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A molecular pathway involved in the generation of microtubule-associated protein 2-positive cells from microglia

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ABSTRACT

We have 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 molecular pathways involved in the generation of microglia-derived MAP2-positive and GFAP-positive cells. Western blot analyses demonstrated that expression levels of Id2 protein, an inhibitory basic helix–loop-helix transcription factor of the inhibitor of differentiation and DNA binding family, and Smad proteins were upregulated under differentiation conditions. Immunocytochemical analyses demonstrated that the generation of MAP2-positive and GFAP-positive cells from microglia was promoted by bone morphogenetic proteins (BMPs) and was inhibited by noggin which is a BMP antagonist, Smad4 siRNA and Id2 siRNA. These results indicate that activation of BMP signaling through Smad and Id2 proteins is one of the molecular pathways involved in the generation of microglia-derived MAP2-positive and GFAP-positive cells.

Microglia are considered to derive from hematopoietic cells that invade the central nervous system early in development [1]. Microglia are generally thought to play an important role in the regulation of phagocytosis, neuronal survival, neuronal cell death and inflammation. We have recently demonstrated that microtubule-associated protein 2 (MAP2)-positive and glial fibrillary acidic protein (GFAP)-positive cells are generated from microglia following treatment with 70% fetal bovine serum (FBS). We observed that the electrophysiological properties (e.g. the spike waveform, firing rate and tetrodotoxin sensitivity of extracellular action potentials evoked by 4-aminopyridine) of microglia-derived MAP2-positive cells are nearly identical to those of cultured cortical neurons [2]. These results suggest that microglia as multipotential stem cells give rise to functional neurons. Our findings are supported by previous studies showing that microglia have the ability to differentiate into neurons, astrocytes or oligodendrocytes [3], and that most microglia within the brain remain in an undifferentiated state [4].

The ability of microglia to differentiate into neurons may substantially promote neural repair since microglia are recruited to lesion sites after brain injury [5]. Importantly, the molecular pathways involved in the differentiation of microglia into neurons are poorly understood. A previous study revealed that members of the basic helix–loop-helix (bHLH) transcription factor family play critical roles in the induction of cell type-specific gene expression [6]. Therefore, we first characterized the expression level of a member of the inhibitory bHLH transcription factors of the inhibitor of differentiation and DNA binding (Id) family, Id2, in microglial cells treated with 70% FBS by Western blot analysis. We also examined the expression levels of Smad proteins because Ids are known to be major target genes for Smad signaling [7]. Next, we examined the effects of bone morphogenetic proteins (BMPs) or the BMP antagonist noggin on the generation of microglia-derived MAP2-positive and GFAP-positive cells by immunocytochemistry because the activation of Smad signaling is known to be a major effect of BMP signaling [7] and because BMPs are known to induce oligodendrocyte precursor cells to reprogram into multipotential neural stem cells [8]. Finally, we investigated whether specific signaling molecules were essential for the generation of MAP2-positive and GFAP-positive cells from microglia by RNAi.

Materials and methods

Microglial cultures. 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 postnatal day 0 to 1

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Wistar rat cortex (Nihon SLC, Shizuoka, Japan), as described previously [9]. 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). The cells were then cultured in DMEM containing 70% FBS and M-CSF for two days (days 4 and 5). Recombinant mouse noggin/Fc chimera (noggin) and recombinant human BMP-2 were purchased from Sigma (St. Louis, MO). Recombinant human BMP-4 was from R&D Systems. Mouse noggin shares 100% amino-acid sequence identity with rat noggin. Human BMP-2 shares 100% amino-acid sequence identity with rat BMP-2. Human BMP-4 shares 98% amino-acid sequence identity with rat BMP-4.

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 include the following: rabbit polyclonal anti-MAP2abc antibody (Chemicon, Temecula, CA), rabbit polyclonal anti-GFAP antibody (DakoCytomation, Glostrup, Denmark), or 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 include 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 three 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 analysis. Western blot analysis was conducted as described previously [9] using rabbit polyclonal anti-Id2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit polyclonal anti-phospho-Smad1/5/8 antibody (Cell Signaling, Danvers, MA), goat polyclonal anti-Smad1/5/8 antibody (Santa Cruz Biotechnology Inc.), mouse monoclonal anti-Smad4 antibody (Santa Cruz Biotechnology Inc.), or mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Ambion, Austin, TX).

