Supporting Information

Multifunctional Chimeric Proteins for the Sequential Regulation of Neural Stem Cell Differentiation

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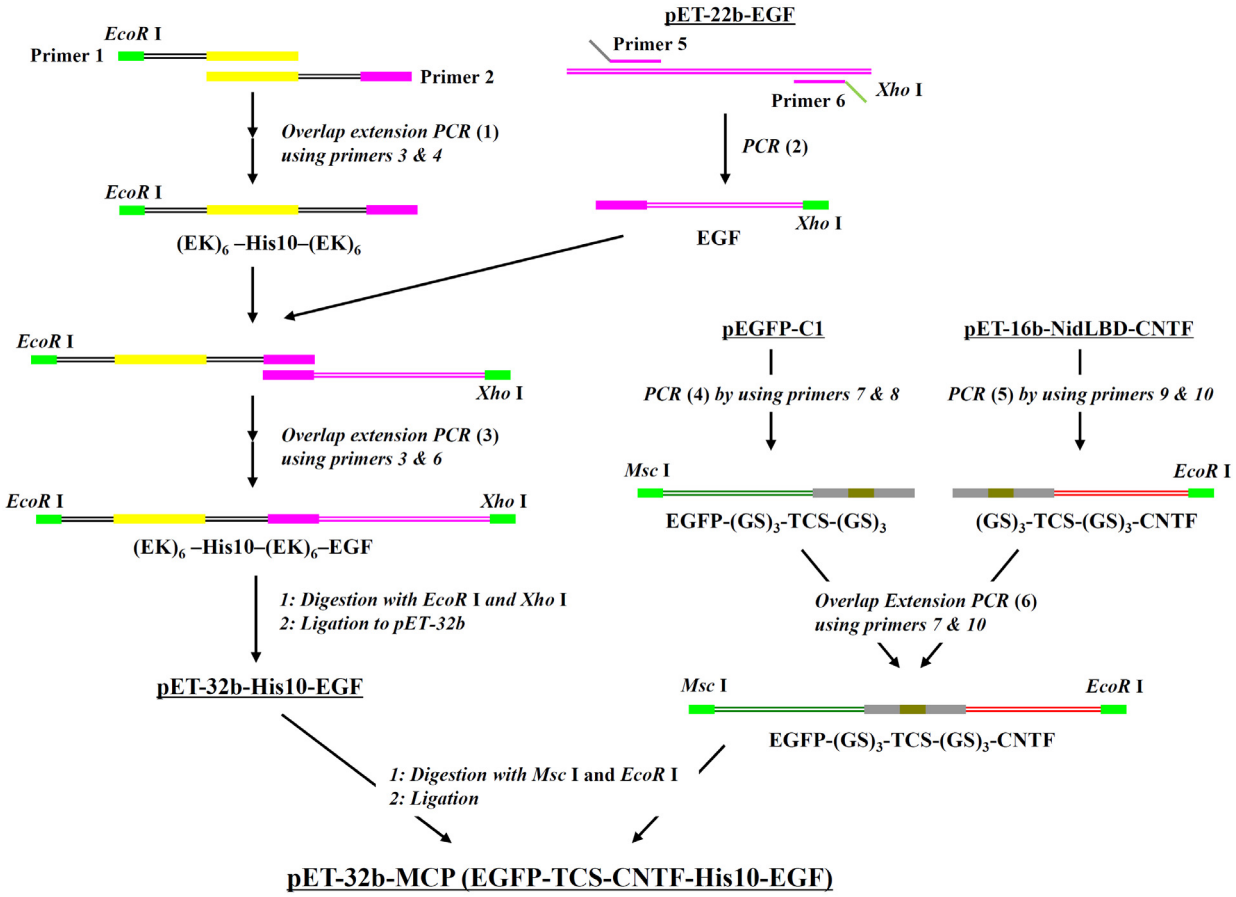

Figure S1. Outline of plasmid construction.

Table S1. Sequences of Primers and Oligonucleotides Used for the Synthesis of Chimeric Genes

