Article title

Epidermal growth factor increases cystathionine ß-synthase expression in cultured embryonic spinal cord cells

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Running head

EGF increases CBS in spinal cord cells

25 **Abstract**

26 In the central nervous system (CNS), cystathionine ß-synthase (CBS) is localized in
27 astrocytes. CBS degrades cytotoxic homocysteine and produces cytoprotective hydrogen
28 sulfide; thus the proper expression of CBS is required to maintain CNS functions. CBS
29 expression is very low at the late embryonic stage and increases after birth. This study
30 examined CBS expression in cultured spinal cord cells derived from fetal rats. Treatment
31 of spinal cord cells with epidermal growth factor (EGF) promoted the proliferation and
32 maturation of astrocytes during development. EGF (30 ng/ml, 4 days) increased CBS
33 protein expression and the number of CBS-expressing astrocytes in the culture. A high
34 cell density also increased CBS expression, and EGF was able to increase CBS
35 expression when cellular proliferation was inhibited. The EGF receptor was
36 predominately expressed in neural stem cells rather than astrocytes. These results
37 suggest that EGF acts on neural stem cells, leading to increase in CBS-expressing
38 astrocytes. This effect may reflect the maturation process of astrocytes during embryonic
39 development.

40

41 **Keywords**: cystathionine ß-synthase, spinal cord, astrocyte, epidermal growth factor,
42 neural stem cell

1. Introduction

Cystathionine ß-synthase (CBS) is the main enzyme that degrades homocysteine. CBS catalyzes the condensation of homocysteine and cysteine to produce hydrogen sulfide (H_2S) (Kimura 2014), and thus reduced activity of CBS increases homocysteine and decreases H_2S levels. An increased level of homocysteine is a well-known risk factor for the pathogenesis of several cardiovascular and neurological diseases. Mild hyperhomocysteinemia occurs in several neurodegenerative disorders, such as spinal cord injury, amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease (Seshadri *et al.* 2002; Lee *et al.* 2006; Valentino *et al.* 2010; Kocer *et al.* 2016). On the other hand, H_2S has antioxidant and anti-inflammatory properties (Szabó 2007). H_2S exerts therapeutic effects in animal models of several neurodegenerative disorders, including those mentioned above (Kida *et al.* 2011; Xuan *et al.* 2012; Campolo *et al.* 2013; Tabassum and Jeong 2019). An imbalance between homocysteine and H_2S levels, induced by CBS deficiency, may contribute to the pathogenesis of these disorders.

In the central nervous system (CNS), CBS is localized in astrocytes (Enokido *et al.* 2005; Miyamoto *et al.* 2015). Exogenous H_2S affects Ca^{2+} signaling and lactate production in astrocytes (Nagai *et al.* 2004; Nii *et al.* 2021a, b), and so it is likely that

61 endogenous H_2S produced by CBS also regulates astrocyte functions. However, how CBS
62 expression is regulated and maintained in astrocytes remains unclear. Astrocyte
63 morphology and the expression profile of functional proteins in astrocytes are influenced
64 by the presence of other cells such as neurons, endothelial cells and neighboring
65 astrocytes (Hatten 1987; Swanson *et al.* 1997; Schlag *et al.* 1998; Li *et al.* 2019; Martinez-
66 Lozada and Robinson 2020). We have previously reported that CBS expression in
67 astrocytes is increased by the presence of neurons (Miyamoto *et al.* 2015), suggesting
68 that cell-to-cell communication is crucial for regulating CBS expression.

69 Proper expression of CBS is also important for normal growth and development
70 of newborns. CBS deficiency shows sever growth retardation and impairs CNS functions
71 (Watanabe *et al.* 1995; Akahoshi *et al.* 2008). CBS expression is very low in the CNS of
72 late-embryonic rodents, and increases after birth (Robert *et al.* 2003; Miyamoto *et al.*
73 2015). Neural stem cells are abundant in the embryonic CNS, and play an important role
74 in maturation during development. The neural stem cells differentiate into neurons and
75 astrocytes; this process is regulated by various transcription factors and growth factors
76 (Temple 2001; Rowitch and Kriegstein 2010). Epidermal growth factor (EGF) is an
77 important regulator of proliferation and differentiation, and expression of the EGF
78 receptor (EGFR) increases in the late embryonic stage (Lillien and Raphael 2000). EGF

stimulates neural stem cells to proliferate and differentiate into astrocytes (Burrows *et al.* 1997). EGF increases CBS expression in cultured astrocytes derived from fetal mouse brain (Enokido *et al.* 2005). These reports suggest that EGF is involved in astrocyte maturation and CBS expression during early development, but it remains to be elucidated whether other cell types, such as neurons and neural stem cells, are also involved in the control of CBS expression. This study investigated the effects of EGF on cultured spinal cord cells derived from fetal rats, which contains astrocytes and other cell types including neurons and neural stem cells.

2. Materials and methods

2.1. Materials

AraC, EGF and PD153035 were purchased from Sigma-Aldrich (St. Louis, MO, USA). PD153035 was dissolved in dimethyl sulfoxide (DMSO).

2.2. Animals

All animal care and experimental protocols were approved by the Animal Care and Use Committee, Hokkaido University (no. 14-0111). Wistar rats were purchased from Clea Japan (Tokyo, Japan) and bred to obtain pups. The rats were fed ad libitum

and kept on a 12 h light–dark cycle at 22±4°C.

2.3. Cell culture

Spinal cords were dissected from embryonic day 16 rats and incubated with papain (10 U/ml, Worthington, Lakewood, NJ, USA) and DNase (0.1 mg/ml, Roche Applied Science, Penzberg, Germany) in divalent cation-free Hanks' balanced salt solution (HBSS) at 37°C for 20 min. The tissues were then suspended in Neurobasal medium supplemented with B-27 (Life Technologies, Carlsbad, CA, USA), 2 mM L-glutamine, 2% horse serum, 100 μg/ml streptomycin, and 100 U/ml penicillin, and were dispersed by pipetting. The cell suspension was seeded onto a poly-L-lysine/laminin-coated 12-well plates or coverslips at a density of 5×10^4 cells/cm^2. After 7 days, the cell cultures were used for experiments. To obtain the neuron-enriched culture, AraC (1 μM) was added 2 days after seeding to inhibit the proliferation of non-neuronal cells. Half of the culture medium was exchanged with fresh medium twice a week.