RNA interference study. All double-strand RNAs were obtained from iGene Therapeutics Inc. (Ibaraki, Japan). The cultured microglia were transfected on day 1 with 75 nM small interfering RNA (siRNA) specific for rat Id2 or rat Smad4 or randomized nonsilencing siRNA (negative control) for 2 h using a Targefect-siRNA transfection kit (Target Systems, San Diego, CA). The targeted sequences of rat Id2 and Smad4 siRNAs were 5′-UUAUGUCGAAUGACAGCAAAGUACUAG-3′ and 5′-AGUUGGAGUGU AAAGGUGAAGGGGAAG-3′, respectively. The transfecting medium was changed to fresh medium 1 day after transfection.

Statistical analyses. Data are shown as means ± 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, USA). Results were considered significant at $p < 0.05$.

Results

Upregulation of Id2 protein and Smad proteins in microglial cells treated with 70% FBS

Members of the bHLH transcription factor family are known to play critical roles in the induction of cell type-specific gene expression [6]. To determine whether positively acting bHLH transcription factors are important for the generation of MAP2-positive and GFAP-positive cells from microglia, we examined the expression level of Id2 during the period of 70% FBS treatment by Western blot analysis (Fig. 1A). Id2 protein was detectable as a band of ~14 kDa. The expression level of Id2 protein increased after 70% FBS treatment. The Id2 protein level was maximal at 18 h after 70% FBS treatment, and it returned to the baseline level at 36 h (Fig. 1B). In contrast, no significant change in Id2 expression was observed after 10% FBS treatment. These results suggest that Id2 contributes to the generation of microglia-derived MAP2-positive and GFAP-positive cells. To characterize the upregulation of Id2 protein after 70% FBS treatment, we examined the activation of Smad signaling since Ids are known to be some of the major target

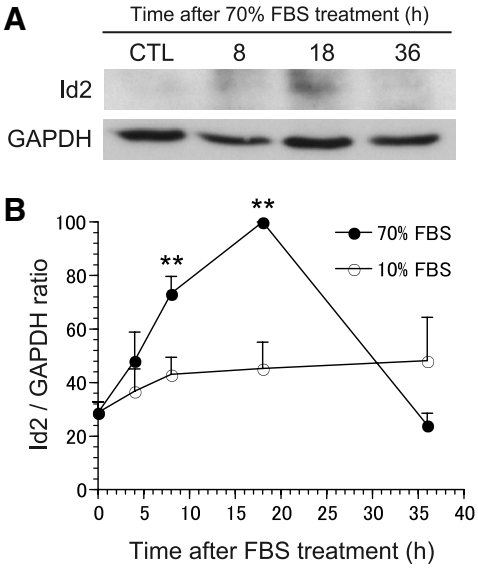

Fig. 1. Expression level of Id2 protein after 70% FBS treatment. The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1–3), and DMEM containing 70% FBS and M-CSF. Cells were harvested at 0 h, 4 h, 8 h, 18 h, and 36 h after 10% or 70% FBS treatment, and the resultant cell lysates were submitted to Western blot analysis. (A) Expression level of Id2 protein in cells at 0 h (CTL), 8 h, 18 h and 36 h after 70% FBS treatment. (B) Quantification of immunoblotting signals. The intensity of each band for Id2 was quantified, normalized to the respective GAPDH signal, which served as an internal standard, and expressed as a percentage of the intensity of the band for Id2 at 18 h after 70% FBS treatment. Data represent means ± SEM from three to six independent experiments. $^{**}p < 0.01$ compared with cells treated with 10% FBS.

genes for Smad signaling [7]. Smad1/5/8 becomes phosphorylated and associates with Smad4 after activation of the upstream receptor. The complex is then translocated into the nucleus, where it is incorporated into a transcriptional unit that modulates gene expression [10]. The phosphorylation of Smad was assessed by Western blot analysis. The expression levels of both phosphorylated Smad1/5/8 and Smad4 proteins were increased after 70% FBS treatment (Fig. 2). In contrast, the expression levels of phosphorylated Smad1/5/8 and Smad4 proteins were not affected after 10% FBS treatment. These results suggest that Smad signaling is activated and leads to a subsequent increase the expression of Id2 protein after 70% FBS treatment.

