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Basic fibroblast growth factor promotes the generation of microtubule-associated protein 2-positive cells from microglia

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Abstract

We recently demonstrated that microglia as multipotential stem cells give rise to microtubule-associated protein 2 (MAP2)-positive and glial fibrillary acidic protein (GFAP)-positive cells and that microglia-derived MAP2-positive cells possess properties of functional neurons. In this study, we investigated the role of fibroblast growth factor (FGF) signaling in the molecular mechanism underlying the generation of microglia-derived MAP2-positive and GFAP-positive cells. Real-time quantitative PCR analyses demonstrated that mRNA levels of a family of three FGF receptors, *Fgfr1-3*, were upregulated in microglia treated with 70% fetal bovine serum (FBS). Immunocytochemical analyses demonstrated that basic FGF (bFGF) promoted the generation of microglia-derived MAP2-positive and GFAP-positive cells, and the FGF receptor tyrosine kinase inhibitor SU5402 and the MEK inhibitor PD98059 both inhibited this process. Western blot analyses demonstrated that bFGF increased phosphorylated ERK1/2 levels without altering total ERK1/2 levels. These results suggest that bFGF promotes the generation of microglia-derived MAP2-positive and GFAP-positive cells via FGF receptors and the ERK-MAP kinase pathway.

Introduction

Microglia are generally considered to play an important role in the regulation of phagocytosis, neuronal survival, neuronal cell death and inflammation [1]. Under certain pathological conditions, microglia express markers of neural stem cells (NSCs) [2], oligodendrocyte precursor cells [3], hematopoietic stem cells [4] and neurons [5], suggesting that microglia possess undifferentiated and multipotential properties. We recently demonstrated that microtubule-associated protein 2 (MAP2)-positive and glial fibrillary acidic protein (GFAP)-positive cells were generated from microglia following treatment with 70% fetal bovine serum (FBS) and that the electrophysiological properties of the microglia-derived MAP2-positive cells were nearly identical to those of cultured cortical neurons [6]. We also demonstrated that activation of the bone morphogenetic protein (BMP) signaling pathway, which is composed of BMP, the Smad 1/5/8 protein, the Smad4 protein and the ID2 protein (a member of the inhibitory basic helix-loop-helix transcription factors of the inhibitor of differentiation and DNA binding family), is involved in the generation of microglia-derived MAP2-positive and GFAP-positive cells [7]. Furthermore, we demonstrated that the SOX2 protein, a high-mobility group DNA binding domain transcription factor, is essential for the generation of microglia-derived MAP2-positive and GFAP-positive cells [8].

Here, we aimed to identify an additional signaling pathway in order to elucidate the molecular mechanisms underlying the generation of microglia-derived MAP2-positive and

GFAP-positive cells. Fibroblast growth factor (FGF) signaling is a candidate pathway, as basic FGF (bFGF) is reported to regulate the proliferation and differentiation of NSCs [9]. We first examined the expression level of a family of three transmembrane receptor tyrosine kinases (FGFR1-3) in microglial cells before and after 70% FBS treatment, because this treatment induces the generation of MAP2-positive and GFAP-positive cells from microglia. We next examined the effects of bFGF on the generation of microglia-derived MAP2-positive and GFAP-positive cells. We also examined the effects of the FGFR tyrosine kinase inhibitor SU5402 on the generation of microglia-derived MAP2-positive and GFAP-positive cells in order to determine whether bFGF promotes the generation of microglia-derived MAP2-positive and GFAP-positive cells via FGF receptors. Finally, we investigated whether the ERK-MAP kinase pathway, which is a major pathway downstream of FGF receptors, is involved in the generation of MAP2-positive and GFAP-positive cells by bFGF.