2.4. Western blotting

Cells were lysed by mechanical homogenization and sonication in radio immunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail

115 (Nacalai Tesque, Kyoto, Japan). After centrifugation and denaturation, the supernatants
116 were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
117 PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The
118 membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1 %
119 Tween-20 (TBST) for 30 min at room temperature, and then incubated overnight at 4ºC
120 with primary antibodies in TBST containing 1% skimmed milk. The antibodies against
121 CBS (1:2,000; #14787-1-AP, Proteintech, Manchester, UK), EGFR (1:5,000; #Ab52894,
122 Abcam, Cambridge, UK) and GFAP (1:100,000; #11051, Immuno-Biological Laboratories,
123 Gunma, Japan), and a peroxidase-conjugated anti-glyceraldehyde-3-phosphate
124 dehydrogenase (GAPDH) antibody (1;150,000; #G9265, Sigma-Aldrich) were used. The
125 membranes were incubated for 1 h at room temperature with horseradish peroxidase-
126 conjugated secondary anti-mouse or anti-rabbit antibodies (1:4,000; GE Healthcare,
127 Chicago, IL, USA) in TBST containing 1% skimmed milk. Antibody binding was
128 visualized by ECL Prime (GE Healthcare). Band intensities were measured with Image
129 J software (National Institutes of Health, Bethesda, MD, USA).
130
131 2.5. Immunofluorescent staining
132 Cells cultured on coverslips were fixed with methanol stored at -20ºC for 1 min,

and then incubated with 10% goat serum in phosphate-buffered saline containing 0.1 % Triton X-100 (PBST) for 30 min at room temperature, followed by overnight incubation at 4ºC with PBST containing 1% goat serum and primary antibodies. Antibodies against CBS (1:200), EGFR (1:500) and GFAP (1:50), MAP2 (1:2,000; #Ab5392, Abcam) and nestin (1:300, #NB100-1604, Novus Biologicals, Centennial, CO, USA) were used. Cells were incubated with Alexa Fluor-conjugated secondary antibodies (1:500; Life Technologies) in PBST containing 1% goat serum for 30 min at room temperature. Coverslips were mounted on glass slides with DAPI Fluoromount G (SouthernBiotech, Birmingham, AL, USA). Immunoreactivity was analyzed with a confocal microscope (LSM700, ZEISS, Jena, Germany).

2.6. Data analysis

Data are expressed mean±SEM (n= number of independent experiments using cell cultures obtained from different animals). All data were obtained from at least three independent experiments from three pregnant rats. Statistical comparisons between two groups were performed using the unpaired Student's t-test. Multiple comparisons were performed using a one-way ANOVA followed by the Dunnett's test. A p value of less than 0.05 was considered statistically significant. All statistical analyses were performed with

151 Ekuseru-Toukei 2008 (Social Survey Research Information, Tokyo, Japan).

152

153 **3. Results**

154 3.1. EGF increases the number of CBS-expressing astrocytes in cultured spinal cord cells

155 derived from fetal rats

156 The effects of EGF on CBS expression was examined in cultured spinal cord

157 cells derived from embryonic day 16 fetal rats. Western blot analysis revealed that CBS

158 protein was expressed in embryonic spinal cord cells. EGF (30 ng/ml, 4 days)

159 significantly increased CBS expression levels (Fig. 1A and B).

160 The CBS-expressing cells in fetal spinal cord cells were examined by

161 immunofluorescent staining with anti-glial fibrillary acidic protein (GFAP; an astrocytic

162 marker), anti-microtubule-associated protein 2 (MAP2; a neuronal marker) and an anti-

163 CBS antibody (Fig. 1C). $GFAP^+$ and $MAP2^+$ cells comprised 17.2 ± 5.2% and 80.0 ± 5.6%

164 (n = 3), respectively, of the CBS^+ cell population. EGF (30 ng/ml, 4 days) increased the

165 proportion of $GFAP^+$ cells in CBS^+ cells (Fig. 1D). EGF also increased the density of

166 $GFAP^+$ cells but did not alter the density of $MAP2^+$ cells (Fig. 1E).

167 As shown in Fig. 1A, western blot analysis showed that the CBS antibody

168 detected not only a canonical 63-kDa band, but also a 50-kDa band. We confirmed that

169 the CBS antibody detected mainly a 50-kDa protein in neuron-enriched culture (Fig. 1F;
170 Supplementary Fig. 1). We also examined the effects of EGF on CBS expression in an
171 astrocyte-enriched culture. Unlike the previous report in cultured astrocytes derived
172 from mouse brain (Enokido *et al.* 2005), CBS expression was almost undetectable, and
173 EGF did not affect CBS expression in astrocyte-enriched cultures derived from rat spinal
174 cord (Supplementary Fig 2).
175
176 3.2. High cell density increases CBS expression in embryonic spinal cord cells
177 As EGF increased the density of embryonic spinal cord cells, we examined
178 whether cell density affected CBS expression. Embryonic spinal cord cells were seeded
179 at a density of either 2.5×10^4, 5×10^4 or 10^5 cells/cm^2, then cultured for 7 days (Fig. 2A).
180 The increase in cell density was dependent on the seeding density (Fig. 2B), but the cell
181 density did not significantly affect the proportion of GFAP$^+$ and MAP2$^+$ cells (Fig. 2C) or
182 GFAP expression levels (Fig 2D, G). CBS expression levels increased in a cell density-
183 dependent manner (Fig. 2D-F). These results suggest that a high cell density causes an
184 increase in CBS expression levels in each astrocyte.
185 Next, we investigated whether EGF increases CBS expression by a mechanism
186 other than increased cellular density. Cells seeded at 5×10^5 cells/cm^2 were cultured for

7 days, and then treated with cytosine arabinoside (AraC) to inhibit proliferation. In the presence of AraC, EGF (30 ng/ml, 4 days) failed to increase cellular density (Fig. 3A) and did not change the proportion of $GFAP^+$ and $MAP2^+$ cells in the culture (Fig. 3B). However, EGF significantly increased CBS expression in AraC-treated cells (Fig. 3C and D).