Primer or oligonucleotide		Sequence
1[a]	$(EK)_6$–His10– $(EK)_6$ sense oligonucleotide	5'-GAAAATGGAATTCGAGAAGGAGAAGGAGAAGGAGA AGGAGAAGGAGAAGCACCATCACCATCACCATCACCA TCACCATGAAAAAGAAAAAG-3'
2[a]	$(EK)_6$–His10– $(EK)_6$ antisense oligonucleotide	5'-CACCATCACCATCACCATCACCATCACCATGAAAAA GAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGGAAAT AGTGACTCTGAATGTCCCCTG-3'
3[a]	$(EK)_6$–His10– $(EK)_6$ Fw primer	5'-GAAAATGGAATTCGAGAAGGAGAAG-3'
4[a]	$(EK)_6$–His10– $(EK)_6$ Rv primer	5'-CAGGGGACATTCAGAGTCACTATTTC-3'
5[a]	EGF Fw primer	5'-GAAAAAGAAAAAGAAAAAGGAAATAGTGACTCTGA ATGTCCCCTG-3'
6[a]	EGF Rv primer	5'-TATCCTCGAGTCAGCGCAGTTCCCACCACTTCAGG-3'
7[a]	EGFP Fw primer	5'-GTTCTGGCCATGTGAGCAAGGGCGAGGAGCTG-3'
8[a]	EGFP Rv primer	5'-TCCGCTTCCGCTTCCGCTTCCAGAACCGCGTGGCACC AGGCTTCCACTGCCCGAACCTCCAGATCTGAGTCCGGA CTTGTAC-3'
9[a]	CNTF Fw primer	5'-GGAGGTTCGGGCAGTGGAAGCCTGGTGCCACGCGGT TCTGGAAGCGGAAGCGGAAGCGGAGCTTTCACAGAGC ATTCACCG-3'
10[a]	CNTF Rv primer	5'-TCTCGAATTCCATTTTCTTGTTGTTAGCAATATAATG-3'
11[b]	CP-2 primer 1 (Fw)	5'-GATCCGAATTCGTGAGCAAGGGCGAGGAGCTG-3'
12[b]	CP-2 primer 2 (Rv)	5'-CCGCTTCCAGAACC**GGC**TGGCACCAGGCTTCCAC-3'
13[b]	CP-2 primer 3 (Fw)	5'-GTGGAAGCCTGGTGCCA**GCC**GGTTCTGGAAGCGG-3'
14[b]	CP-2 primer 4 (Rv)	5'-TATCCTCGAGTCAGCGCAGTTCCCACCACTTCAGG-3'
15[c]	CP-3 primer (Rv)	5'-CATGGTGATGGTGATGGTGATG-3'
16[d]	CP-4 Fw primer	5'-GCCCATATGGAATTCGCTTTCACAGAGCATTCACC-3'
17[d]	CP-4 Rv primer	5'-GGTGCTCGAGCATTTTCTTGTTGTTAGCAATATAATG-3'

[a]Primers and oligonucleotides for synthesis of CP-1 gene. [b]Primers for synthesis of CP-2 gene. Sequences in bold represent mutation in the TCS site. [c]Primer for synthesis of CP-3 gene. CP-3 gene was amplified from CP-1-expression plasmid using this primer and CP-2 primer 1 (primer 11). [d]Primers for the amplification of CNTF gene. Fw: forward, Rv: reverse.

Table S2. Thermal Cycling Conditions for Overlap Extension PCR

PCR No.[a]	Oligonucleotides, primers or genes[a,b]	Annealing temperature (^{o}C)[c]	Number of thermal cycles[c]
1	Primers 1 and 2	63	10
	Primers 3 and 4	52	35
2	Primers 5 and 6	55	35
3	$(EK)_6$-His10-$(EK)_6$ and EGF genes	63	10
	Primers 3 and 6	52	35
4	Primers 7 and 8	56	35
5	Primers 9 and 10	56	35
6	EGFP-$(GS)_3$-TCS-$(GS)_3$ and $(GS)_3$-TCS-$(GS)_3$-CNTF genes	63	10
	Primers 7 and 10	56	35

[a]Refer to PCR number shown in Figure S1. [b]Primer and oligonucleotide sequences are shown in Table S1. [c]PCR was performed under the following conditions; denaturation: 94 ^{o}C, 30 sec, annealing: 30 sec, elongation: 72 ^{o}C, 30 sec. Annealing temperature and cycle numbers are shown above.

Table S3. Conditions for the Refolding of Chimeric Proteins[a]

Order	Composition of dialysis solution	pH	Time (hr)
1	50 mM Tris-HCl, 10 mM NaCl, 0.2 M L-arginine, 8 M urea, 0.2 mM dithiothreitol	8.5	Overnight
2	50 mM Tris-HCl, 10 mM NaCl, 0.2 M L-arginine, 8 M urea	8.5	5
3	50 mM Tris-HCl, 10 mM NaCl, 0.2 M L-arginine, 4 M urea, 1 mM reduced glutathione, 0.1 mM oxidized glutathione	8.5	4
4	50 mM Tris-HCl, 10 mM NaCl, 0.2 M L-arginine, 2 M urea, 3.75 mM reduced glutathione, 0.375 mM oxidized glutathione	8.5	4
5	50 mM Tris-HCl, 10 mM NaCl, 0.2 M L-arginine, 1 M urea, 3.75 mM reduced glutathione, 0.375 mM oxidized glutathione	8.5	4
6	50 mM Tris-HCl, 10 mM NaCl, 0.2 M L-arginine, 3.75 mM reduced glutathione, 0.375 mM oxidized glutathione	8.0	4
7	50 mM Tris-HCl, 10 mM NaCl, 0.2 M L-arginine, 0.5 mM reduced glutathione, 0.3 mM oxidized glutathion	8.0	4
8	50 mM Tris-HCl, 10 mM NaCl, 0.1 M L-arginine	8.0	10
9	Phosphate buffered saline	7.4	Overnight
10	Phosphate buffered saline	7.4	Overnight