Materials and methods

Microglial culture. The use of experimental animals in this study was conducted in accordance with the ethical guidelines of the Kyoto University Animal Experimentation Committee. Microglial cells were obtained from the cortwx of postnatal day 0 to 1 Wistar rats (Nihon SLC, Shizuoka, Japan), as previously described [6]. For the induction of differentiation, the enriched microglial cells were cultured in Dulbecco's modified Eagle's

medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Dainippon-pharm,

Osaka, Japan) and 10 ng/ml macrophage colony stimulating factor (M-CSF; R&D Systems,

Minneapolis, MN) for three days (days 1-3), in DMEM containing 70% FBS and M-CSF for

two days (days 4 and 5), and then in serum-free DMEM in the presence of 20 ng/ml bFGF

(PeproTech, London, UK) for four days (days 6-9). The FGF receptor tyrosine kinase

inhibitor SU5402 and the MEK inhibitor PD98059 were purchased from Calbiochem (San

Diego, CA).

Real-time quantitative PCR. Total RNA was isolated using ISOGEN (Nippon Gene Co., Ltd.,

Tokyo, Japan) according to the manufacturer's instruction. Total RNA (2 μg) was

reverse-transcribed using a mixture of primers including oligo (dT) primer and random

hexamers and the PrimeScript™ RT reagent Kit (TAKARA BIO INC., Shiga, Japan).

Real-time reverse transcription PCR was performed using the SYBR Premix EX Taq™ Kit

(TAKARA BIO INC.). The pairs of primers for rat *Fgfr2* mRNA were designed using the

ProbeFinder software (http://www.roche-applied-science.com), and the nucleotide sequences

of the forward and reverse PCR primers used were 5'-GGG CGA CTT CCA GTC AAG

T-3'and 5'-TAA CAC CCC GAA GGA CCA G-3', respectively. The pairs of primers for rat

Fgfr1 (RA017051), rat *Fgfr3* (RA01241) and rat peptidylprolyl isomerase A (*Ppia*,

RA015371) mRNA were purchased from TAKARA BIO INC. PCR amplification and

fluorescence detection were performed using the Thermal Cycler Dice Real Time System (TP850; TAKARA BIO INC.). All of the PCR conditions were 10 s at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C.

Immunocytochemistry. Cells were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde, washed with PBS and blocked with 5% normal goat serum (Vector Laboratories Inc., Burlingame, CA) in PBS. Cultures were then incubated at 4 °C overnight with primary antibodies diluted in PBS containing 1% normal goat serum. The primary antibodies included the following: rabbit polyclonal anti-MAP2abc antibody (Chemicon, Temecula, CA), rabbit polyclonal anti-GFAP antibody (DakoCytomation, Glostrup, Denmark), and mouse monoclonal anti-CD11b antibody (Serotec, Oxford, UK). Cells were then incubated for 90 min at room temperature with secondary antibodies diluted in PBS containing 1% normal goat serum. The secondary antibodies included the following: Cy™2-conjugated AffiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) and Cy™3-conjugated AffiniPure goat anti-rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories). Cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR). No immunoreactivity was observed when the primary antibodies were omitted during immunofluorescent staining. Immunoreactive cells were quantified in at least four

independent experiments. For each experiment, immunoreactive cells were counted in eight randomly chosen fields for each experiment under 200× magnification, and the results were expressed as a percentage of the total number of cells within the same field. Staining of individual nuclei with DAPI was used to determine the total number of cells per field of view. Labeled cells were visualized and photographed with an Olympus IX81 photomicroscope (Olympus Optical, Tokyo, Japan).

Western blot analyses. Western blot analysis was conducted as previously described [10] using rabbit polyclonal anti-ERK1/2 antibody (Cell Signaling, Danvers, MA), rabbit polyclonal anti-phospho-ERK1/2 (Thr202/Tyr204) antibody (Cell Signaling) or mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Ambion, Austin, TX).

Statistical analyses. Each value represents the mean ± SEM. Statistical comparisons were made using Student's *t*-test, Aspin–Welch's *t*-test or one-way analysis of variance, followed by Dunnett's multiple comparison test using the SPSS version 12.0 program (SPSS Inc., Chicago, IL). Results were considered significant at $p < 0.05$.

