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A role for SOX2 in the generation of microtubule-associated protein 2-positive cells from microglia

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ABSTRACT

We recently demonstrated that, as a type of multipotential stem cells, microglia give rise to microtubule-associated protein 2 (MAP2)-positive and glial fibrillary acidic protein (GFAP)-positive cells. In this study, we investigated the role of SOX2, a high-mobility group DNA binding domain transcription factor, in the generation of microglia-derived MAP2-positive and GFAP-positive cells. Western blot analysis demonstrated that expression of SOX2 was upregulated by treatment with 70% fetal bovine serum treatment. Immunocytochemical analyses demonstrated that SOX2 expression was evident in the nuclei of microglia-derived MAP2-positive and GFAP-positive cells, whereas it was not present in the nuclei of microglia. These assays also showed that *Sox2* siRNA inhibited the generation of MAP2-positive and GFAP-positive cells from microglia. Interestingly, this activity was also inhibited by *Smad4* siRNA, which reduces SOX2 expression. These results indicate that SOX2 upregulation is involved in the generation of microglia-derived MAP2-positive and GFAP-positive cells through SMAD4.

Microglia are considered the resident immune cells of the central nervous system [1]. It is generally accepted that microglia play important roles in the regulation of phagocytosis, neuronal survival, neuronal cell death, and inflammation. In addition, microglia express markers of neural stem cells [2], hematopoietic stem cells [3], and neurons [4] under certain pathological conditions, 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 are generated from microglia following treatment with 70% fetal bovine serum (FBS) and 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 [5]. These observations are supported by previous studies showing that microglia have the ability to differentiate into neurons, astrocytes, or oligodendrocytes [6] and that most microglia within the brain remain in an undifferentiated state [7]. Furthermore, we demonstrated that microglia contribute to the integrity of the microenvironment of neural stem cells and that activated microglia release interleukin-6 and leukemia inhibitory factor, which promote astrocytic differentiation of neural stem cells via activating the Janus kinase/signal transducer and activation of transcription (JAK/STAT) and the mitogen-activated protein kinase (MAPK) pathways [8]. Our findings suggest that, as a type of multipotential stem cells, microglia give rise to functional neurons and that microglia control the behavior of neural stem cells.

The molecular mechanisms by which microglia give rise to functional neurons have not been largely characterized. SOX2, a high-mobility group DNA binding domain transcription factor, is a candidate regulatory molecule because (i) it controls the expression of genes required for the self-renewal and pluripotency of embryonic stem (ES) cells [9]; (ii) it helps maintain neural stem/progenitor cell identity [10,11]; and (iii) it is one of the key factors that can reprogram differentiated cells into pluripotent stem cells [12]. We therefore investigated the possibility that SOX2 is involved in the generation of microglia-derived MAP2-positive and GFAP-positive cells. We characterized the expression level and cellular localization of SOX2 in microglial cells treated with 70% FBS by Western blot and immunocytochemical analyses, respectively. We then used siRNA to determine whether SOX2 is essential for generating MAP2-positive and GFAP-positive cells from microglia. Finally, we investigated whether SMAD4 is involved in this SOX2 pathway, because a previous study revealed that the expression level of SMAD4 was increased after 70% FBS treatment [13].

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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 postnatal day 0 to 1 Wistar rat cortex (Nihon SLC, Shizuoka, Japan), as described previously [5,8]. To induce 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). In our microglial cultures, the cell purity was 99.4 ± 0.2%, expressed as the percentage of CD11b-positive cells.