[a]Dialysis was carried out at 4 ^{o}C.

Table S4. Primers and PCR Conditions for RT-PCR

Gene	Primer sequences[b]	Length of amplified gene (bp)	Annealing temperature (^{o}C)[c]	Number of thermal cycles[c]
EGFR[a]	5'-GCTGGGGAAGAGGAGAGGAGA-3' 5'-ACGAGTGGTGGGCAGGTGTCTT-3'	205	56	30
CNTFR[a]	5'-GCCATGTGGGACAGCAAGTT-3' 5'-GGAGTCACGGTGGAAACAGG-3'	151	56	30
LIFR[a]	5'-TGGCTGTGGCTGTCATTGTT-3' 5'-GCGTTGCTTCCCTCACAGAC-3'	151	55	30
gp130[a]	5'-GGTCCACATGGCAGCATACA-3' 5'-GAACAAGACTCCCAGCAGCG-3'	151	56	30
EGF	5'-AACAGTAACACAGGATGCCCGC-3' 5'-GCGCAGCTTCCACCAACGTAAG-3'	159	56	31
CNTF	5'-GCAAACACCTCTGACCCTTCAC-3' 5'-CTTGGAAGGTACGGTAAGCCTG-3'	239	56	31
Fibronectin	5'-GAAGATTTTGTGGACTCCTCAG-3' 5'-CTGTTTTCTGTCTTCCTCTGAG-3'	406	55	35
Laminin γ1 chain	5'-ATGTAGACCAAAATCTGATGG-3' 5'-CTGCCAGGGTCCTCAAAAGCAG-3'	381	55	25
Nestin[a]	5'-AGTGTGAAGGCAAAGATAGC-3' 5'-TCTGTCAGGATTGGGATGGG-3'	317	56	31
β-Tubulin III	5'-CCTCCGCAGCCCGCTGTCCGC-3' 5'-GCAGGCAGTCACAATTCTCAC-3'	443	56	24
GFAP[a]	5'-GAAACCAACCTGAGGCTGGAG-3' 5'-GGCGATAGTCATTAGCCTCG-3'	388	56	31
GAPDH[a]	5'-TGATGGGTGTGAACCACGAG-3' 5'-CTCCTGTTGTTATGGGGTCTG-3'	758	55	21

[a]Same sequences as those used by Cai et al [J. Cai, et al. (2004) *J. Neurochem.88*, 212–226]. Other primer sequences were from Protein Data Bank. EGFR: epidermal growth factor receptor, CNTFR: ciliary neurotrohic factor, LIFR: leukemia inhibitory factor, GFAP: glial fibrillary acidic protein, GAPDH: glycelardehyde-3-phosphate dehydrogenase. [b]Upper: Forward primer, lower: reverse primer. [c]PCR was performed under the following conditions; denaturation: 94 ^{o}C, 30 sec, annealing: 30 sec, elongation: 72 ^{o}C, 30 sec. Annealing temperature and cycle numbers are shown above.

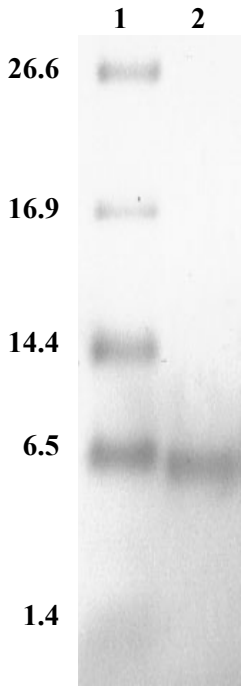

Figure S2. SDS-PAGE analysis of CP-5. CP-5 was electrophoresed in 16% polyacrylamide gel at 200 V for 45 min and visualized by CBB staining. Lane 1: molecular weight standard, Lane 2: CP-5.