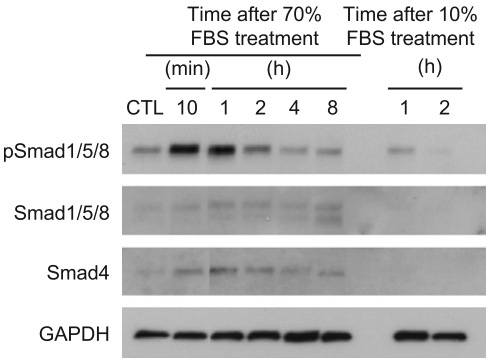

Fig. 2. Activation of Smad signaling after 70% FBS treatment. The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1–3), and DMEM containing 70% FBS and M-CSF. Cells were harvested at 0 min (CTL), 10 min, 1 h, 2 h, 4 h and 8 h after 70% FBS treatment, or 1 h and 2 h after 10% FBS treatment. The resultant cell lysates were submitted to Western blot analysis with anti-phospho-Smad1/5/8, anti-Smad1/5/8, or anti-Smad4 antibody.

Essential role of BMP signaling in the generation of microglia-derived MAP2-positive and GFAP-positive cells

Since microglia are recruited to lesion sites after brain injury, it is important to identify molecules that are essential for the generation of microglia-derived MAP2-positive and GFAP-positive cells. A previous study revealed that simultaneous treatment with BMP-2 and BMP-4 can induce reprogramming of oligodendrocyte precursor cells into multipotential neural stem cells [8]. In addition, the activation of Smad proteins is known to be a major effect of BMP signaling [7]. Therefore, we investigated the possibility that the BMP signaling mimics the effects of 70% FBS treatment. Treatment with 10 ng/ml BMP-2 and BMP-4 significantly increased the percentage of MAP2-positive and GFAP-positive cells on day 5 (Fig. 3A, B, E, F, I, and J). In contrast, 100 ng/ml BMP antagonist noggin significantly decreased the percentage of MAP2-positive and GFAP-positive cells induced by 70% FBS treatment from 5.5 ± 0.5% to 2.7 ± 0.7% and from 3.3 ± 0.4% to 0.4 ± 0.2%, respectively (Fig. 3C, D, G, H, I, and J). To determine whether the expression level of Id2 protein was upregulated by activation of BMP signaling, we examined the expression level of Id2 protein at 18 h after BMP-2/BMP-4 treatment by Western blot analysis (Fig. 3K). A significant increase in the expression level of Id2 protein occurred after BMP-2/BMP-4 treatment. In contrast, the BMP antagonist noggin significantly reduced the expression level of Id2 protein induced by 70% FBS treatment. Collectively, our results suggest that BMPs, Id2 protein and Smad proteins play an important role in the generation of microglia-derived MAP2-positive and GFAP-positive cells. To test this hypothesis, an RNAi approach was used to knock-down the expression of Id2 and Smad4 proteins. Western blot analyses revealed that transfection of 75 nM Id2 siRNA significantly decreased the expression level of Id2 protein at 18 h after BMP-2/BMP-4 treatment to 48.7 ± 10.0% compared to the level observed following transfection of control siRNA. Moreover, transfection of 75 nM Smad4 siRNA significantly decreased the expression level of Smad4 protein at 1 h after BMP-2/BMP-4 treatment to 53.6 ± 7.3% compared to the level observed following transfection of control siRNA (data not shown). Control siRNA did not affect the expression levels of Id2 protein, Smad4 protein, or GAPDH protein (data not shown). The enriched microglial cells were transfected with Id2 siRNA, Smad4 siRNA, or control siRNA at 2 days before BMP-2/BMP-4 treatment, fixed at 2 days after BMP-2/BMP-4

