Journal of Materials Chemistry B

Electronic Supporting Information

Advantages of anchoring growth factors to materials for neural stem/progenitor cell proliferation

T. Nakaji-Hirabayashi,[*a,b,c] K. Fujimoto,[a] Y. Kato,[b] H. Kitano,[a,d] Y. Inoue[e] and K. Ishihara[e]

a. Department of Applied Chemistry, Graduate School of Science and Engineering, University of Toyama, 3190 Gofuku, Toyama-shi, Toyama 930-8555, Japan
b. Department of Applied Chemistry, Faculty of Engineering, University of Toyama, 3190 Gofuku, Toyama-shi, Toyama 930-8555, Japan
c. Frontier Research Core for Life Sciences, University of Toyama, 3190 Gofuku, Toyama-shi, Toyama 930-8555, Japan
d. Institute for Polymer-Water Interfaces, 84 Fukujima, Yatsuo, Toyama-Shi, Toyama 939-2376, Japan
e. Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

*Correspondence - nakaji@eng.u-toyama.ac.jp

Results

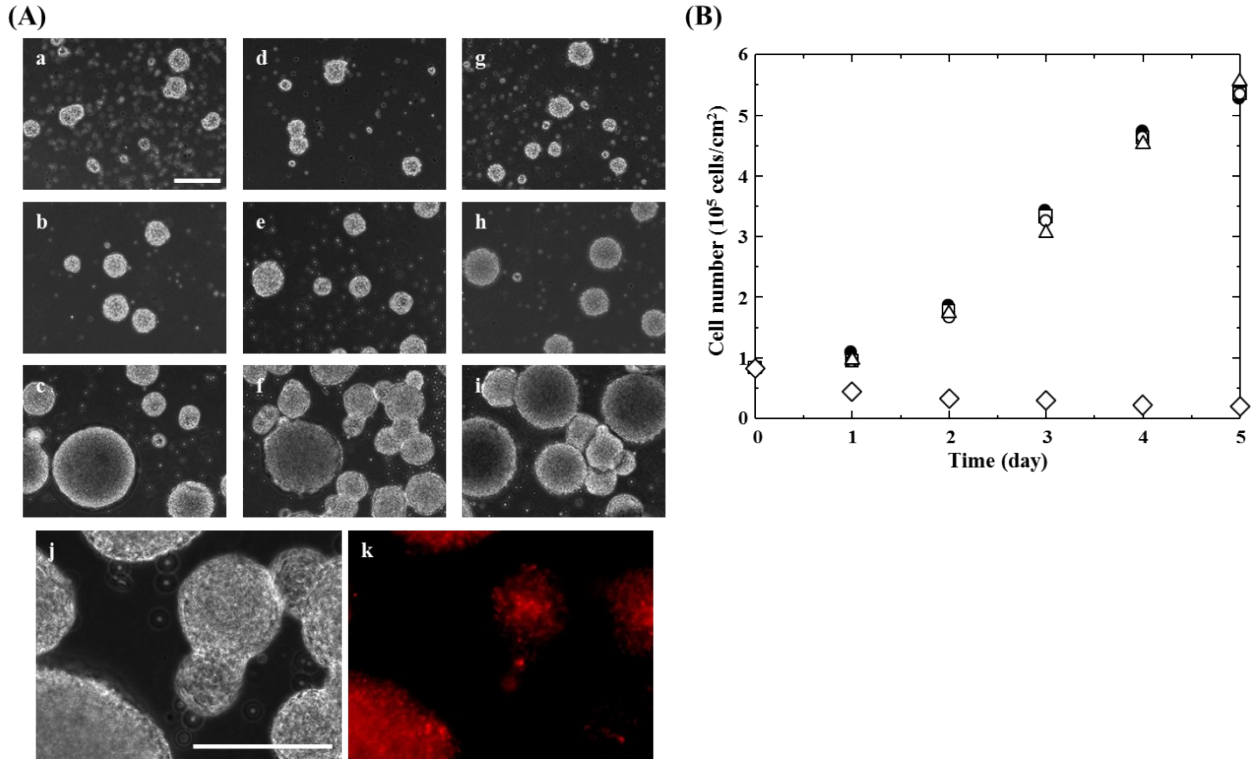

Fig. S1. Bioactivity assay of (a–c) EGF-His, (d–f, j, k) A594-EGF-His, and (g–i) EGF-biotin. (A) Phase contrast (a–i) and fluorescence (j, k) images of neural stem/progenitor cells (NSPCs) cultured for 1 (a, d, g), 2 (b, e, h), 3 (c, f, i), and 4 (j, k) days using the neurosphere method. Scale bar = 200 μm. (B) Growth curve of NSPCs

cultured in medium with EGF-His (open circle), A594-EGF-His (open square), EGF-biotin (open triangle), and rhEGF (closed circle), and without growth factor (open diamond).