3.3. EGF receptor is mainly expressed in neural stem cells and involved in CBS expression

The EGF receptor (EGFR) inhibitor PD153035 (1 μM) suppressed EGF-mediated increases in CBS expression and cell density (Fig. 4A-C), indicating that both of these effects were mediated via EGFR. Western blot analysis indicated the EGFR protein was expressed in embryonic spinal cord cells, and that this expression was significantly higher than that in neuron-enriched cultures (Fig. 4D and E).

We then examined the proportion of EGFR-expressing cells that were astrocytes or neurons by immunofluorescent staining. $GFAP^+$ and $MAP2^+$ cells comprised 17.0 ± 1.6 % (n = 9) and 24.5 ± 2.2% (n = 9), respectively, of the total cell population, and no cells expressed both markers. $EGFR^+$ cells comprised 6.8 ± 1.3% (n = 3) of the total cell population. $GFAP^-/MAP2^-$ cells comprised 73.9%, $GFAP^+$ cells comprised 17.4%, and

205 MAP2$^+$ cells comprised 8.7% of the EGFR$^+$ cell population (Fig. 4F and G).

206 Primary cultures of spinal cord cells derived from fetal rats contain cells
207 expressing nestin (a neural stem cell marker; Sato *et al.* 2006). We thus performed
208 immunofluorescent staining with antibodies to GFAP, EGFR and nestin (Fig.5A). About
209 one-fourth (23.4%) of cultured embryonic spinal cord cells strongly expressed nestin, and
210 most of these were GFAP$^-$ cells. More than half (57.9%) of EGFR$^+$ cells were nestin$^+$. The
211 percentage of nestin$^+$ cells was markedly higher in EGFR$^+$ cells than EGFR$^-$ cells (Fig.
212 5B). These results suggest that EGFR is mainly expressed on neural stem cells. EGF (30
213 ng/ml, 4 days) increased the proportion of EGFR$^+$ cells (Fig. 5C). In the presence of AraC,
214 EGFR$^+$ cells disappeared after EGF treatment (Fig. 5D), and EGF significantly reduced
215 EGFR expression levels (Fig. 5E and F).

216

217 **4. Discussion**

218 CBS expression has been reported to localize to astrocytes in the CNS (Enokido
219 *et al.* 2005). In this study, the immunoreactivity of an anti-CBS antibody was detected
220 in both neurons and astrocytes, while western blot analysis showed that the canonical
221 63-kDa CBS was detected in astrocytes, but not in neurons. We have previously reported
222 that cultured spinal cord neurons derived from fetal rats have low CBS enzyme activity

even though a 50-kDa protein can be detected by an anti-CBS antibody (Miyamoto *et al.* 2015). Therefore, it is unlikely that the detected 50-kDa protein is a catalytically-active truncated form of CBS as previously reported (Skovby *et al.* 1984).

EGF failed to increase CBS expression in the astrocyte-enriched culture derived from rat spinal cord. In the cultured astrocytes derived from mouse brain, however, EGF reportedly increases CBS expression (Enokido *et al.* 2005). In the present study, astrocyte-enriched cultures were obtained from postnatal days 0-3 rat spinal cord with shaking to remove non-astrocytic cells, and were subsequently cultured in a medium containing 10% fetal bovine serum and 30 ng/ml EGF. On the other hand, in the previous report, the astrocyte cultures are prepared from embryonic mouse cortex and hippocampus without shaking, and are cultured in a medium containing 5% horse serum, which supports the proliferation of neural stem cells (Fredoroff and Hall 1979), along with 50 ng/ml EGF. Therefore, the astrocyte cultures used in the previous study may contain not only astrocytes but also neural stem cells. Alternatively, the differences may be attributed to the regional and/or species differences. In the rat cortex, EGFR is expressed on neural stem cells during embryonic development (Lillien and Raphael 2000) and promotes proliferation and differentiation of neural stem cells into astrocytes (Burrows *et al.* 1997). In our study, the majority of $EGFR^+$ cells were identified as $nestin^+$

241 neural stem cells, and EGF increased both the density of embryonic spinal cord cells and
242 the proportion of astrocytes. EGF also increased the proportion $EGFR^+$ cells, and
243 decreased the proportion of $EGFR^+$ cells, when proliferation was inhibited. Based on
244 these results, we suggest that EGF promotes the proliferation of $EGFR^+$ neural stem
245 cells and their differentiation into $EGFR^-$ astrocytes.

246 In cultured cells isolated from the cerebral hemisphere of embryonic rats, a high
247 cell density promotes the development of astrocytes associated with the upregulation of
248 GFAP expression (Adachi *et al.* 2002). In our study, although the cell density did not
249 affect the proportion of astrocytes or GFAP expression, CBS expression increased in a
250 cell density-dependent manner. Since the ratio of CBS to GFAP expression increased, it
251 is likely that CBS expression is upregulated in individual astrocytes. Astrocytic
252 expression of functional proteins is regulated by the presence of neurons (Swanson *et al.*
253 1997; Schlag *et al.* 1998). Adhesion molecules in the plasma membrane, such as ephrin
254 and neuroligin, mediate communication between astrocytes and neurons (Stogsdill *et al.*
255 2017; Tyzack *et al.* 2017). Astrocyte to astrocyte communication occurs via gap junction
256 channels, and the expression of gap junction channels is regulated by the presence of
257 neurons (Rouach *et al.* 2000; Koulakoff *et al.* 2008). These factors may be involved in the
258 cell density-dependent upregulation of CBS in astrocytes. Further investigation is

needed to elucidate the relationship between cell-density and CBS expression.

