TEM image of RGO sheets on a TEM grid and (B) High-resolution TEM image of RGO sheets in RGO/chitosan substrata. The (*) and (**) indicate RGO and chitosan, respectively. The red dotted circles indicate randomly distributed small crystalline regions.

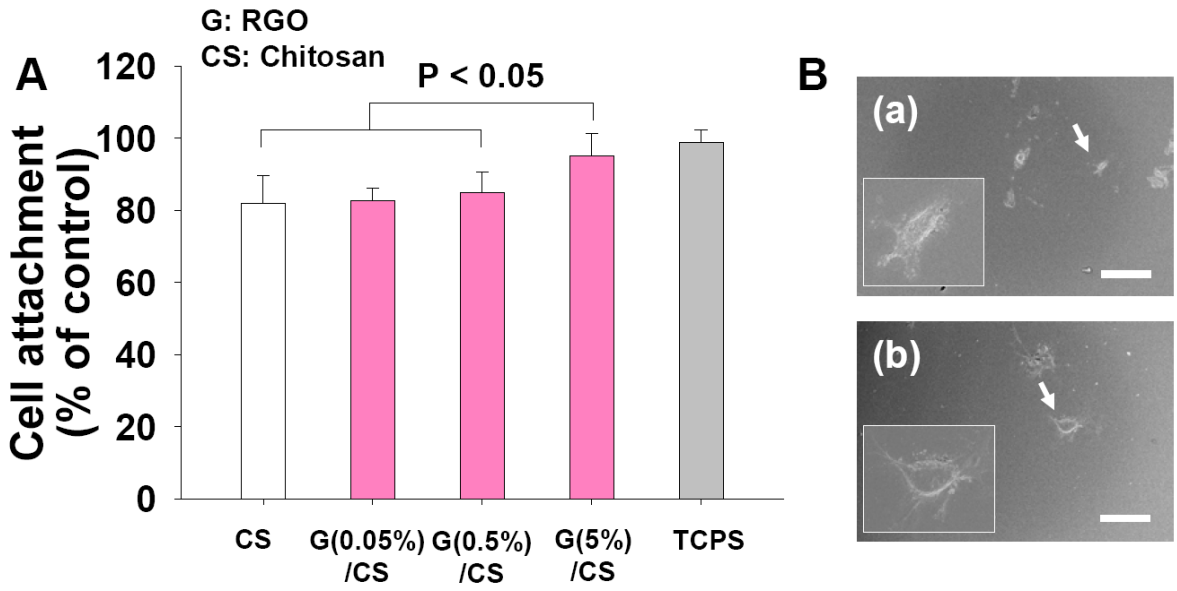

Fig. S6 Attachment of hMSCs on chitosan RGO/chitosan substrata. (A) Quantification of the adhered hMSCs on RGO/chitosan substrata, and also on chitosan substrata and TCPS at 6 hour after seeding of hMSCs. Error bars represent the SD about mean (n = 3 for each group). The control means the initial cell density seeded on the samples. (B) FESEM images of hMSCs on (a) chitosan and (b) 5% RGO/chitosan substrata at 6 hour; the insets in (a) and (c) are high magnification images of adhered cells (white arrows) on the sample surfaces. The scale bars represent 60 μm.

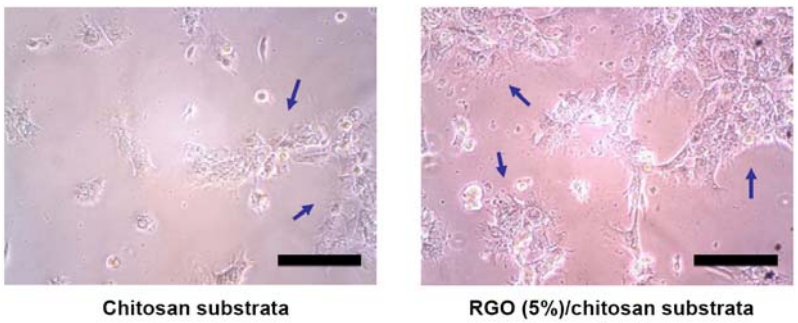

Fig. S7 Phase contrast micrographs of hMSCs adhered on chitosan and 5% RGO/chitosan substrata at 6 hour; all scale bars represent 200 μm.