Table S1. Surface density of EGF-His and laminin immobilized to the mixed SAM surface with Ni^{2+}-NTA-modified COOH-thiol and CH_3-thiol.

Ni-NTA content (%)[1)]	0	15	33	50	67	85	100
Surface density of EGF-His [2)*] (ng/cm^2)	–	19.1 ± 3.3	69.8 ± 6.6	165 ± 5.7	248 ± 3.3	298 ± 9.9	349 ± 22
Number of EGF-His molecules ($pmol/cm^2$)	–	119	437	1030	1550	1860	2190
Surface density of laminin [2)*] (ng/cm^2)	688 ± 92	643 ± 23	620 ± 41	549 ± 19	572 ± 45	516 ± 34	–
Number of laminin molecules ($pmol/cm^2$)	69.1	65.1	62.8	55.5	57.7	52.7	–

[1)] Surface COOH-thiol and CH_3-thiol contents in the mixed SAM were investigated in previous research.[S1]
[2)] Surface densities of all immobilized proteins were determined by MicroBCA assay on the substrate.[S2]
* These data are expressed as the mean ± standard deviation for n = 3.

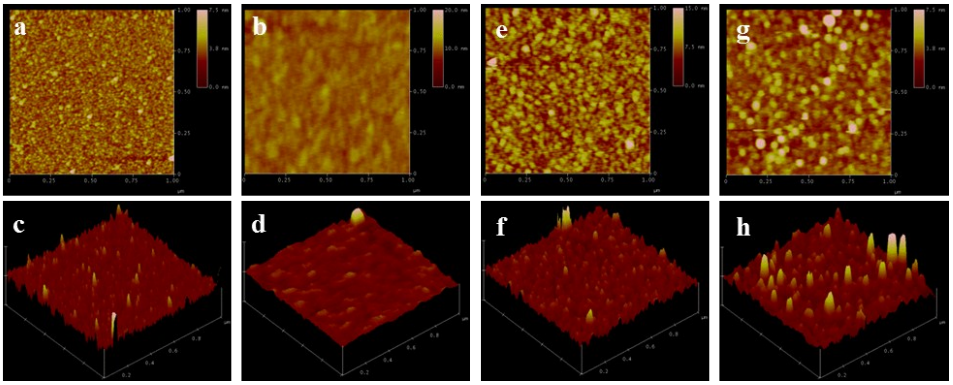

Fig. S2. Atomic force microscope images of the protein-immobilized surfaces. (a–d) EGF-His immobilized on a Ni-NTA-modified substrate (Ni-NTA content% = 100), (e–h) EGF-His and laminin immobilized on Ni-NTA terminal and CH3 terminal mixed SAM substrates (COOH content% = 50). (a, c, e, f) dry conditions, (b, d, g, h) wet conditions.