Results

Upregulation of Fgfr1, Frfr2 and Fgfr3 mRNA in microglial cells treated with 70% FBS

The proliferation and differentiation of NSCs are regulated by various growth factors including bFGF. FGF signaling is transduced via a family of four transmembrane receptor tyrosine kinases (FGFR1-4), and FGFR1-3 are expressed in NSCs [9,11]. To elucidate the role of FGF signaling in the generation of MAP2-positive and GFAP-positive cells from microglia, we first examined the expression levels of *Fgfr1*, *Fgfr2* and *Fgfr3* mRNA before (day 3) and after (day 5) 70% FBS treatment, because 70% FBS treatment induces the generation of MAP2-positive and GFAP-positive cells from microglia [6-8]. Real-time quantitative PCR analyses showed that the expression levels of *Fgfr1*, *Fgfr2* and *Fgfr3* mRNA significantly increased after 70% FBS treatment (*Fgfr1*: 3.2 ± 0.7-fold, *Fgfr2*: 51.6 ± 14.5-fold, and *Fgfr3*: 5.1 ± 0.6-fold; Fig. 1). These results suggest that FGF receptors contribute to the generation of microglia-derived MAP2-positive and GFAP-positive cells.

Characterization of the generation of MAP2-positive and GFAP-positive cells by bFGF

We next examined the effect of bFGF on the generation of microglia-derived MAP2-positive and GFAP-positive cells. On day 5, cells were cultured in the absence or presence of 20 ng/ml bFGF for four days. Cells were then fixed on day 9, and immunostained for CD11b (green), MAP2 (red), and GFAP (red) as markers of microglia, neurons, and astrocytes respectively (Fig. 2A-D). Both MAP2-positive and GFAP-positive cells had longer branches in the presence of bFGF than those in the absence of bFGF. The percentage of CD11b-positive cells per DAPI-positive cells significantly decreased from $91.6 \pm 0.8\%$ to $57.1 \pm 2.9\%$ (Fig. 2E). In contrast, the percentage of MAP2-positive cells significantly increased from $3.0 \pm 0.6\%$ to $35.2 \pm 3.1\%$ and the percentage of GFAP-positive cells also significantly increased from $4.8 \pm 0.8\%$ to $27.3 \pm 3.0\%$ (Fig. 2F and G). To determine whether bFGF induce the generation of microglia-derived MAP2-positive and GFAP-positive cells via FGF receptors, we examined the effect of the FGFR tyrosine kinase inhibitor SU5402 on the generation of microglia-derived MAP2-positive and GFAP-positive cells. In the presence of bFGF, treatment with 20 μM SU5402 significantly increased the percentage of CD11b-positive cells to $90.3 \pm 2.4\%$ and significantly decreased the percentage of MAP2-positive and GFAP-positive cells to $7.4 \pm 4.0\%$ and $4.1 \pm 1.7\%$, respectively (Fig. 3). These values roughly correspond to those of cells in the absence of bFGF (Fig. 2). These results suggest that bFGF has the ability to promote the generation of microglia-derived MAP2-positive and GFAP-positive cells, and this ability is largely via FGF receptors. Activation of the

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ERK-MAP kinase pathway is a response common to all FGF receptors [9]. We therefore examined the effect of the MEK inhibitor PD98059 on the generation of microglia-derived MAP2-positive and GFAP-positive cells in the presence of bFGF. In the presence of bFGF, treatment with 20 μM PD98059 significantly increased the percentage of CD11b-positive cells to $88.4 \pm 2.2\%$ and significantly decreased the percentage of MAP2-positive and GFAP-positive cells to $6.7 \pm 4.6\%$ and $7.8 \pm 3.0\%$, respectively (Fig. 4A-C). Western blot analyses revealed that bFGF increased the phosphorylated ERK1/2 protein levels without altering the total ERK1/2 protein levels (Fig. 4D). These results suggest that activation of the ERK-MAP kinase pathway is at least involved in the generation of microglia-derived MAP2-positive and GFAP-positive cells in the presence of bFGF.