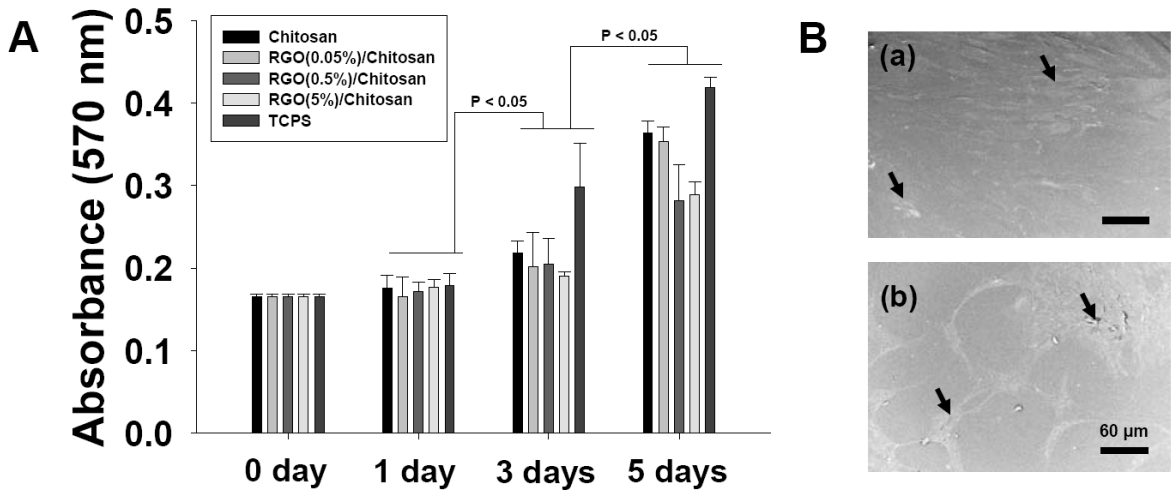

Fig. S8 Proliferation of hMSCs on chitosan RGO/chitosan substrata. (A) Quantification of the time-dependent viability of hMSCs on RGO/chitosan substrata, and also on chitosan substrata and TCPS as controls at 1, 3, and 5 days after seeding of hMSCs. Error bars represent the SD about mean (n = 3 for each group). (B) FESEM images of hMSCs on (a) chitosan substrata and (b) 5% RGO/chitosan substrata at 5 days; all scale bars represent 60 μm.

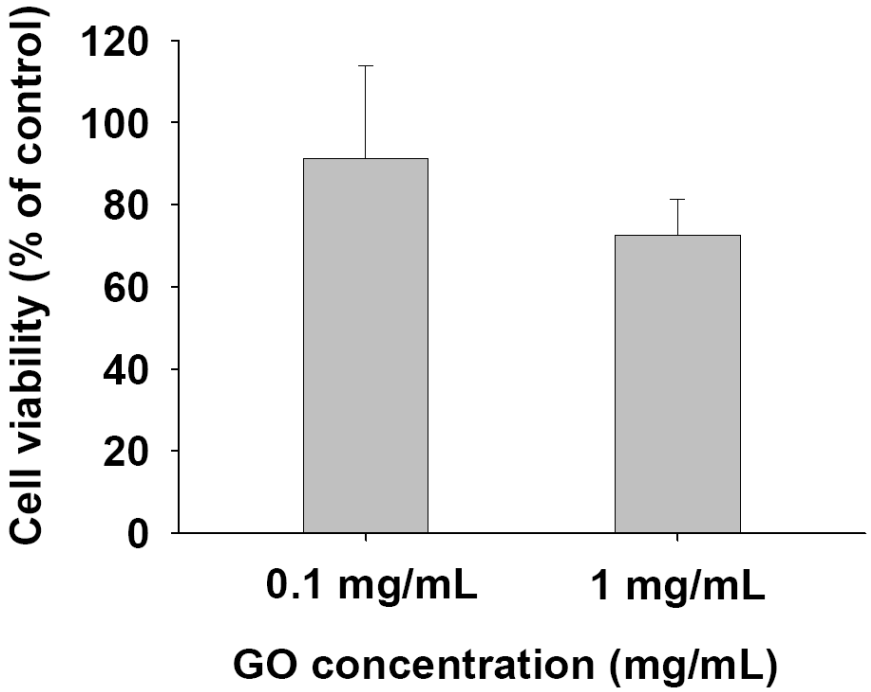

Fig. S9 Indirect cytotoxicity of GO. Viability of hMSCs where the hASCs were cultured with extraction media from GO at different concentrations and TCPS for 24 hour where the absorbance was normalized with that of the control (TCPS), which was taken as 100%.