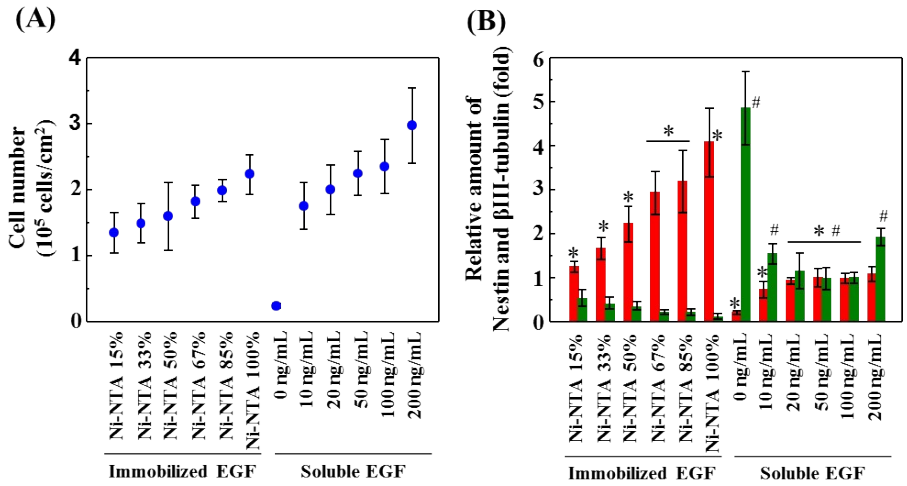

Fig. S3. Relationship between the concentration of EGF-His and growth/undifferentiated state of neural stem cells. (A) Number of cells cultured for 3 days on the EGF-immobilized and laminin-adsorbed substrates. (B) Amount of nestin and βIII-tubulin expressed in cells cultured for 3 days on the EGF-immobilized and laminin-adsorbed substrates. The amounts of these marker proteins were determined with fluorescent intensity normalized by the cell number. The relative amount is shown in comparison with the amount of marker proteins in cells cultured on the laminin surface in medium containing 20 ng/mL EGF (usual culture condition). Data are expressed as the mean ± standard deviation for n = 3. *Statistically significant differences for nestin ($p < 0.05$, Tukey's HSD test). #Statistically significant differences for βIII-tubulin ($p < 0.05$, Tukey's HSD test).

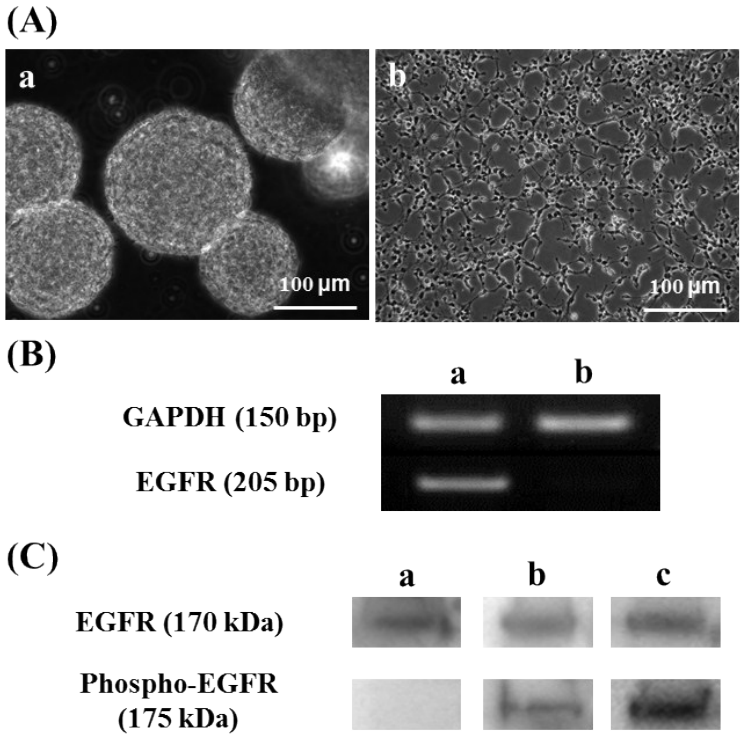

Figure S4. Signal transduction by EGF was investigated with mRNA expression and phosphorylation of the EGF receptor using RT-PCR and western blotting, respectively. (A) Phase-contrast images of cells cultured for 4 days using the neurosphere method (a) and on the substrate with immobilized EGF (b). (B) RT-PCR analysis of genes expressed in cells cultured for 4 days with neurosphere culture (a) and on the EGF-immobilized substrate (b). (C) Result of the western blotting analysis for phosphorylated EGFR and total EGFR. Lysates were prepared from cells cultured for 4 days with neurosphere culture (a, b) and on the EGF-immobilized substrate (c). (a) Cells were cultured for 4 days using the neurosphere method. (b) Cells were cultured for 4 days using the neurosphere method and then stimulated for 30 min with 1 μg/mL EGF-His.