Discussion

In this study, we found that activation of FGF signaling promotes the generation of microglia-derived MAP2-positive and GFAP-positive cells. This conclusion is based on the following results: (i) FGFRs mRNA levels were upregulated by 70% FBS treatment; (ii) the generation of microglia-derived MAP2-positive and GFAP-positive cells was promoted by bFGF; and (iii) the generation of microglia-derived MAP2-positive and GFAP-positive cells

in the presence of bFGF was inhibited by the FGFR tyrosine kinase inhibitor SU5402 in a concentration-dependent manner.

When the expression levels of FGFR1-3 mRNA were low in cells before 70% FBS treatment (day 3), the generation of MAP2-positive and GFAP-positive cells from microglia was not promoted by bFGF (data not shown). This also supports our conclusion that activation of FGF signaling promotes the generation of microglia-derived MAP2-positive and GFAP-positive cells. Thus, it is possible that 70% FBS treatment increases the expression levels of FGF receptors and thereby enhances FGF signaling. Although it is unclear whether microglia differentiate into MAP2-positive and GFAP-positive cells directly or whether they differentiate into MAP2-positive and GFAP-positive cells through stem/progenitor-like cells, there are at least three possible mechanisms whereby bFGF promotes the generation of microglia-derived MAP2-positive and GFAP-positive cells. First, bFGF could promote the proliferation of microglia-derived stem/progenitor-like cells. Second, bFGF could promote the differentiation of microglia-derived stem/progenitor-like cells. Finally, bFGF could promote the transdifferentiation of microglia into MAP2-positive and GFAP-positive cells. With regard to NSCs, bFGF is reported to control both proliferation and differentiation of NSCs. Basic FGF maintains the proliferation of NSCs via Notch signaling [12] or by regulating Cyclin D2 [13], and it also induces the differentiation of NSCs by controlling transcription factors such as C/EBP [14] and Foxg1 [15]. In addition, bFGF is reported to

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induce the transdifferentiation of non-neuronal cells, such as oligodendrocyte precursor cells [16], bone marrow stromal cells [17] and retina epithelium [18], into neuronal cells.

We recently demonstrated that both the ID2 protein (a member of the inhibitory basic helix-loop-helix transcription factors of the inhibitor of differentiation and DNA binding family) and the SOX2 protein (a high-mobility group DNA binding domain transcription factor) play important roles in the generation of MAP2-positive and GFAP-positive cells from microglia [7,8]. We speculated that the ID2 protein initially inhibits the differentiation of microglia, and thereafter the SOX2 protein determines the direction of differentiation into neural lineages. Interestingly, it has been reported that the expression levels of Sox2 mRNA in osteoblasts is elevated by FGF and reduced by SU5402 [19]. It has also been reported that bFGF induces the differentiation of neuroblastoma cells via the ID2 protein [20]. In contrast, no publication to date has addressed the regulation of bFGF/FGFR expression levels by the SOX2 and/or ID2 proteins. Thus, it is possible that activation of FGF signaling promotes the generation of microglia-derived MAP2-positive and GFAP-positive cells by increasing the expression levels of the SOX2 and ID2 proteins. This notion is supported by the fact that the expression levels of SOX2 protein were upregulated in cells treated with bFGF for four days (data not shown).

In conclusion, this is the first report showing that bFGF promotes the generation of MAP2-positive and GFAP-positive cells from microglia. Our findings provide a new understanding of the molecular mechanism underlying the generation of microglia-derived MAP2-positive and GFAP-positive cells, and fundamental insights that may lead to therapeutic interventions for central nervous system disorders.