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 used include the following: rabbit polyclonal anti-MAP2abc antibody (Chemicon, Temecula, CA), mouse monoclonal anti-MAP2 antibody (Sigma, St. Louis, MO), rabbit polyclonal anti-GFAP antibody (DakoCytomation, Glostrup, Denmark), mouse monoclonal anti-GFAP antibody (Sigma), mouse monoclonal anti-CD11b antibody (Serotec, Oxford, UK), and rabbit polyclonal anti-SOX2 antibody (Chemicon). Cells were subsequently incubated for 90 min at room temperature with secondary antibodies diluted in PBS containing 1% normal goat serum. The following secondary antibodies were used: 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 Immuno-Research 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 under 200× magnification, and the results were expressed as the percentage of immunoreactive cells per the total number of cells within the same field. DAPI-positive cells were counted 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 analyses were conducted as described previously [5,8] using a rabbit polyclonal anti-SOX2 antibody (Chemicon), a mouse monoclonal anti-SMAD4 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or a 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 *Sox2*, rat *Smad4*, or randomized non-silencing siRNA (negative control) for 2 h using a Targefect-siRNA transfection kit (Target Systems, San Diego, CA). The targeted sequences of rat *Sox2* and *Smad4* siRNAs were 5′-ACC AAG ACG CUC AUG AAG AAG GAU AAG-3′ and 5′-AGU UGG AGU GUA AAG GUG AAG GGG AAG-3′, respectively. The transfection medium was changed to fresh medium one day after transfection.

Statistical analyses. Data are shown as means ± SEM. Statistical comparisons were made using Student's *t*-test, Aspin–Welch's *t*-

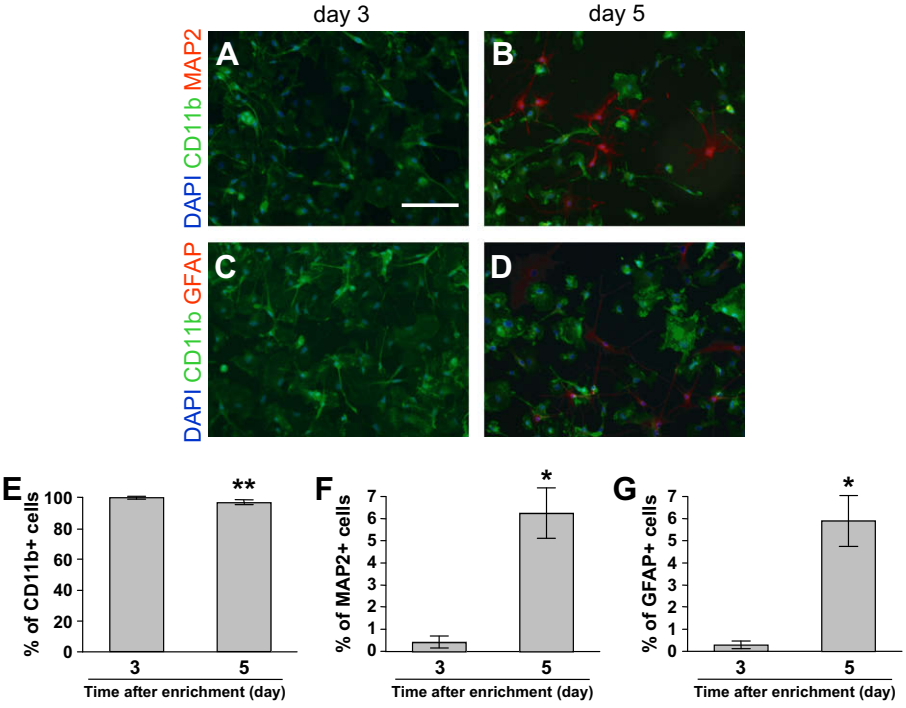

Fig. 1. Immunocytochemistry of microglia-derived MAP2-positive and GFAP-positive cells. (A–D) Representative immunofluorescent images of cells on day 3 (A,C), and 5 (B,D). The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1–3), and then in DMEM containing 70% FBS and M-CSF for two days (days 4 and 5). Cells were fixed on days 3 and 5, 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. (E–G) Quantification of CD11b- (E), MAP2- (F), and GFAP- (G) positive cells (expressed as the percentage of DAPI-positive cells). Data represent means ± SEM from four independent experiments. The numbers of cells counted per experiment were 1085 ± 60 and 537 ± 58 on days 3 and 5, respectively. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with cells on day 3. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

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

SOX2 is upregulated during the generation of microglia-derived MAP2-positive and GFAP-positive cells