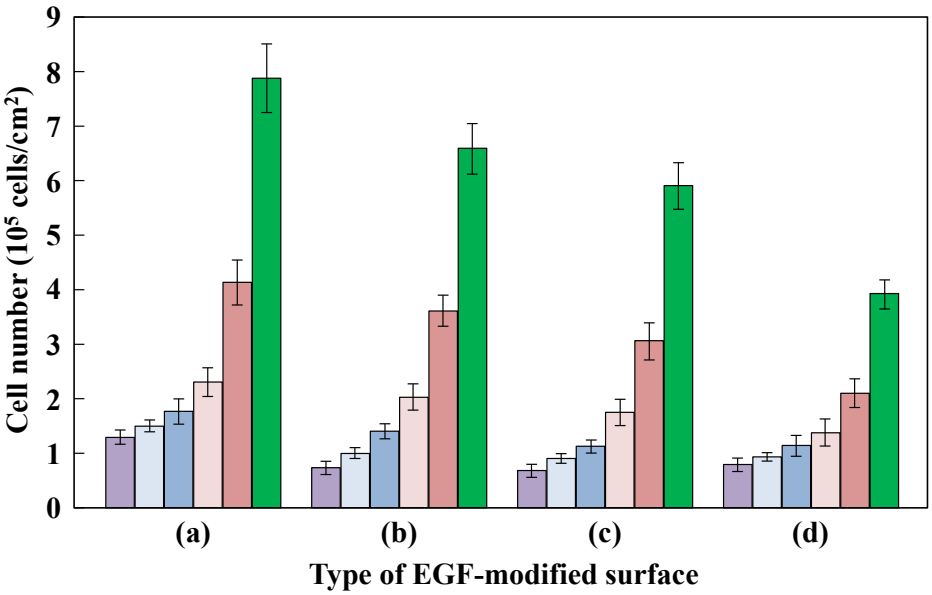

Figure S5. Number of cells cultured for 36 (purple), 48 (light blue), 60 (blue), 72 (pink), 84 (red), 96 (green) hours on the EGF-anchored surface with (a) SAv-bound biotin-modified NH_2 SAM, (b) Ni^{2+}-NTA-modified NH_2 SAM, (c) Zn^{2+}-NTA-modified NH_2 SAM, and (d) Mg^{2+}-NTA-modified NH_2 SAM.

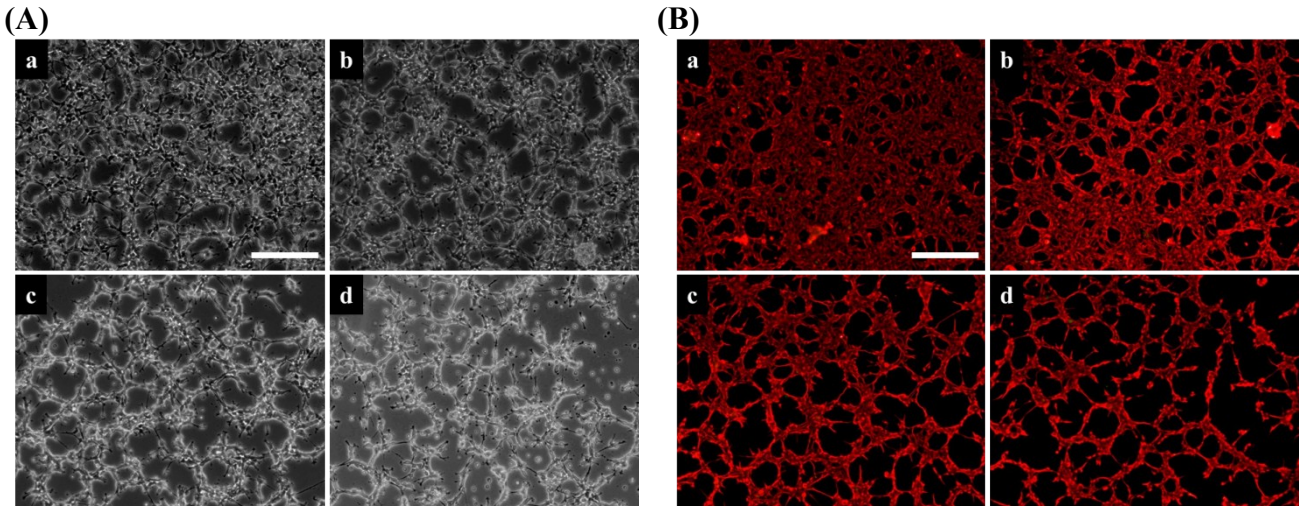

Fig. S6. (A) Phase-contrast images and (B) fluorescence images of cells cultured for 4 days on surfaces with EGF anchored through (a) biotin-SAv interaction, (b) oligohistidine-Ni^{2+}NTA interaction, (c) oligohistidine-Zn^{2+}NTA interaction, and (d) oligohistidine-Mg^{2+}NTA interaction. Scale bar: 200 μm.