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Figure legends

Fig. 1. The expression levels of *Fgfr1*, *Fgfr2* and *Fgfr3* mRNA. The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1-3), and then in DMEM containing 70% FBS and M-CSF for two days (days 4 and 5). Total RNA was isolated from cells on days 3 and 5. The expression levels of *Fgfr1* (A), *Fgfr2* (B), *Fgfr3* (C) and *Ppia* (internal standard) mRNA were determined by real-time quantitative PCR analysis. Each value represents the mean ± SEM from six independent experiments and is expressed in reference to cells on day 3. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with cells on day 3.

Fig. 2. Effects of bFGF on the generation of microglia-derived MAP2-positive and GFAP-positive cells. The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1-3), in DMEM containing 70% FBS and M-CSF for two days (days 4 and 5), and then in serum-free DMEM in the absence (control) or presence of bFGF for four days (days 6-9). Cells were fixed on day 9, immunostained for a microglial marker (CD11b, green), a neuron marker (MAP2, red; A,B), and an astrocyte marker (GFAP, red; C,D), and counterstained with DAPI (blue). Scale bar for all: (in A) 100 μm. Quantification of CD11b- (E), MAP2- (F), and GFAP- (G) positive cells (expressed as the percentage of DAPI-positive cells). Each value represents the mean ± SEM from five

independent experiments. The number of cells counted per experiment in the absence or presence of bFGF was 413 ± 21 or 405 ± 25, respectively. $^{**}p < 0.01$ compared with the control.

Fig. 3. Effects of SU5402 on the generation of MAP-positive and GFAP-positive cells in the presence of bFGF. The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1-3), in DMEM containing 70% FBS and M-CSF for two days (days 4 and 5), and then in serum-free DMEM containing bFGF (A,D), bFGF plus 10 μM SU5402 (B,E), or bFGF plus 20 μM SU5402 (C,F) for four days (days 6-9). Cells were fixed on day 9, immunostained for a microglial marker (CD11b, green), a neuron marker (MAP2, red; A-C), and an astrocyte marker (GFAP, red; D-F), and counterstained with DAPI (blue). Scale bar for all: (in A) 100 μm. Quantification of CD11b- (G), MAP2- (H), and GFAP- (I) positive cells (expressed as the percentage of DAPI-positive cells). Each value represents the mean ± SEM from five independent experiments. The number of cells counted per experiment ranged from 296 ± 37 to 304 ± 42. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with cells treated with bFGF alone.