To induce the microglia to generate MAP2-positive and GFAP-positive cells, the enriched microglial cells were cultured in DMEM containing 10% FBS for three days (days 1–3) and then in DMEM containing 70% FBS for two days (days 4 and 5) as described previously [5,13]. Cells were fixed on day 3 or 5, and immunostained for CD11b (green), MAP2 (red), and GFAP (red) as markers of microglia, neurons, and astrocytes, respectively (Fig. 1A–D). The percentage of CD11b-positive cells per DAPI-positive cells significantly decreased from 99.4 ± 0.2% on day 3 to 93.2 ± 1.0% on day 5 (Fig. 1E). In contrast, the percentage of MAP2-positive cells significantly increased from 0.4 ± 0.3% on day 3 to 6.3 ± 1.1% on day 5 (Fig. 1F). The percentage of GFAP-positive cells also significantly increased from 0.3 ± 0.2% on day 3 to 5.9 ± 1.5% on day 5 (Fig. 1G). To determine whether SOX2 activity is important when generating of MAP2-positive and GFAP-positive cells from microglia, we examined the expression level and cellular localization of SOX2 in response to 70% FBS treatment. Western blot analysis revealed that the SOX2 expression increased after 70% FBS treatment. Significant upregulation was detected at 12 h after the onset of 70% FBS treatment and was maintained at 48 h (Fig. 2A). In contrast, no significant change in SOX2 expression was observed after 10% FBS treatment. Because SOX2 must translocate into the nucleus in order to regulate transcription [14], it is important to determine the level of nuclear SOX2 expression in addition to the total levels assessed by Western blot analysis. Immunocytochemistry revealed that SOX2 expression was evident in the nuclei of microglia-derived MAP2-positive and GFAP-positive cells 48 h after 70% FBS treatment (Fig. 2F–M). In contrast, nuclear expression of SOX2 in microglial cells was obscure (Fig. 2B–E). These results suggest that SOX2 contributes to the generation of microglia-derived MAP2-positive and GFAP-positive cells.

Essential roles for SOX2 and SMAD4 in the generation of microglia-derived MAP2-positive and GFAP-positive cells

We next used an RNAi technique to investigate whether SOX2 is essential for the generation of MAP2-positive and GFAP-positive cells from microglia. The enriched microglial cells were transfected with *Sox2* siRNA or control siRNA two days before 70% FBS treatment and were analyzed by Western blot and immunocytochemical analyses two days after 70% FBS treatment. Western blot analyses revealed that transfection of *Sox2* siRNA significantly decreased the expression of SOX2 protein to 53.8 ± 13.9% of the level observed following transfection of control siRNA (Fig. 3A). Immunocytochemical analyses revealed that transfection with *Sox2* siRNA significantly increased the percentage of CD11b-positive cells and decreased the percentage of both MAP2-positive and GFAP-positive