Experimental

EGF chimeric protein synthesis

EGF-His, prepared by genetic engineering as reported previously,[S3,S4] was used in the present study.

A_{594}-EGF-His, synthesized with Alexa Fluor® 594 Labeling Kit (Life Technologies, Carlsbad, CA, USA), was used to trace the immobilized EGF by fluorescence.

Moreover, EGF-biotin was synthesized to evaluate the correlation between binding affinity and cell regulation. EGF-His (1.5 mg/mL) in phosphate buffer saline (PBS) was reacted for 1 h with biotin-AC_5 sulfo-OSu (Cat. No.: B320, Dojindo Laboratories, Kumamoto, Japan) at a molar ratio of 1:2 (EGF:biotin), and the reaction solution was purified with a G-10 column (GE Healthcare, Buckinghamshire, UK) to remove the unreacted biotin-AC_5 sulfo-OSu.

rhEGF (Wako Pure Chemical Industries Ltd., Osaka, Japan) was used as a control protein. These proteins were evaluated by SDS-PAGE, CD spectroscopy, and cell assays. The labelling ratios of A_{594}-EGF-His and EGF-biotin were determined with absorbances measured using a Nanodrop-2000 (Thermo Fisher Scientific, Waltham, MA, USA) and GPC measurement using a 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) equipped with a G-30 column (Wako Pure Chemical Industries Ltd.).

Preparation of the EGF-anchored substrate

Preparation of a mixed SAM array on Au-evaporated glass surface

Au-evaporated glass substrates (22 mm × 26 mm × 0.5 mm, Au:Cr = 25:5 nm) were purchased from Jitsugisyoukou Co. Ltd (Kanagawa, Japan). This substrate was immersed overnight in an ethanol solution containing 1 mM hydroxyl-(ethylene glycol)-undecanethiol (HO-$(EG)_6$-SH, Dojindo Laboratories). After washing with ethanol and drying with N_2 gas, the SAM of HO-$(EG)_6$-SH was photolytically patterned to create an array of 5 × 5 circular spots (1.5 mm in diameter and 3.0 mm in centre-to-centre distance) on bare gold (SP-9, USIO Inc., Tokyo, Japan). Then, the mixed SAM of 11-mercaptoundecanoic acid (COOH SAM) and 1-dodecane thiol (CH_3 SAM) at molar ratios of 0:100, 15:85, 33:67, 50:50, 67:33, 85:15, and 100:0 (final concentration of COOH SAM and CH_3 SAM: 1 mM) formed in the spots.[S5-S7] After a 1-h reaction, the substrate was washed with ethanol.

The composition of COOH SAM and CH_3 SAM and the contents of Ni-NTA SAM were determined by infrared reflection adsorption spectroscopy (IRRAS) using infrared spectrometer (FTLA2000, ABB Corporation, Zürich, Switzerland) equipped with Seagull™ variable angle reflection accessory (SEA-B05, HARRICK Scientific, NY, USA).[S2]

Preparation of SAM on glass and silicon wafer surfaces

Glass substrates (22 mm × 26 mm × 0.14 mm) for cell culture, prepared for the immunostaining assay and TIRFM and CLSM observation, and silicon wafers (10 mm × 10 mm × 1 mm and 25 mm × 25 mm × 1 mm), prepared for AFM observation and ellipsometry measurements, were used. These substrates were immersed in a dry toluene solution (−20 °C) containing 3-carboxymethyl-propyl trichlorosilane ($COOCH_3$ SAM) and reacted for 1 h at −20 °C. Then, these substrates were washed with cool toluene and cool methanol. After this, these substrates were incubated for 1.5 h in 1 M HCl aqueous solution at 65 °C for the deprotection of $COOCH_3$ (changing to COOH groups). Finally,

the acid-treated substrates were carefully washed with double-distilled water (DDW) and methanol.