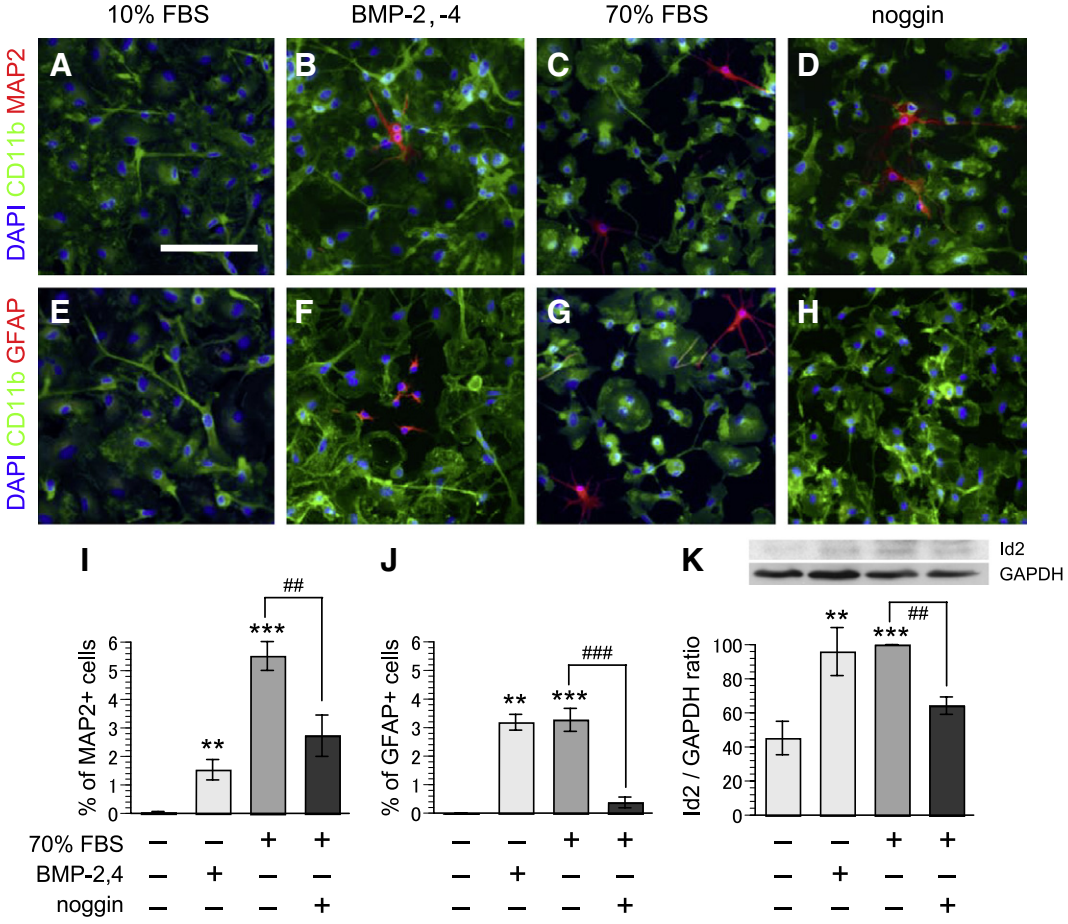

Fig. 3. Effects of BMPs and noggin on the generation of microglia-derived MAP2-positive and GFAP-positive cells. (A–H) Representative immunofluorescent images of cells on day 5. The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1–3) and in DMEM containing 10% FBS and M-CSF (A,E), DMEM containing 10% FBS and M-CSF in the presence of 10 ng/ml BMP-2 plus BMP-4 (B,F), DMEM containing 70% FBS and M-CSF (C,G), or DMEM containing 70% FBS and M-CSF in the presence of 100 ng/ml noggin (D,H) for two days (days 4 and 5). Cells were fixed on day 5, immunostained for a microglia marker, CD11b (green; A–H), a neuron marker, MAP2 (red; A–D), and an astrocyte marker, GFAP (red; E–H), and counterstained with DAPI (blue). Scale bar for all (A–H): (in A) 100 μm. Quantification of MAP2- (I), and GFAP- (J) positive cells (expressed as a percentage of DAPI-positive cells). Data represent means ± SEM from six to eight independent experiments (the numbers of cells counted per experiment ranged from 710 ± 61 to 979 ± 94). (K) Expression level of Id2 protein. Culture conditions were as described above. Cells were harvested at 18 h after 10% FBS, 70% FBS, BMP-2/BMP-4, or noggin treatment, and the resultant cell lysates were submitted to Western blot analysis. The intensity of each band for Id2 was quantified, normalized to the respective GAPDH band signal, which served as an internal standard, and expressed as a percentage of the intensity of the bands for Id2 at 18 h after 70% FBS treatment. Data represent means ± SEM from four to five independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with cells in DMEM containing 10% FBS and M-CSF, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