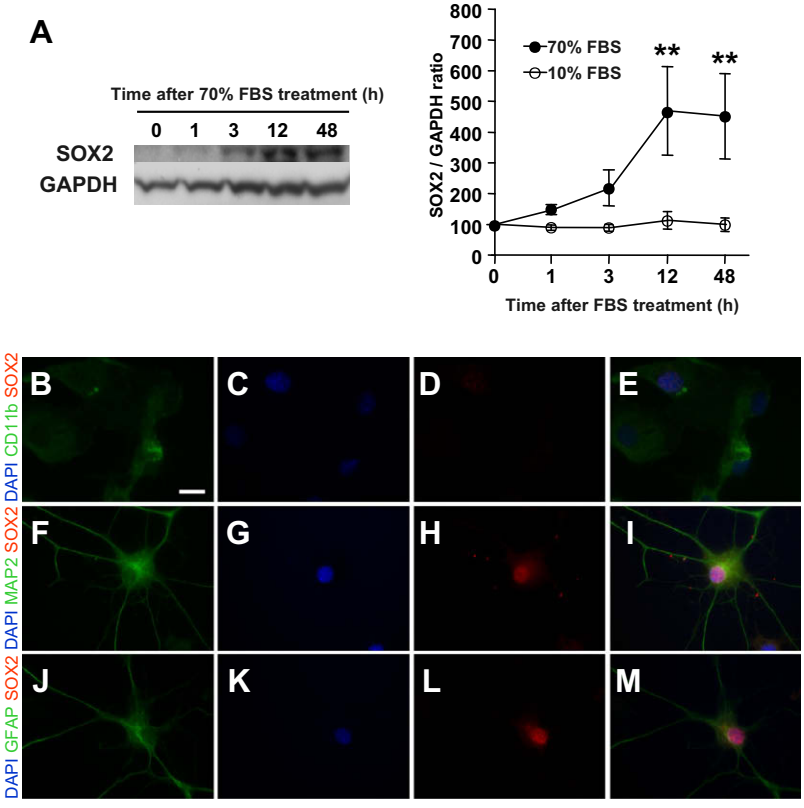

Fig. 2. SOX2 expression level and cellular localization after 70% FBS treatment. The enriched microglial cells were cultured in DMEM containing 10% FBS and M-CSF for three days (days 1–3), and then in DMEM containing 70% FBS and M-CSF. Cells were harvested at 0, 1, 3, 12, and 48 h after the onset of 70% FBS treatment, and the resultant cell lysates were analyzed by Western blotting using an antibody to SOX2 (A, left). The intensity of each band for SOX2 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 SOX2 at 0 h (A, right). Data represent means ± SEM from six independent experiments. $^{**}p < 0.01$ compared with cells at 0 h. (B–M) Representative immunofluorescent images of cells at 48 h after 70% FBS treatment. Cells were fixed, immunostained for Sox2 (red; D,E,H,I,L,M), microglial marker CD11b (green; B,E), a neuron marker MAP2 (green; F,I), and an astrocyte marker GFAP (green; J,M), and counterstained with DAPI (blue; C,E,G,I,K,M). Scale bar for all: (in B) 10 μm. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