To prepare the biotin-modified SAM substrate, we prepared a glass substrate on which the NH_2-terminal SAM was constructed. The glass substrate was immersed for 2 h in a methanol solution containing 1 v/v% 3-aminopropyl triethoxysilane and 0.5 v/v% triethylamine at 40 °C. Then, the substrate was washed with methanol and DDW.

Modification of SAM surfaces

The substrates modified with COOH-terminal SAM were immersed for 45 min in an aqueous solution containing 50 mM *N*-hydroxy succinimide and 50 mM 1-ethyl-3,3-dimethyl aminopropyl carbodiimide hydrochloric acid salt to facilitate the conversion of COOH-terminal groups to active esters. After this, the substrate was reacted for 3 h with 10 mM *N*-(5-amino-1-carboxypropyl) iminodiacetic acid (NTA, Dojindo Laboratories) solution. NTA-modified substrates were immersed in 40 mM $NiSO_4$, $ZnSO_4$, and $MgSO_4$ aqueous solutions for Ni^{2+}, Zn^{2+}, and Mg^{2+} coordination, respectively.

In contrast, the substrate modified with NH_2-terminal SAM was reacted for 45 min with an aqueous solution containing 25 mM biotin-AC_5 sulfo-OSu, and then the reacted substrate was carefully washed with methanol and DDW.

The modification of surfaces by Metal^{2+}-chelation and biotinylation was estimated by the water contact angle (Fig. S7).

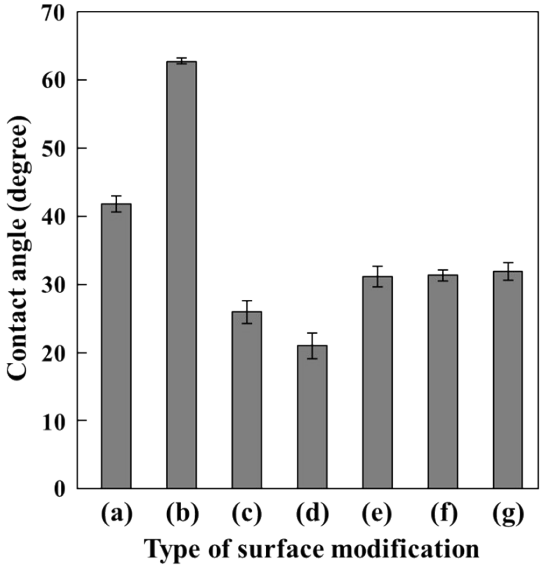

Fig. S7. Water contact angles on a glass surface with (a) NH_2-terminal SAM (3-aminopropyl triethoxysilane), (b) biotin-modified NH_2 SAM, (c) SAv-bound biotin-modified NH_2 SAM, (d) NTA-modified NH_2 SAM, (e) Ni^{2+}-NTA-modified NH_2 SAM, (f) Zn^{2+}-NTA-modified NH_2 SAM, and (g) Mg^{2+}-NTA-modified NH_2 SAM.

Protein immobilization on the mixed SAM array substrate

A PBS solution containing 3 μM EGF-His and 100 μg/mL laminin was mounted on the mixed Ni-NTA/CH_3 SAM spots (~1 μL/spot), and this substrate was incubated at 4 °C to avoid vaporization of the spotted solution. After a 2-h reaction, the spotted solution was removed by aspiration, and this substrate was immersed for 1 h into PBS containing 2% bovine serum albumin (BSA) to prevent the non-specific adsorption of proteins to the area with HO-

$(EG)_6$ SAM around spots. Then, these substrates were washed ten times with fresh PBS. Until they were used in experiments, the protein-immobilized substrates were maintained in cell culture medium.

Protein immobilization onto SAM substrate

$Metal^{2+}$-chelated-NTA SAM surfaces were reacted with a PBS solution containing 3 μM EGF-His or A_{594}-EGF-His. After reaction for 2 h, these substrates were washed ten times with fresh PBS.

To anchor EGF by biotin-SAv interaction, the biotin-modified surface was immersed for 1 h in PBS solution containing 50 μg/mL SAv (Jackson Immuno Research Labs, West Grove, PA, USA). After washing with PBS, PBS solution containing 3 μM EGF-biotin was added to the SAv-modified surface and reacted for 1 h. Then, the EGF-anchored substrate was washed ten times with fresh PBS. Until they were used in experiments, these protein-immobilized substrates were maintained in cell culture medium.