In cultured spinal cord cells, EGF increased CBS expression under conditions in which cell density was kept relatively low by AraC. In the presence of AraC, EGF did not increase the proportion of astrocytes, supporting the notion that EGF upregulates CBS in individual astrocytes. EGFR is a receptor tyrosine kinase that regulates cell functions including proliferation and differentiation. The activation of EGFR influences gene transcription via several signaling pathways such as Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC) and signal transducer and activator of transcription (STAT) pathways (Wieduwilt and Moasser 2008). Notably, the MAPK and STAT pathways may contribute to the transcriptional regulation of CBS; however, further studies are needed to elucidate the detailed mechanisms downstream of EGFR. It has been reported that EGF diminishes Ca^{2+} response to H_2S in the cultured astrocytes derived from rat brain, and that de novo protein synthesis is required for this effect (Tsugane *et al.* 2007). H_2S increases intracellular Ca^{2+} concentration in astrocytes mainly by 1) Ca^{2+} influx through the transient receptor potential A1 channels (Kimura *et al.* 2013) and 2) Ca^{2+} release from the intracellular Ca^{2+} stores (Nii *et al.* 2021a, b). EGF may also affect the expression of molecules responsible for these Ca^{2+} signalling pathways in astrocytes. Further

277 experiments are needed to address these issues.

278 EGF is crucial for neural stem cells to proliferate and differentiate into
279 astrocytes during embryonic development (Temple 2001). We have previously confirmed
280 that CBS expression is very low in the spinal cord from embryonic day 16 rats, but is
281 markedly higher in neonatal P0-P3 rats (Miyamoto *et al.* 2015). CBS expression levels
282 may reflect astrocyte maturity. CBS degrades cytotoxic L-homocysteine, increased levels
283 of which are a risk factor for the development of various neurological disorders (Regland
284 *et al.* 1995; Seshadri *et al.* 2002; Valentino *et al.* 2010). On the other hand, CBS activity
285 produces H_2S, which is cytoprotective against DNA damage and oxidative stress (Kimura
286 and Kimura 2004; Whiteman *et al.* 2005; Zhao *et al.* 2014). Upregulation of CBS
287 expression together with astrocyte maturation could bestow cells with tolerance to
288 cytotoxic stimuli, and so maintain normal CNS function.

289

290 **5. Conclusion**

291 This study demonstrated that EGF increased CBS expression in cultured spinal
292 cord cells derived from fetal rats. EGF increased CBS^+ astrocytes, which is suppressed
293 by EGFR inhibitor, while the majority of $EGFR^+$ cells were neural stem cells. Taken
294 together, EGF is likely to act on neural stem cells (that do not express CBS) to promote

their proliferation and differentiation into CBS-expressing astrocytes. This process may play an important role in the maturation and /or development of the spinal cord cells.

Abbreviations

AraC, cytosine arabinoside; CNS, central nervous system; CBS, cystathionine ß-synthase; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; H_2S, hydrogen sulfide; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2

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Contributions

Ryota Eguchi: Writing – original draft, Visualization, Validation, Investigation, Formal analysis. Yuya Higashida: Visualization, Validation, Investigation, Formal analysis. Mizuki Oouchi: Visualization, Validation, Investigation, Formal analysis. Soichiro Yamaguchi: Writing – review & editing, Conceptualization. Ken-ichi Otsuguro: Writing

– review & editing, Conceptualization, Funding acquisition, Supervision.

Declaration of competing interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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431

432 **Figure legends**

433 **Fig. 1.** The effect of EGF on CBS expression in cultured spinal cord cells derived from
434 fetal rats. The spinal cord cell culture was treated with EGF (30 ng/ml) for 4 days. (A, B)
435 Western blot analysis of CBS expression. Representative western blots of CBS (63 kDa)
436 and GAPDH (37 kDa) expression are shown in (A). Summarized data of CBS expression
437 normalized to GAPDH expression (B) (n = 3). (C-E) Immunofluorescent staining of CBS-
438 expressing cells. Representative images of CBS (green), GFAP (red) and MPA2 (white)

staining are shown in (C). Arrowheads indicate $CBS^+/MAP2^+$ cells and arrows indicate $CBS^+/GFAP^+$ cells. Scale bars, 50 μm. Summarized data indicating the percentage of CBS^+ cells that are $GFAP^+$ (D), and the cell density of $GFAP^+$ and $MAP2^+$ cells (E) (n = 3). (F) Representative western blots of CBS and GAPDH expression in the spinal cord (SC) cell culture and neuron-enriched cell culture. Data are mean±SEM. *$p < 0.05$ (unpaired Student's t-test).

Fig. 2. Cell density-dependent CBS expression in embryonic spinal cord cells. Cells were seeded at 2.5×10^4, 5×10^4 and 10^5 cells/cm^2, then cultured for 7 days. (A-C) Immunofluorescent staining of spinal cord cells. Representative images of GFAP (red) and MPA2 (white) staining are shown in (A). Scale bars, 50 μm. Summarized data of the cell density (B) and the percentage of $GFAP^+$ or $MAP2^+$ cells (C) (n = 4). (D-G) Western blot analysis of CBS and GFAP expression. Representative western blots of CBS (63 kDa), GFAP (50 kDa) and GAPDH (37 kDa) expression are shown in (D). Summarized data of CBS expression normalized to GAPDH (E) or GFAP (F) expression, and GFAP expression normalized to GAPDH expression (G) (n = 4). Data are mean±SEM. *$p < 0.05$, **$p < 0.01$ versus 2.5×10^4 cells/cm^2 (Dunnett's t-test).

Fig. 3. EGF increases CBS expression independently of cellular proliferation. Embryonic spinal cord cells were treated with EGF (30 ng/ml) for 4 days in the presence or absence of AraC (1 μM), and then immunofluorescently stained for GFAP and MAP2 expression. Summarized data of cell density (A) and the percentage of $GFAP^+$ or $MAP2^+$ cells (B) (n = 3). (C, D) Western blot analysis of CBS expression. Representative western blots of CBS (63 kDa) and GAPDH (37 kDa) expression are shown in (C). Summarized data of CBS expression normalized to GAPDH expression (D) (n = 4). Data are mean±SEM. *p < 0.05 (unpaired Student's t-test).

Fig. 4. EGFR-mediated increase in CBS expression in embryonic spinal cord cells. Cells were treated with EGF (30 ng/ml) for 4 days in the presence of the EGFR tyrosine kinase inhibitor PD153035 (PD, 1 μM) or vehicle (DMSO). (A, B) Western blot analysis of CBS expression. Representative western blots of CBS (63 kDa) and GAPDH (37 kDa) expression are shown in (A). Summarized data of CBS expression (B) (n = 5). (C) Cell density was calculated from the number of $DAPI^+$ cells determined by immunofluorescent staining (n = 3). (D, E) Western blot analysis of EGFR expression. Representative western blots of EGFR (180 kDa) and GAPDH (37 kDa) expression in spinal cord (SC) cell culture and a neuron-enriched culture are shown in (D).