Detail Experimental

Synthesis of Reduced Graphene Oxide

Graphite powder (< 20 μm) was purchased from Sigma-Aldrich and used as received. Graphene oxide (GO) was synthesized using a modified Hummers method. Synthesized GO was suspended in water (0.05 wt.%) to give a brown dispersion. Exfoliation of GO was achieved by ultrasonication for 3 h, and the mixture was subsequently dialyzed (12–14 kDa cut-off) for 6 h to remove residual salts and acids. The obtained solution was then subjected to 20 min of centrifugation at 3000 rpm to remove unexfoliated GO. The resulting homogeneous dispersion (100 mL) was mixed with 1 mL of hydrazine solution (35 wt.% in water) and 7 mL of ammonia solution (28 wt.% in water). The reduced GO (RGO) powder was then obtained by filtration and drying in vacuum.

Preparation of Graphene-Incorporated Chitosan Substrata

Chitosan (molecular weight: 200,000, deacetylation degree: 89%) was purchased from Taehoon Company (Korea). The chitosan was dissolved using an acetic acid solution (1 wt.%). Then, an RGO/chitosan composite solution was prepared by blending RGO with chitosan solution. RGO concentrations in the mixed solution were controlled by 0, 0.05, 0.5, and 5% w/w. The RGO/chitosan composite solution was spin-coated on glass (Malenfeld Gmbh & Co.KG, Germany) at 4000 rpm followed by drying for 12 h in a vacuum oven at 50°C.

Imaging of Graphene-Incorporated Chitosan Substrata

Graphene-incorporated chitosan nanocomposite substrata were characterized using an atomic

force microscope (NanostationII; Surface Imaging Systems).

Contact Angle Measurements of Graphene-Incorporated Chitosan Substrata

Water contact angles on the fabricated substrata were measured using a video contact angle analyzer (VCA; Phoenix 600, Seoul, South Korea). Ten microliters of water was dropped onto the surface of each sample. The water contact angle was measured as the tangent to the interface of the droplet on the sample surfaces. Measurements were repeated at least 3 times for each sample and averaged. All experiments were performed at room temperature.

Raman spectra of Graphene-Incorporated Chitosan Substrata

The Raman spectra of the samples were obtained with a T64000 (Horiba Jobin Yvon) at an excitation wavelength of 514.54 nm.

TEM observation of Graphene-Incorporated Chitosan Substrata

TEM samples were prepared by dropping a small amount of the RGO, chitosan, and RGO/chitosan solution on a lacey carbon-coated TEM Cu grid and allowing the solution to dry at ambient temperatures. TEM analysis was performed in a JEM-2100 microscope operating at 200 kV with a LaB6 filament. The point-to-point resolution and the lattice resolution were 0.23 and 0.19nm, respectively.

Isolation and Culture of hMSCs

Adipose tissues were isolated from patients undergoing ear surgeries under sufficient informed consent at the Ajou University School of Medicine (Suwon, Korea). The experimental protocol was approved by the Institutional Review Board of the Ajou University

School of Medicine (Suwon, Korea). Tissues were washed with PBS and digested with 100 Unit/mL collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) with low glucose Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) and incubated for 8 h to lyse the adipose tissues. The stromal fraction was collected by centrifugation and then passed through a cell strainer (100 μm size) to remove any large cell clumps and particles. For cell culture and expansion of adipose-derived cells, cell were grown in low glucose DMEM with 10% FBS and 1% penicillin-streptomycin (Gibco, Milan, Italy) at 37°C in a 5% CO_2 atmosphere.

Cytotoxicity of Graphene Oxide on hMSCs

GO at different concentrations in DMEM without any serum or antibiotics were used. The extraction media were then prepared by immersing the samples in a 96-well culture plate and incubating for 24 h. The hMSCs were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated in culture medium. The culture medium was removed after 24 h and then the prepared extraction media was added to the 96-well culture plate. The cells were incubated for 24 h and the number of viable cells was quantified by the 3-(4,5-dimethyl-2-thiazoly(2,5-d-iphenyl-2H- terazolium bromide)) (MTT) assay. Three independent experiments were conducted.

Quantification of Adhesion and Proliferation of hMSCs on Graphene-Incorporated Chitosan Substrata

For the quantitative analysis of adhesion and proliferation of hMSCs on graphene/chitosan substrata, chitosan substrata, and TCPS, the hMSCs on the sample surface were cultured for 0.25, 1, 3, and 5 days, and were observed using a phase contrast microscope. After careful

removal of the unattached hMSCs by washing the sample surfaces with PBS, we conducted MTT assays to quantify hMSC adhesion and proliferation onto the graphene/chitosan substrata.