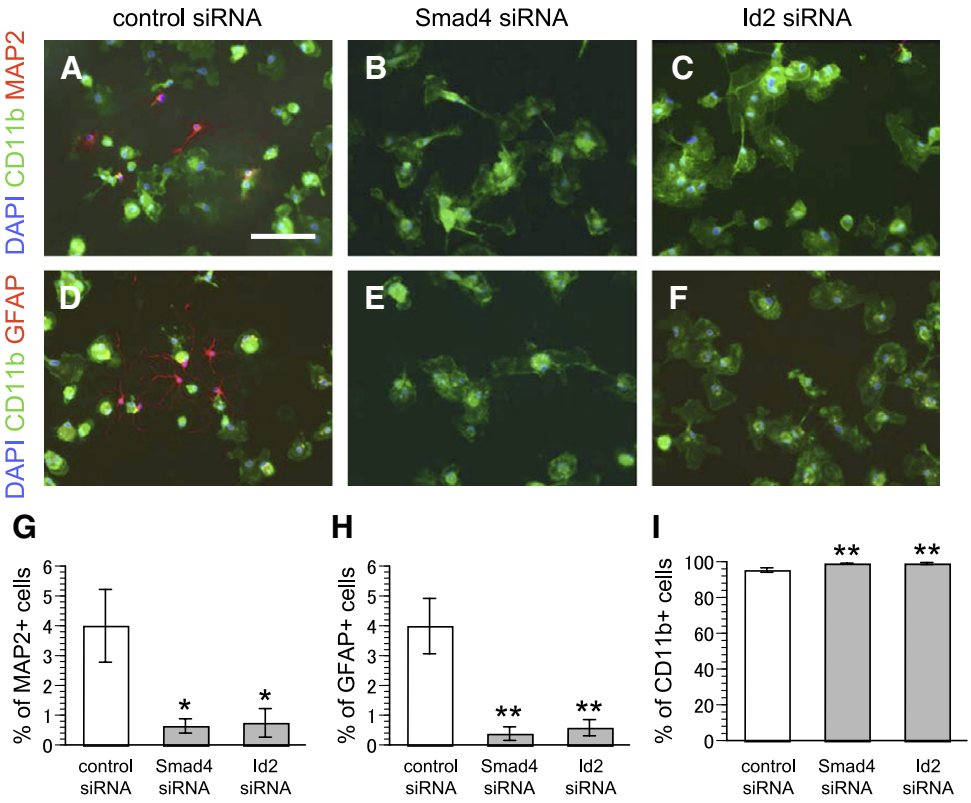

Fig. 4. Effects of Smad4 siRNA and Id2 siRNA transfection on the generation of microglia-derived MAP2-positive and GFAP-positive cells. (A–F) Representative immunofluorescent images of cells on day 5. The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1–3), and DMEM containing 10% FBS and M-CSF in the presence of 10 ng/ml BMP-2 plus BMP-4 for two days (days 4 and 5). Microglial cells were transfected with 75 nM control siRNA, Smad4 siRNA or Id2 siRNA at 2 days before BMP-2/BMP-4 treatment. Cells were fixed at 2 days after BMP-2/BMP-4 treatment, immunostained for either a microglia marker, CD11b (green; A–F), a neuron marker, MAP2 (red; A–C), or an astrocyte marker, GFAP (red; D–F), and counterstained with DAPI (blue). Scale bar for all: (in A) 100 μm. Quantification of MAP2- (G), GFAP- (H), and CD11b- (I) positive cells (expressed as a percentage of DAPI-positive cells). Data represent means ± SEM from five to six independent experiments. The numbers of cells counted per experiment ranged from 246 ± 36 to 299 ± 49. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with cells transfected with control siRNA.

treatment, and immunostained for CD11b (green), MAP2 (red), and GFAP (red) (Fig. 4A–F). Compared with cells transfected with control siRNA, Id2 siRNA, or Smad4 siRNA transfection significantly decreased the percentage of both MAP2-positive and GAFP-positive cells, and increased the percentage of CD11b-positive cells relative to the total number of DAPI-positive cells (Fig. 4G–I). These results indicate that activation of BMP signaling through Smad and Id2 proteins is one of the molecular pathways involved in the generation of microglia-derived MAP2-positive and GFAP-positive cells.

Discussion

In this study, we found that activation of BMP signaling promoted the generation of MAP2-positive and GFAP-positive cells from microglia. This notion is based on the following results: (i) the generation of MAP2-positive and GFAP-positive cells from microglia was promoted by BMPs and was inhibited by a BMP antagonist noggin; (ii) the expression level of Id2 was upregulated by BMPs; and (iii) the generation of MAP2-positive and GFAP-positive cells from microglia was inhibited by Smad4 siRNA and Id2 siRNA. Importantly, the expression of BMP receptor type 1A in microglia was confirmed by immunocytochemistry and Western blot analysis (data not shown).