475 Summarized data of EGFR expression normalized to GAPDH expression (E) (n = 3). (F,
476 G) Immunofluorescent staining of EGFR$^-$expressing cells. Summarized data of the
477 proportion of GFAP$^-$/MAP2$^-$, GFAP$^+$ and MAP2$^+$ cells in the EGFR$^+$ cell population.
478 Numbers represent the number of cells (F) (n = 3). Representative images of EGFR
479 (green), GFAP (red) and MPA2 (white) staining are shown in (G). Arrowheads indicate
480 EGFR$^+$/GFAP$^-$/MAP2$^-$ cells and arrows indicate EGFR$^+$/GFAP$^+$ cells. Scale bar, 50 μm.
481 Data are mean±SEM. *$p < 0.05$, **$p < 0.01$ (unpaired Student's t-test).
482
483 **Fig. 5.** EGFR expression in neural stem cells. Embryonic spinal cord cells were treated
484 with EGF (30 ng/ml) for 4 days in the presence or absence of AraC (1 μM). (A-D)
485 Immunofluorescent staining of nestin-expressing cells in embryonic spinal cord cell
486 culture. Representative images of EGFR (green), GFAP (red) and nestin (white) staining
487 are shown in (A). Arrowheads indicate EGFR$^+$/nestin$^+$ cells. Scale bar, 50 μm.
488 Summarized data of the percentage of nestin$^+$ cells in the EGFR$^+$ or EGFR$^-$ cell
489 population (B), and the percentage of EGFR$^+$ cells in the total cell population in the
490 presence or absence of EGF (C) (n = 3). Representative images of EGFR staining (green)
491 in the presence of AraC and EGF are shown in (D). (E, F) Western blot analysis of EGFR
492 expression. Representative western blots of EGFR (180 kDa) and GAPDH (37 kDa)

493 expression are shown in (E). Summarized data of EGFR expression normalized to