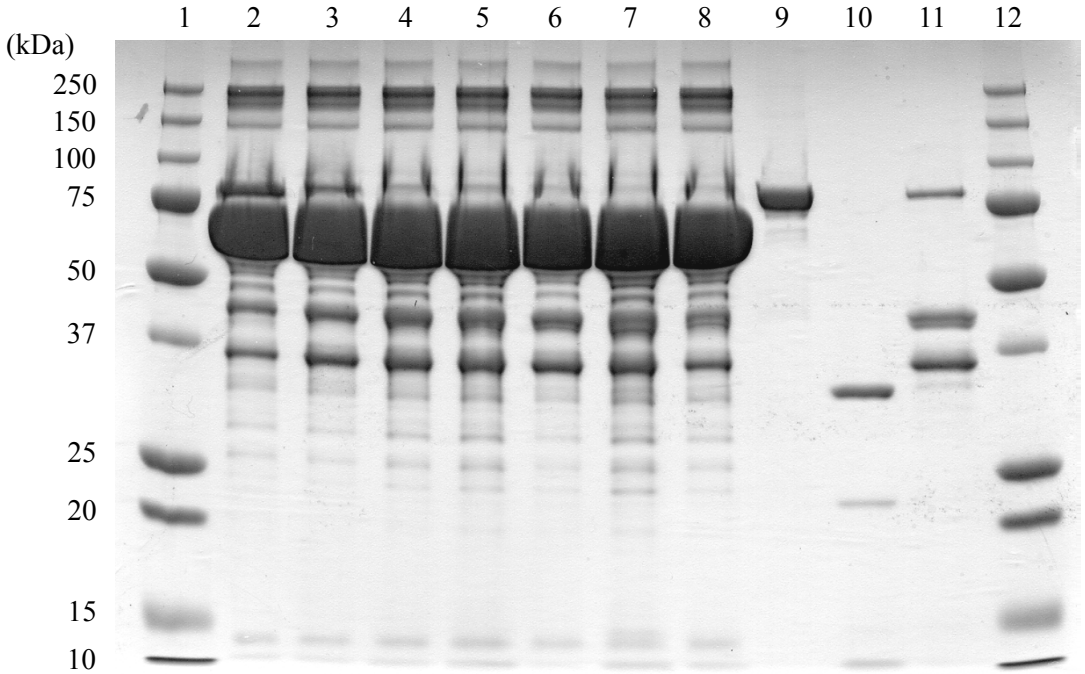

Figure S3. SDS-PAGE analysis of MPC digested by thrombin in DMEM/F12 containing 2% B27. Thrombin (0.6 unit/mL) was added to the medium containing 50 μg MCP, and digestion was allowed to proceed at 37 ^{o}C for (lane 2) 10, (lane 3) 15, (lane 4) 20, (lane 5) 30, (lane 6) 45, (lane 7) 60, and (lane 8) 90 min. Lanes 9 and 10 represent the result for pristine MPC and thrombin, respectively. Lane 11 represents the result for MPC digested with thrombin in buffer solution at 37 ^{o}C for 10 min. Molecular weight standard was electrophoresed in lanes 1 and 12. Note that the band intensity of MPC (75 kDa) decreases with digestion time, indicating that MPC was digested by thrombin even in the medium.

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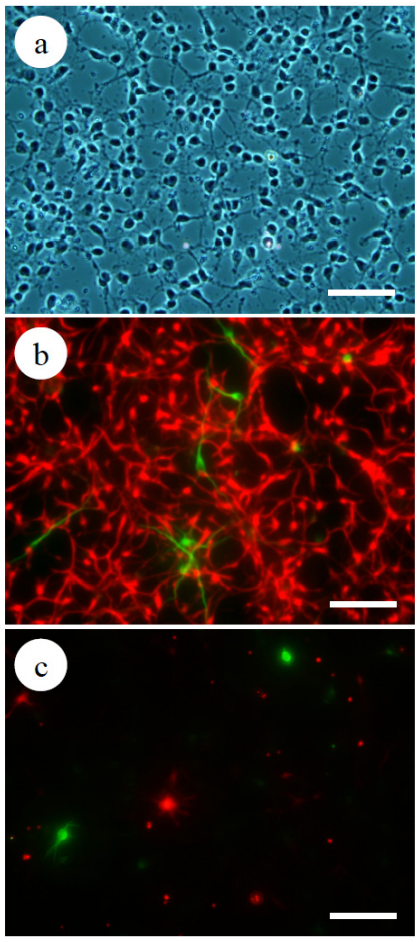

Figure S4. Phase contrast (a) and immunofluorescent (b, c) images of cells cultured for 5 days on the CP-5-immobilized surface. (b) Nestin (red) and βIII (green). (c) GFAP (red) and βIII (green). The results shown in this figure are in good accordance with the our observation previously reported [Nakaji-Hirabayashi, T., Kato, K., Arima, Y., and Iwata, H. (2007) *Biomaterials 28*, 3517–3529]. Scale bar: 100 μm.