Cell culture, assay, and evaluation

NSPCs derived from the striatum of Fischer 344 rat foetuses (E15) were used in this study.[S3] To trace proteins after the interaction between immobilized EGF and its cell receptors using TIRFM (IX81 and IX2-RFAEVAM, Olympus Co. Ltd., Tokyo, Japan) and CLSM (IX81 and Fluoview confocal system, Olympus Co. Ltd.), enhanced green fluorescent protein-expressing NSPCs (EGFP-NSPCs) derived from the striatum of EGFP-SD rat foetuses (E15) were used.[S8]

The striatum was isolated from foetuses (E15) of Fischer 344 or EGFP-SD rats and dissociated into single cells using a 0.05% trypsin solution containing 0.53 mM 2,2',2",2"'-(ethane-1,2-diyldinitrilo) tetraacetic acid. All experiments were performed according to the guidelines of the Animal Welfare Committee of the university. The cells obtained from rats were suspended in DMEM/F12 (1:1) containing 2% B27 supplement, 5 μg/mL heparin, and antibiotics (NSPC base medium), supplemented with 20 ng/mL bFGF and 20 ng/mL EGF, and cultured for 5 days to form neurospheres. These neurosphere cells were used in experiments as NSPCs.

Neurospheres at passage 2, dissociated into single cells by trypsinization, were used in all experiments. The single cells were suspended in an NSPC base medium and seeded onto the protein-immobilized substrates at a density of 3×10^4 cells/cm^2. The cells were cultured for 4 days in the NSPC base medium supplemented with 2% B27 without growth factors (37 °C, 5% CO_2). As a control, the cells were cultured in medium containing soluble rhEGF using the same method as described above.

The assays for bioactivity of EGF-His and A_{594}-EGF-His were carried out by the neurosphere method. The cells were cultured for 5 days in the NSPC base medium containing 2% B27 and 20 ng/mL rhEGF, EGF-His, or A_{594}-EGF-His. The obtained neurosphere cells were dissociated into single cells by trypsinization and counted by trypan blue staining.

Evaluation of mRNA expression of the EGF receptor using RT-PCR

The messenger RNA (mRNA) expression of EGF receptors (EGFR) was qualitatively and quantitatively evaluated by RT-PCR. The specific genes encoding the EGF receptor (EGFR) were detected by PCR using the primers shown in Table S2.

Table S2. Primer sequences for gene expression of EGFR and GAPDH.

Type of primer	Primer sequence
EGFR (sense)	5'-GCTGGGGAAGAGGAGAGGAGA-3'
EGFR (antisense)	5'-ACGAGTGGTGGGCAGGTGTCTT-3'
GAPDH (sense)[1]	5'-TGAACGGGAAGCTCACTGG-3'
GAPDH (antisense)[1]	5'-TCCACCACCCTGTTGCTGTA-3'

1) GAPDH was used as an internal standard.

References

S1. T. Nakaji-Hirabayashi, K. Kato, Y. Arima, H. Iwata, *Bioconjugate Chem.*, 2008, **19**, 516–524.

S2. T. Nakaji-Hirabayashi, K. Kato, Y. Arima, H. Iwata, *Biomaterials*, 2007, **28**, 3517–3529.

S3. Y. Asal, M. Mie, E. Kobatake, *Biomaterials*, 2013, **34**, 3315–3323.

S4. T. Nakaji-Hirabayashi, K. Kato, H. Iwata, *Biomaterials*, 2008, **29**, 4403–4408.

S5. T. Nakaji-Hirabayashi, K. Kato, Y. Arima, H. Iwata, *Bioconjugate Chem.*, 2008, **19**, 516–524.

S6. M. Toda, T. Kitazawa, I. Hirata, Y. Hirano, H. Iwata, *Biomaterials*, 2008, **29**, 407–417.

S7. Y. Arima, H. Iwata, *J. Mater. Chem.*, 2007, **17**, 4079–4087.

S8. T. Nakaji-Hirabayashi, K. Kato, H. Iwata, Bioconjugate Chem., 2013, 24, 1798–1804.