494 GAPDH expression (F) (n = 3). Data are mean±SEM. *p < 0.05, **p < 0.01 (unpaired

495 Student's t-test).

Fig. 1

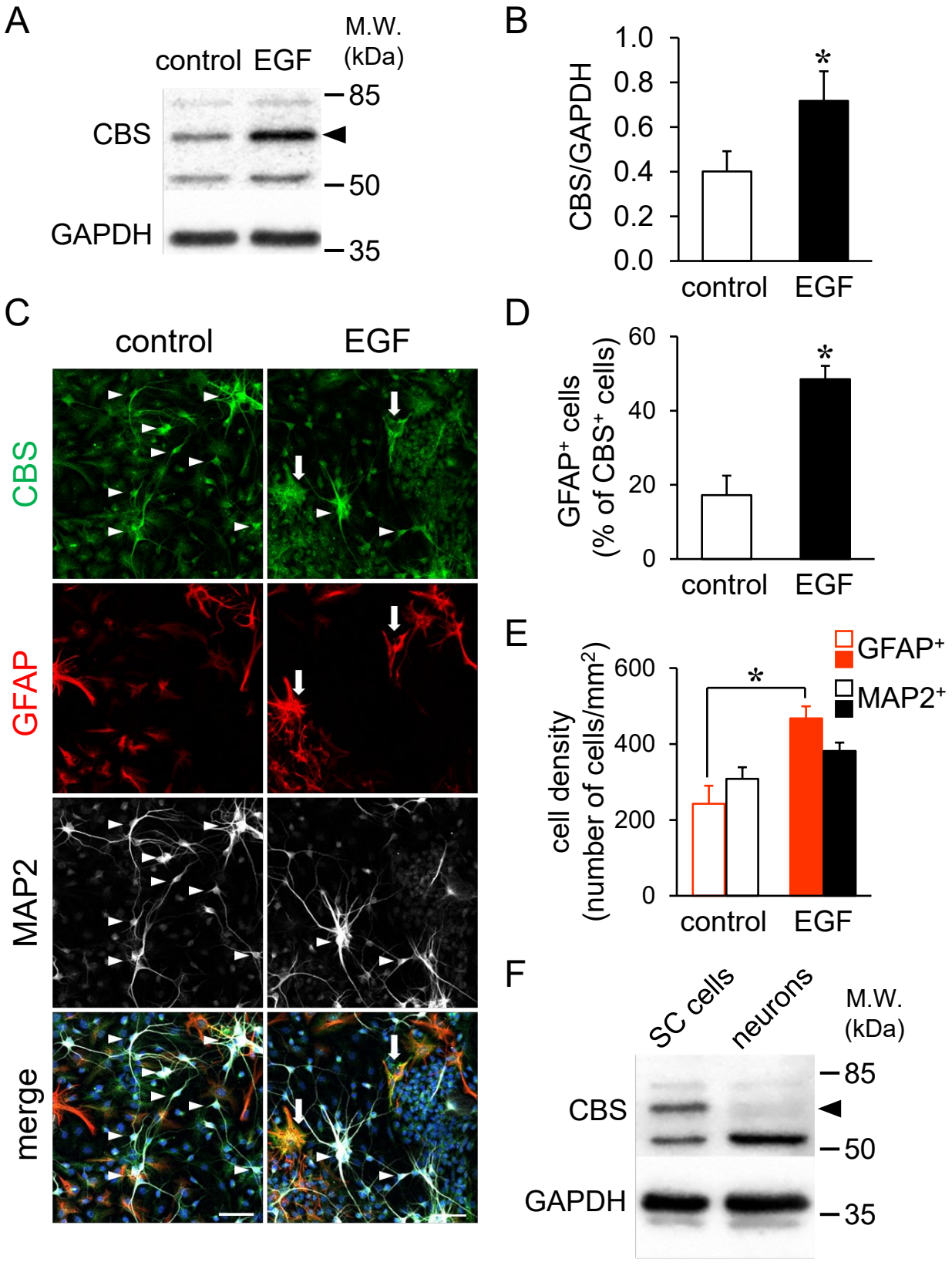
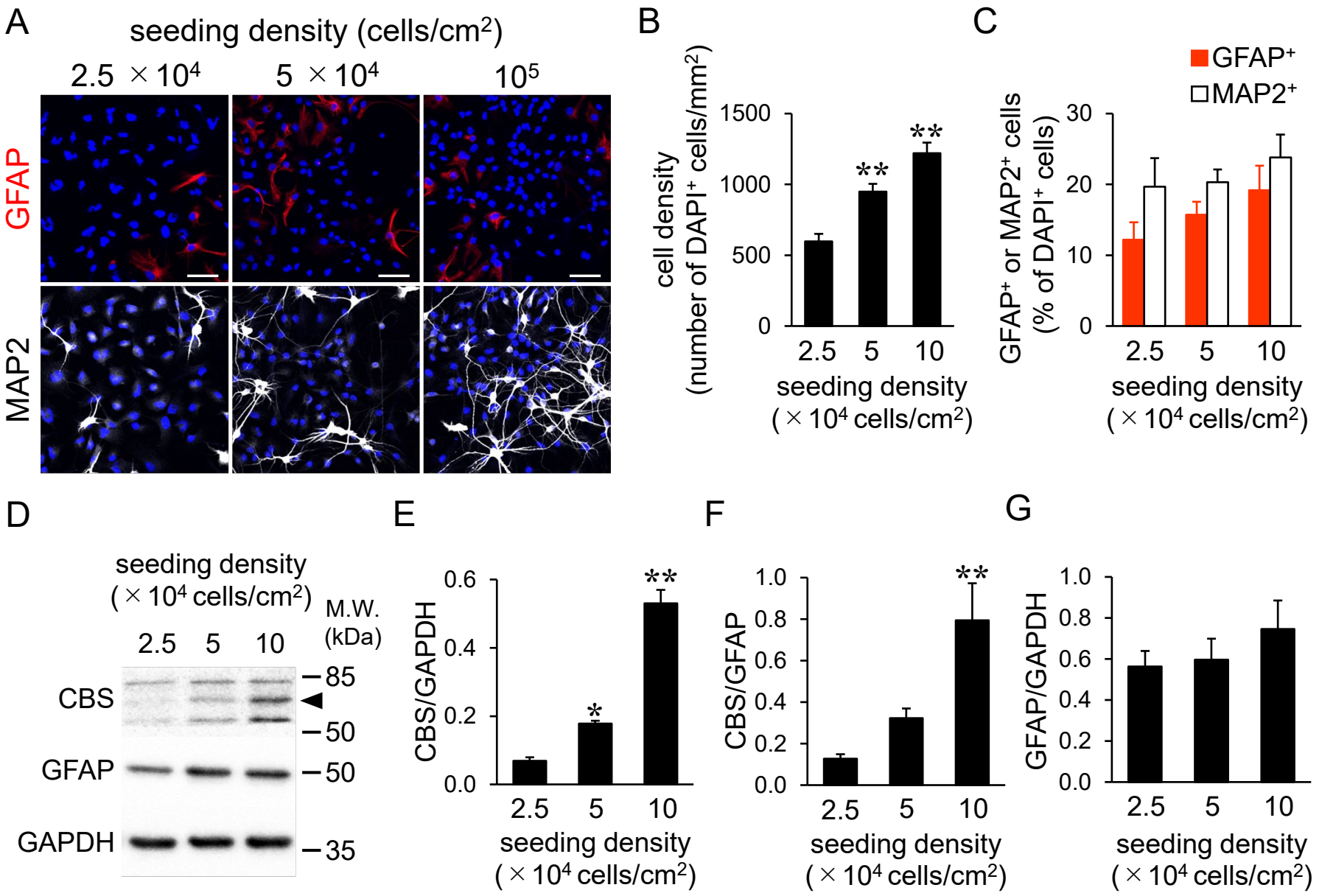
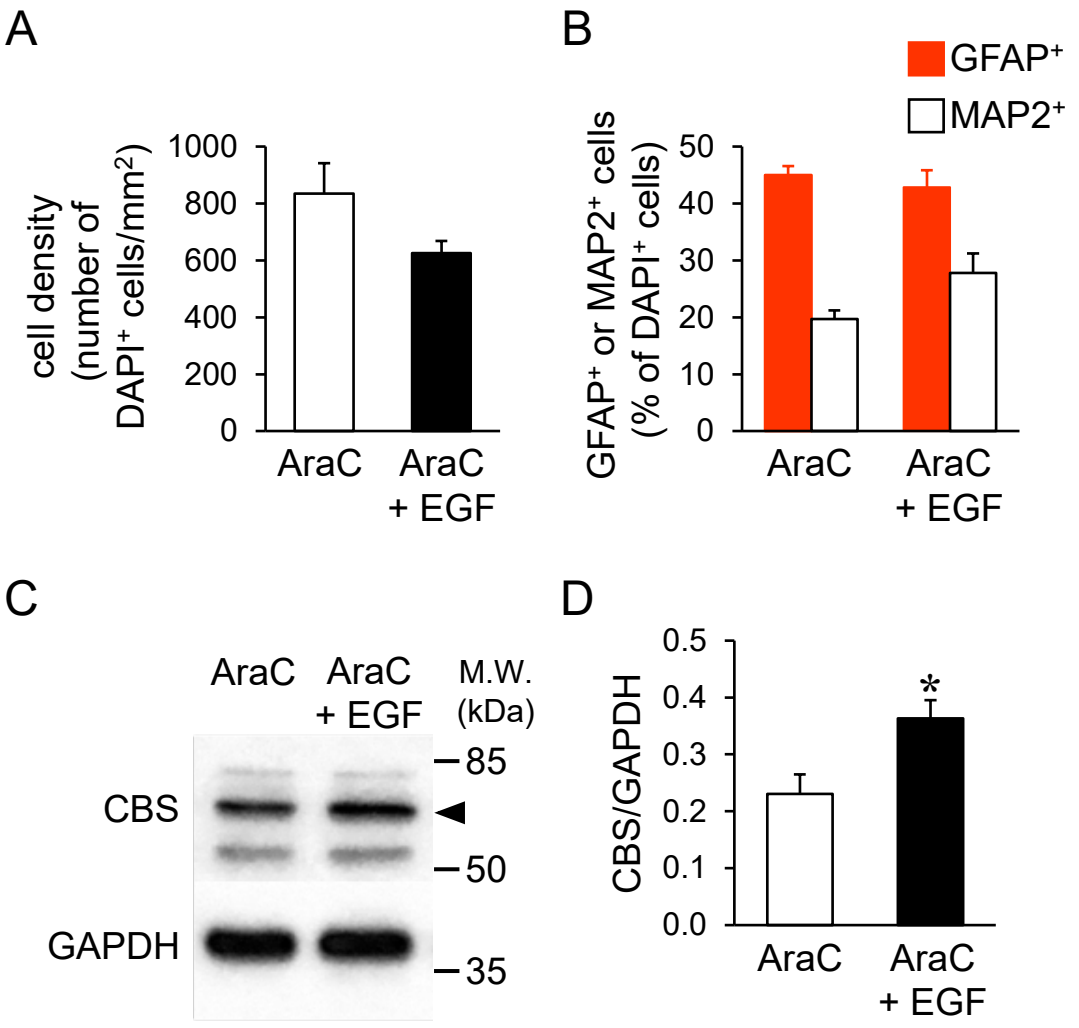

