Electronic Supplementary Information for

Neural differentiation on aligned fullerene C_{60} nanowhiskers

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Experimental Section

Materials

Pristine fullerene C_{60} powder with purity > 99.5%was purchased from Materials Technologies Research (MTR), Ltd. (Cleveland,OH, USA). Toluene and IPA with purity > 99% were purchased from the Wako Chemical Corp., Japan. Allchemicals were used as received.

Preparation and purification of C_{60} NWs (see main text)

C_{60} NWs were prepared using the modified LLIP method. Xylene (or toluene) was used as the solvent and IPA was used as the non-solvent.

Alignment of C_{60} NWs (see main text)

C_{60} NWs were aligned at the air-water interface with a constant reciprocal linear shaking motion at 3 cm/sec contained in a 100 mL beaker. Glass microscope slides were used to collect and deposit the aligned C_{60} NWs. A typical linear shaker apparatus used was the reciprocal shaker from Major Science (Taiwan).

Characterization of C_{60} NWs

The morphology and size distribution of the C_{60} NWs were observed by a scanning electron microscope (SEM; Hitachi S-4800, Japan). Raman spectra were recorded by Raman scattering (UniD2G, UniNano Tech Co., Korea) operating at a 532 nm excitation wavelength with the power level set to 1.0 mW. The crystallinity of C_{60} NWs was analyzed by the X-ray powder diffraction (XRD, X'Pert, PANalytical, Netherland) over the 2θ range from 10 to 30° at a rate of 0.02°/s, using Cu-Kα radiation (40 kV, 40 mA).

Culture of murine neural stem cells (NSCs)

Murine NSCs were isolated from adult mouse brain and transfected with the promoter F1B-green fluorescence protein (F1B-GFP). NSCs were plated onto T75 flask in chemically defined neurosphere medium [F12 nutrient mix, DMEM (both Gibco, USA), 400 μg/ml G418 (Invitrogen), and 100 U/ml Pen-Strep (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) as growth factors] in an incubator set at 37 °C and 5% CO_2 in a humidified atmosphere.

Cell viability

NSCs were seeded at a density of 1.5×10^5 cells per well in the 6-well tissue culture polystyrene (TCPS) plates or on C_{60} NW substrates and were incubated at 37 °C for 72 h. The cell viability on various C_{60} NWs substrates at 72 h was evaluated by the

Nucleocounter® NC-3000™. The numbers of live (healthy)/live (unhealthy)/dead cells on C_{60} NWs were counted based on staining with fluorophore VB-48, acridine orange (AO), and propidium iodide (PI) solution. Finally, the percent viable cells were normalized to that on the TCPS dish (control).

Gene expression analysis

The total RNA was extracted by the Trizol® reagent (Invitrogen, USA) from NSCs. The purity of total RNA were checked by a ratio of A260/A280 (>1.9). Total RNA (1 μg) was used to synthesize cDNA in 20 μl reaction solution using a kit of RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Then 2 μl of cDNA was used for qPCR assay in triplicates with DyNAmo Flash SYBR Green qPCR Kit (Finnzymes Oy, Espoo, Finland). PCR consisting of denaturing at 95 °C for 10 min, annealing and extension for 30 s at 60 °C was operated by an Applied Biosystems® StepOne™. The expression was normalized to that of GAPDH. The primer sequences for each gene used in this study are shown in **Table S1**.

Immunofluorescent staining for NSCs on C_{60} NWs substrates

The protein expression of Nestin, glial fibrillary acidic protein (GFAP), β-tubulin, and Microtubule-associated protein 2 (MAP2) was analyzed by immunofluorescent staining. NSCs were seeded on C_{60} NWs film and washed by wash buffer and fixed with 4% paraformaldehyde solution for 30 min. The samples blocked with 2% Goat serum for 1 h and stained with Nestin (GeneTex, GTX26142), β-tubulin (Proteintech, 10068-1-AP), MAP2 (BioLegend, B206152), and GFAP for 1 h at 25°C. The samples were washed with wash buffer and incubated with a secondary goat anti-rabbit or anti-mouse IgG antibody (GeneTex) for 1 h at 25 °C. The samples were washed with wash buffer and observed by the fluorescence microscope.

Statistical analyses

Data were analyzed by one-way ANOVA as appropriate. The data of qPCR and invasion assay are presented as mean ± SEM. The rest of data is presented as mean ± SD. A probability value ≤ 0.05 was regarded as significant.

Supplemental tables and figures:

Table S1. The primer sequences used for real-time RT-PCR analysis.