Imaging of hMSCs on Graphene-Incorporated Chitosan Substrata using FESEM

Cells adhered to the sample surfaces were fixed with modified Karnovsky's fixative consisting of 2% paraformaldehyde and 2% glutaraldehyde (Sigma-Aldrich) in a 0.05 M sodium cacodylate buffer (Sigma-Aldrich) for 4 h. The samples were washed with 0.05 M sodium cacodylate buffer 3 times for 10 min and fixed with 1% osmium tetroxide (Sigma-Aldrich). The samples were then washed with distilled water and dehydrated with graded concentrations (50, 70, 80, 90, and 100% v/v) of ethanol. Then, the samples were treated with hexamethyldisilazane (Sigma-Aldrich) for 15 min. Finally, the samples were coated with gold prior to cell shape observation by FESEM (JEOL, JSM-5410LV, Japan).

Osteogenesis of hMSCs on Graphene-Incorporated Chitosan Substrata

hMSCs (4×10^4 cells/sample) were cultured for 7, 14 or 21 days on RGO/chitosan substrata, chitosan substrata, and TCPS in normal media (DMEM with 10% FBS and 1% antibiotics) or osteogenic differentiation media (100 nM dexamethsone, 50 μM ascorbic acid, and 10 mM glycerol 2-phosphate in normal media). Alizarin Red S (Sigma-Aldrich) staining was used for confirming osteogenic differentiation of hMSCs on the sample surfaces. The degree of mineralization was measured by Alizarin Red S staining of hMSCs cultured on the sample surfaces, and this measurement was were used for quantification of the osteogenic differentiation of hMSCs. Cells were stained with Alizarin Red S and were destained with cetylpyridinium chloride (Sigma-Aldrich), and then the extracted stains were measured using

an ELISA reader (VERSAMAX reader, Molecular Devices, Sunnyvale) at 540 nm.

Neurogenesis of hMSCs on Graphene-Incorporated Chitosan Substrata

hMSCs (2×10^4 cells/sample) were cultured on RGO/chitosan substrata, chitosan substrata, and TCPS in neurogenesis media (NPBM media (Bio Whittaker) with 5 mM cAMP, 5 mM IBMX, 25 ng/ml NGF, 10 ng/ml BDNF, 2.5 mg/ml insulin, and 50μ g/ml ascorbic acid (Sigma-Aldrich)). To check neurogenesis of hMSCs, analysis of the immunostaining of TUJ1 (early neural marker) and Neun (later neural marker) were used.

Western Blot Analysis

Total cellular protein was extracted by RIPA lysis buffer (62.5mM Tris-HCL, 2% SDS, 10% glycero, pH 7.5) with added proteinase inhibitor cocktail (Invitrogen). Cell lysates were incubated on ice for 30 min and then centrifuged at 13,000rpm for 30 min at 4℃. Supernatant (protein lysate) was collected and protein concentration was determined by a micro bicinchoninic acid (BCA) Protein Assay Kit (Bio-rad, Hercules, Calif). 25 μg aliquots of the cell lysates were separated by 8% SDS-PAGE under reducing conditions. Separated proteins were transferred to a PVDF membrane (Millipore, Corporation, Bedford, MA) at 30 V for 1 h. After blocking with 5% skim milk in PBST, the membrane were incubated overnight in primary antibody at 4℃. Primary antibody was purchased from the company (Integrin β1 (Santa Cruz, Biotechnology, Santa Cruz, CA); Connexin 43 (Cell Signaling Technology, Beverly, MA); Osteopontin (Abcam, Cambridge, MA). After washing, the membranes were probed with horseradish peroxidase-conjuaged anti-IgG (Invitrogen), and proteins were visualized using the ECL chemiluminescence detection system (GenDEPOT, Hous-ton, TX, USA). Protein expression was normalized versus β-actin antibody (Cell Signaling Technology,

Beverly, MA) on he same blot membrane. Quantification of the Western blot was performed using the Image J software with a normalization of the level of the entire protein.

Immunohistochemical Analysis

Adhered cells on samples were fixed with a 4% paraformaldehyde solution (Sigma-Aldrich, Milwaukee, WI, USA) for 20 min, permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, WI, Milwaukee, USA) for 15 min, and stained with TRIT conjugated phalloidin (Millipore, Billerica, MA, USA) and 4, 6-diamidino-2-phrnykinodole (DAPI; Millipore, Billerica, MA, USA) for 1 h. Images of the stained cells were taken using a fluorescence microscope (Zeiss, Germany).

Statistical Analysis

All quantitative results are presented as mean ± standard deviation (SD) and unpaired Student's t-tests were used for statistical analysis.