A
control
EGF
M.W. (kDa)
CBS
GAPDH
85
50
35
B
CBS/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
control
EGF
C
control
EGF
CBS
GFAP
MAP2
merge
D
GFAP+ cells (% of CBS+ cells)
60
40
20
0
control
EGF
E
cell density (number of cells/mm2)
600
400
200
0
GFAP+
MAP2+
control
EGF
F
SC cells
neurons
M.W. (kDa)
CBS
GAPDH
85
50
35

Fig. 2


A
seeding density (cells/cm²)
2.5 ×10⁴
5 ×10⁴
10⁵
GFAP
MAP2
B
cell density
(number of DAPI⁺ cells/mm²)
1500
1000
500
0
**
**
2.5
5
10
seeding density
(×10⁴ cells/cm²)
C
GFAP⁺
MAP2⁺
GFAP⁺ or MAP2⁺ cells
(% of DAPI⁺ cells)
30
20
10
0
2.5
5
10
seeding density
(×10⁴ cells/cm²)
D
seeding density
(×10⁴ cells/cm²)
M.W.
(kDa)
2.5
5
10
CBS
GFAP
GAPDH
85
50
50
35
E
CBS/GAPDH
0.6
0.4
0.2
0.0
*
**
2.5
5
10
seeding density
(×10⁴ cells/cm²)
F
CBS/GFAP
1.0
0.8
0.6
0.4
0.2
0.0
**
2.5
5
10
seeding density
(×10⁴ cells/cm²)
G
GFAP/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
2.5
5
10
seeding density
(×10⁴ cells/cm²)


A
cell density
(number of
DAPI+ cells/mm2)
1000
800
600
400
200
0
AraC
AraC
+ EGF
B
GFAP+
MAP2+
GFAP+ or MAP2+ cells
(% of DAPI+ cells)
50
40
30
20
10
0
AraC
AraC
+ EGF
C
AraC
AraC
+ EGF
M.W.
(kDa)
CBS
GAPDH
85
50
35
D
CBS/GAPDH
0.5
0.4
0.3
0.2
0.1
0.0
*
AraC
AraC
+ EGF