Interestingly, the contribution of BMP signaling to newly generated MAP2-positive and GFAP-positive cells derived from microglia was different. The percentage of GFAP-positive cells generated by BMP treatment was comparable to that generated by 70% FBS treatment. Furthermore, the percentage of cells generated by 70% FBS treatment was inhibited almost completely by the BMP antagonist noggin. In contrast, the percentage of MAP2-positive cells generated by BMP treatment was clearly lower than that generated by 70% FBS treatment and the percentage of cells generated by 70% FBS treatment was not completely inhibited by noggin. These results indicate the existence of molecular pathways other than BMP signaling that promote the generation of MAP2-positive cells from microglia. Fibronectin, laminin and/or integrins may contribute to additional molecular pathways since they are present in FBS and are capable of modulating activity of the Id gene promoter/enhancer [11]. Retinoic acid [12], MEK-ERK signaling modulator [13], and direct cell–cell interaction [14] are also known to induce transdifferentiation toward the neural lineage from the mesodermal lineage. BMPs have important roles in directing cell fate choices of mesenchymal and endothelial cells via the induction of Id genes [15]. BMP-induced Id protein blocks the activity of lineage-specific transcription factors (e.g. Mash1 and Ngn2) and sustains the self-renewal response of embryonic stem cells in collaboration with signal transducer and activator of transcription 3. In addition, BMPs mediate the switch of neural progenitor cells [16] and neuroepithelial cells [17] from neurogenesis to astrocytogenesis via the upregulation of Id proteins. Recent studies have revealed that Id is involved not only in the regulation of differentiation but also in transdifferentiation. For example, epithelial-mesenchymal transdifferentiation induced by transforming growth factor-β was coupled to a decreased level of Id proteins [18], and ectopic Id3 induction repressed transdifferentiation of astrocytes to a neuronal lineage [19].

The application of neural stem cells and embryonic stem cells to treat central nervous system disorders is a promising therapeutic

intervention. The generation of functional neurons from microglia is also considered to be attractive because microglia themselves migrate to lesion sites. In this study, we have demonstrated that BMPs, Smad4 protein and Id2 protein promote the generation of MAP2-positive and GFAP-positive cells from microglia. These molecules may become future drug targets to restore neural functions.

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