Fig. 4. Involvement of the ERK-MAP kinase pathway in the generation of MAP2-positive and GFAP-positive cells in the presence of bFGF. The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1-3), in DMEM containing 70% FBS and M-CSF for two days (days 4 and 5), and then in serum-free DMEM containing bFGF or bFGF plus 20 μM PD98059 for four days (days 6-9). Cells were fixed on day 9, immunostained for a microglial marker (CD11b), a neuron marker (MAP2), and an astrocyte marker (GFAP), and counterstained with DAPI. Quantification of CD11b- (A), MAP2- (B), and GFAP- (C) positive cells (expressed as the percentage of DAPI-positive cells). Each value represents the mean ± SEM from four independent experiments. The number of cells counted per experiment ranged from 225 ± 56 to 241 ± 18. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with cells treated with bFGF alone. (D) Expression of phospho-ERK1/2 and ERK1/2 protein. The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1-3), in DMEM containing 70% FBS and M-CSF for two days (days 4 and 5), and then in serum-free DMEM in the absence (-) or presence (+) of bFGF for one hour, followed by Western blot analyses. GAPDH was used as an internal standard.
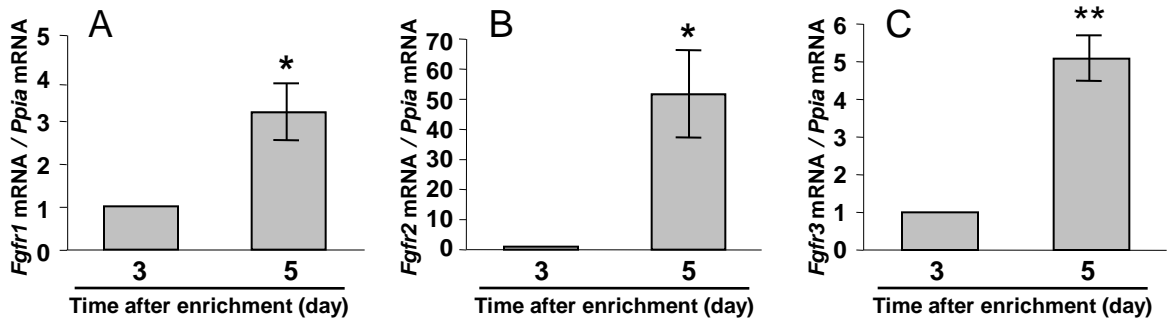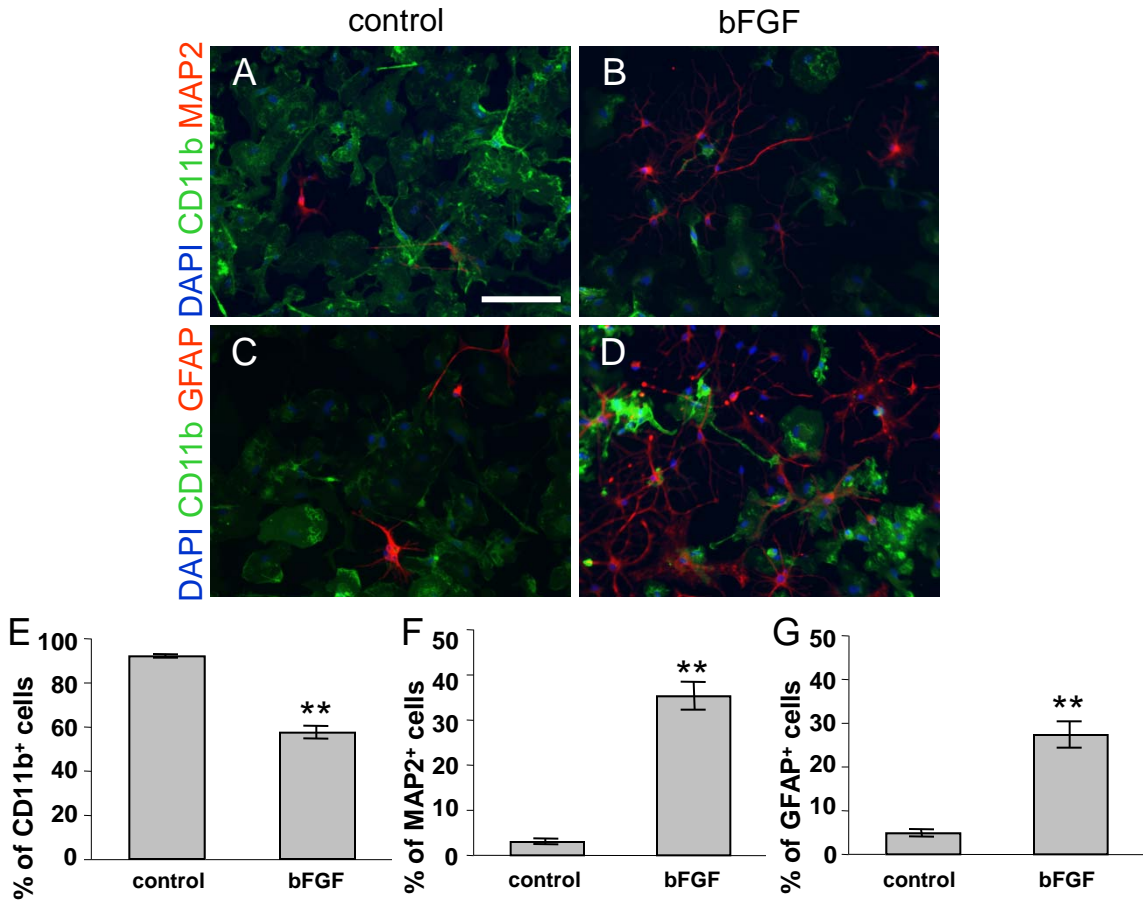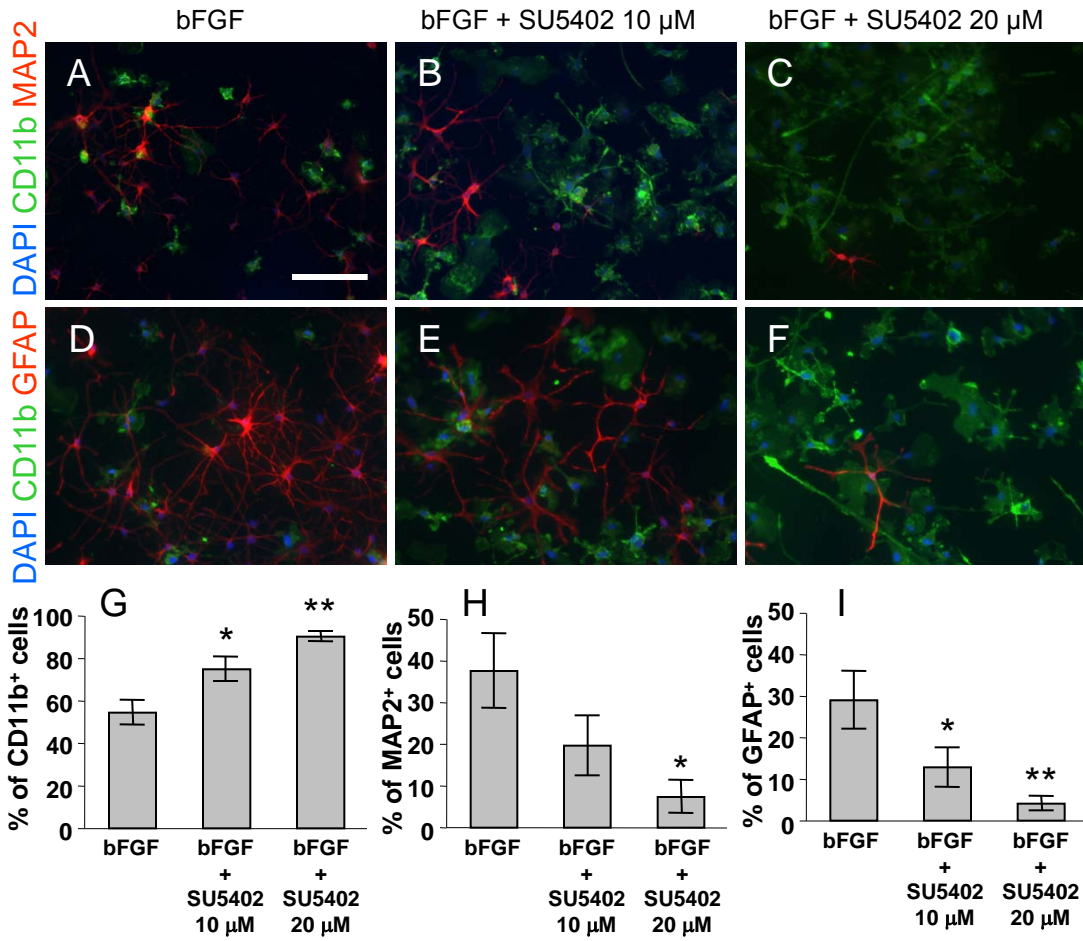
Figure 1

Figure 2

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