Genes	Primer sequences	Annealing temperature (°C)
GAPDH	Forward: AGAACATCATCCCTGCATC Reverse: ACTCCTCAGCAACTGAGGG	60
nestin	Forward: GTGGCCTCTGGGATGATG Reverse: TTGACCTTCCTCCCCCTC	60
β-tubulin	Forward: AGCTGTTCAAACGCATCTCG Reverse: GACACCAGGTCATTCATGTTGC	60
MAP2	Forward: TTCTCCACTGTGGCTGTTTG Reverse: GAGCCTGTTTGTAGACTGGAAGA	60
GFAP	Forward: CCTTCCTTCCCTGGTTTTCT Reverse: TGCTCATCTTTCCTCTTCCC	60

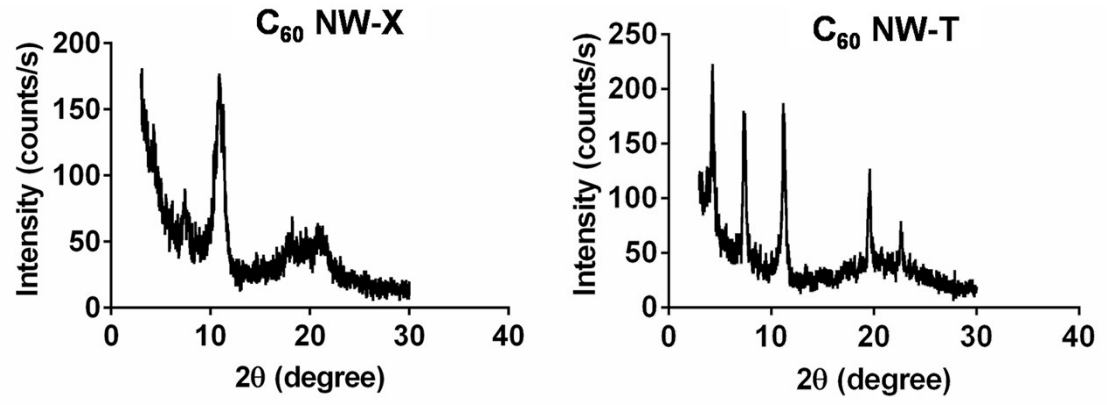

Figure S1. XRD patterns for the aligned fullerene NWs. According to the data, we found that the NWs made from m-xylene showed higher crystallinity in (111) planes than those prepared by toluene. Moreover, the pattern of fullerene NWs made from m-xylene was almost the same with that of the pristine fullerene. These results indicated that the fullerene NWs made from m-xylene had a more horizontally aligned structure in (111) planes. This may account for the better effect of C_{60} NW-X on promoting NSC differentiation into neurons.

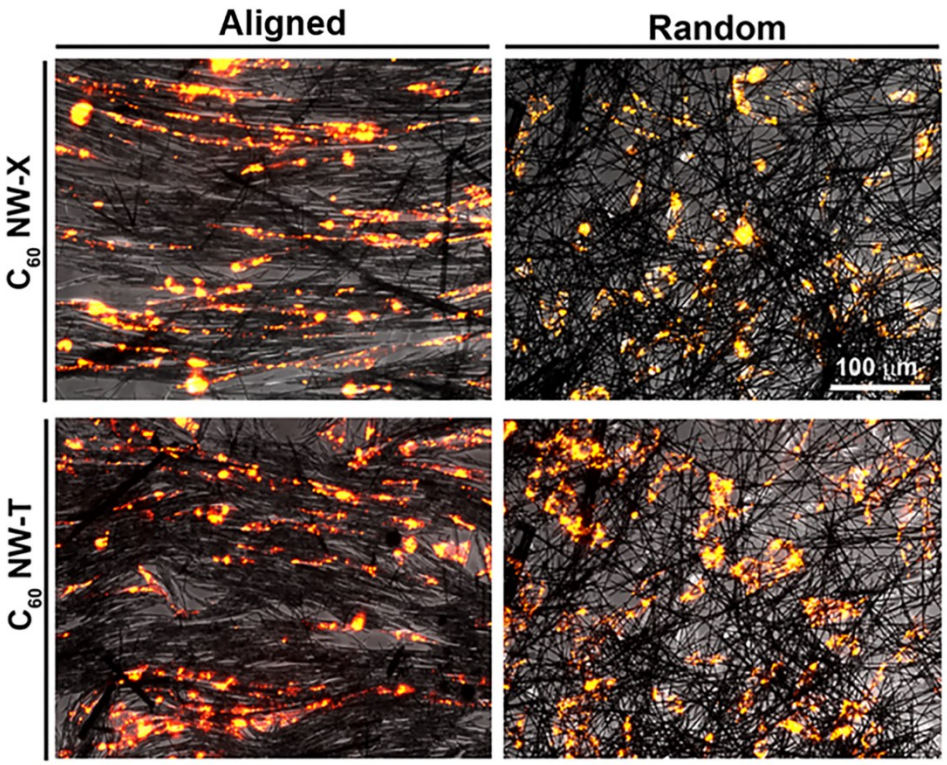

Figure S2. The morphology of NSCs grown on the aligned or random C_{60} NWs for 24 h. NSCs on the aligned C_{60} NWs were observed to orient with the C_{60} NWs.