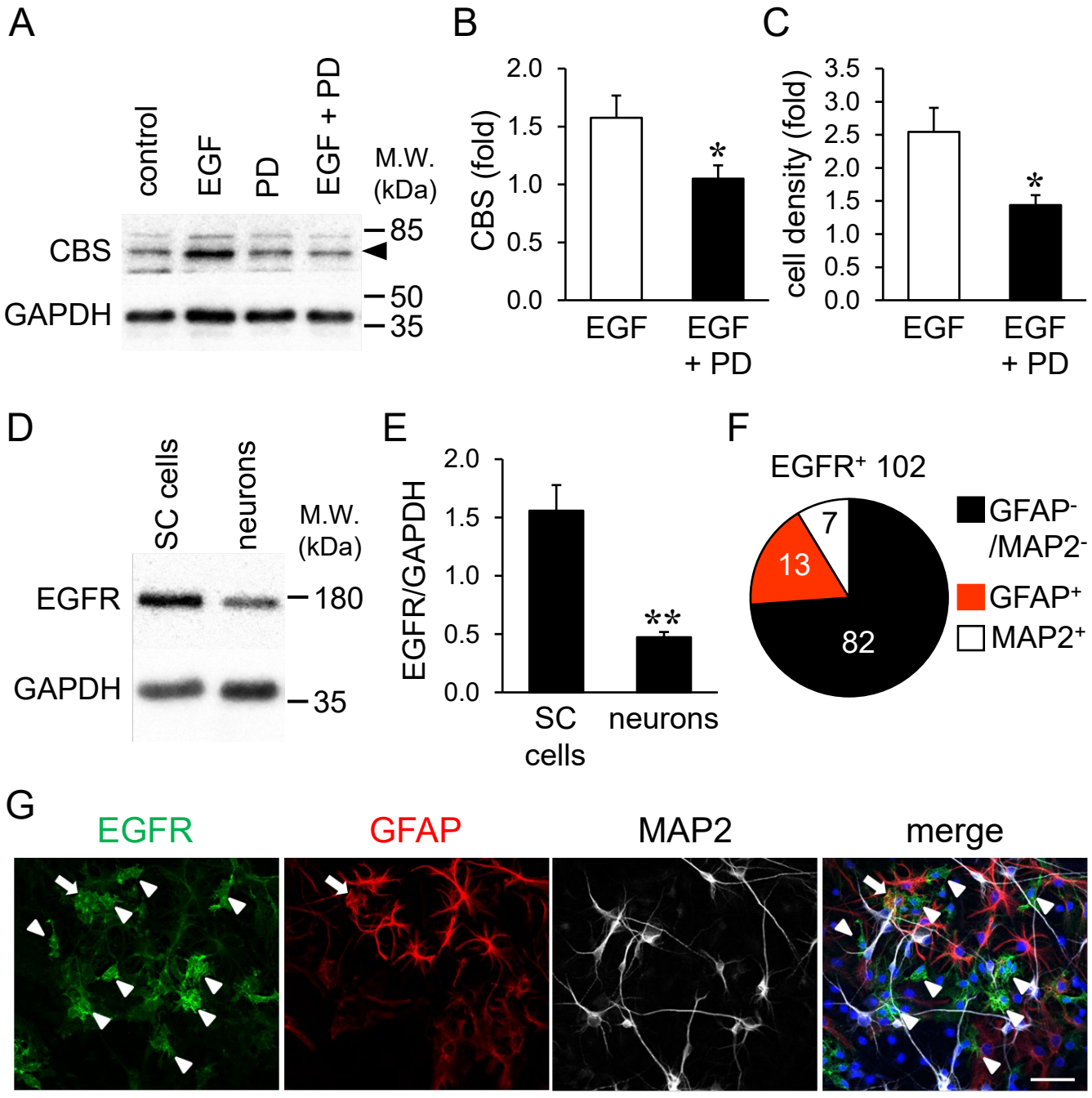
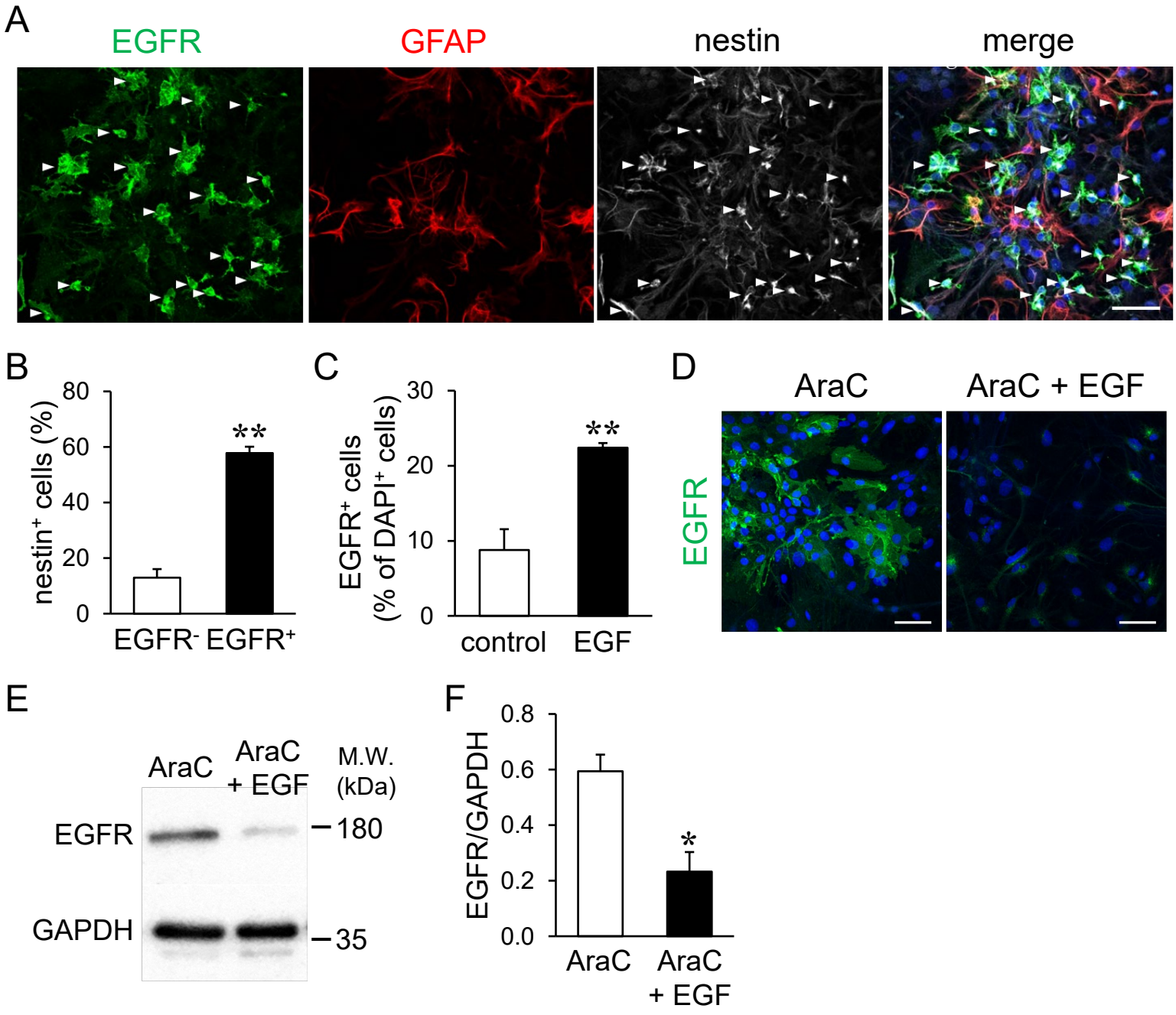

A
control
EGF
PD
EGF + PD
M.W. (kDa)
CBS
GAPDH
85
50
35
B
CBS (fold)
2.0
1.5
1.0
0.5
0.0
*
EGF
EGF + PD
C
cell density (fold)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
EGF
EGF + PD
D
SC cells
neurons
M.W. (kDa)
EGFR
GAPDH
180
35
E
EGFR/GAPDH
2.0
1.5
1.0
0.5
0.0
**
SC cells
neurons
F
EGFR+ 102
7
13
82
GFAP-/MAP2-
GFAP+
MAP2+
G
EGFR
GFAP
MAP2
merge

Fig. 5


A
EGFR
GFAP
nestin
merge
B
nestin+ cells (%)
80
60
40
20
0
**
EGFR- EGFR+
C
EGFR+ cells (% of DAPI+ cells)
30
20
10
0
**
control
EGF
D
AraC
AraC + EGF
EGFR
E
AraC
AraC + EGF
M.W. (kDa)
EGFR
180
GAPDH
35
F
EGFR/GAPDH
0.8
0.6
0.4
0.2
0.0
*
AraC
AraC + EGF