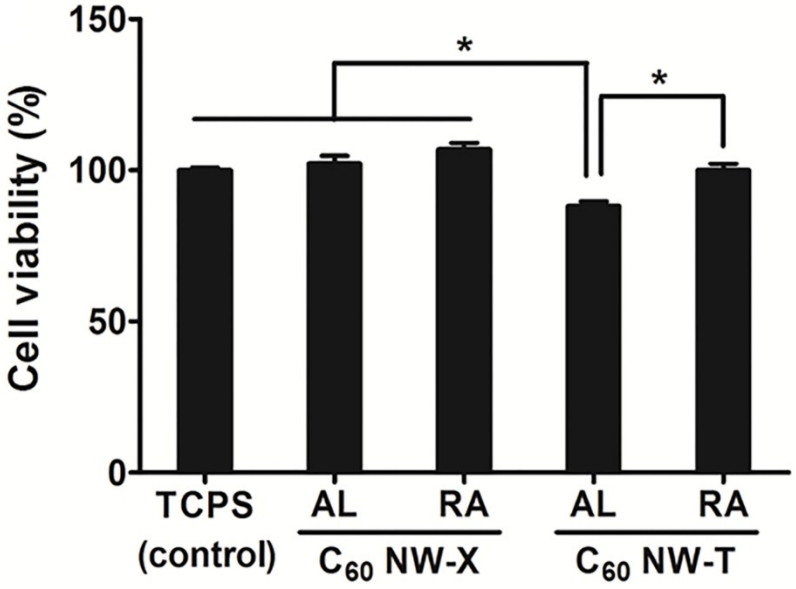

Figure S3. The cell viability for NSCs grown on C_{60} NWs after 72 h, compared to the control TCPS.

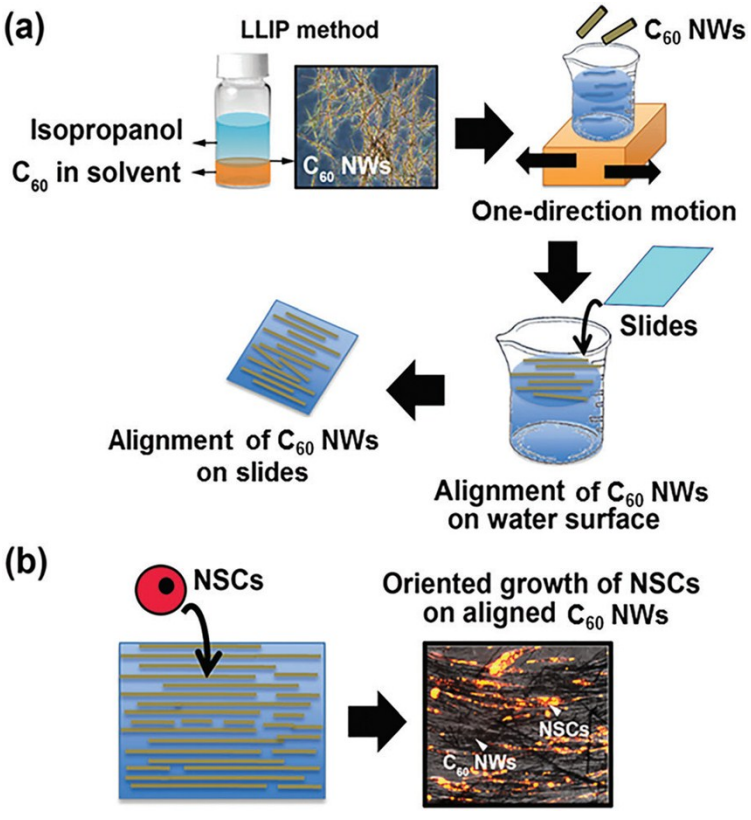

Figure S4. Schematic illustration for the preparation of highly aligned C60 NWs and growing neural stem cells (NSCs) on the NW scaffolds. (a) The alignment of C60 NWs was obtained at an air–water interface by the

reciprocal one-directional shaking flow. (b) The aligned C60 NWs were used as scaffolds for directing NSC growth.