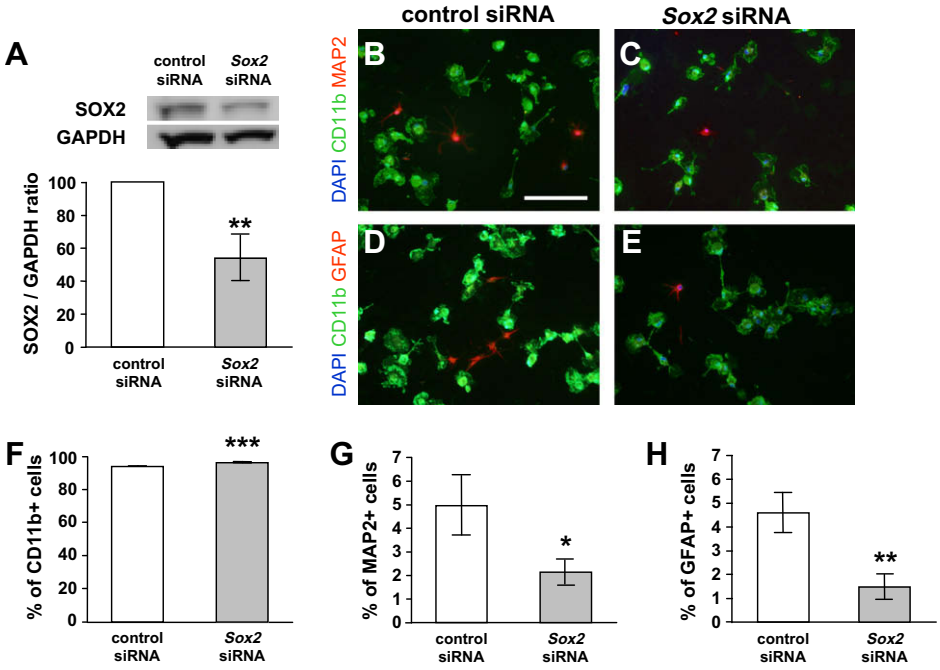

Fig. 3. Effects of *Sox2* siRNA transfection on the generation of microglia-derived MAP2-positive and GFAP-positive cells. Culture conditions as in Fig. 1. Microglial cells were transfected with 75 nM control or *Sox2* siRNA on day 1. Cells were harvested on day 5, and the resultant cell lysates were analyzed by Western blotting using an antibody to SOX2 (A, upper). The intensity of each band for SOX2 was quantified, normalized to the respective GAPDH signal, and expressed as a percentage of the intensity of the band for SOX2 of cell lysates transfected with control siRNA (A, bottom). Data represent means ± SEM from five independent experiments. $^{**}p < 0.01$ compared with cells transfected with control siRNA. (B–E) Representative immunofluorescent images of cells on day 5. Cells were fixed on day 5, immunostained for microglial marker CD11b (green), a neuron marker MAP2 (red; B,C), and an astrocyte marker GFAP (red; D,E), and counterstained with DAPI (blue). Scale bar for all: (in B) 100 μm. (F–H) Quantification of CD11b- (F), MAP2- (G), and GFAP- (H) positive cells (expressed as a percentage of DAPI-positive cells). Data represent means ± SEM from eight independent experiments. The numbers of cells transfected with control and *Sox2* siRNA counted per experiment were 203 ± 25 and 155 ± 15, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with cells transfected with control siRNA. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

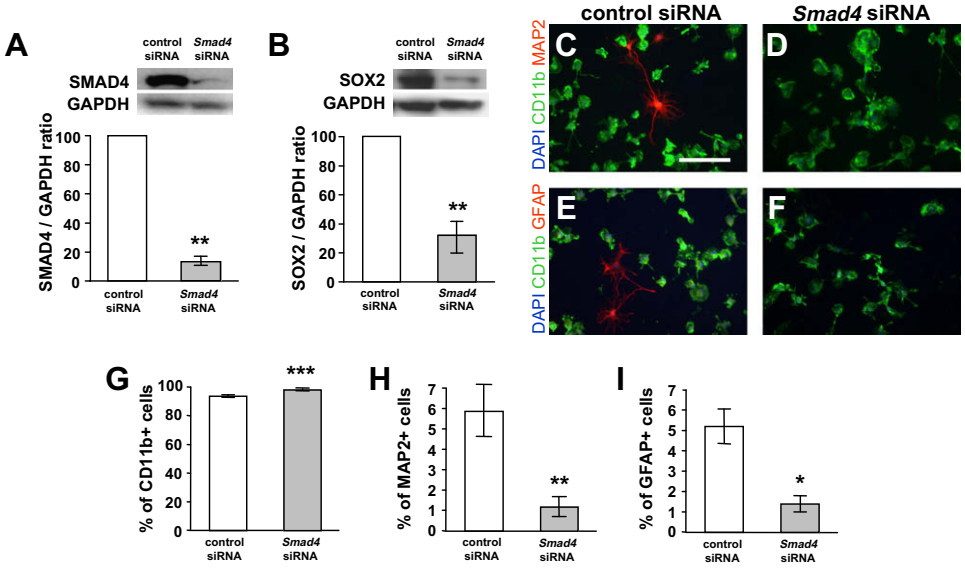

Fig. 4. Effects of *Smad4* siRNA transfection on the generation of microglia-derived MAP2-positive and GFAP-positive cells. Culture conditions as in Fig. 1. Microglial cells were transfected with 75 nM control or *Smad4* siRNA on day 1. Cells were harvested at 1 h (A) or 48 h (B) after 70% FBS treatment, and the resultant cell lysates were analyzed by Western blotting using an antibody to either SMAD4 (A) or SOX2 (B). The intensity of each band was quantified, normalized to the respective GAPDH signal, and expressed as a percentage of the intensity of the bands for SMAD4 and SOX2 of cell lysates transfected with control siRNA. Data represent means ± SEM from five independent experiments. $^{**}p < 0.01$ compared with cells transfected with control siRNA. (C–F) Representative immunofluorescent images of cells on day 5. Cells were fixed on day 5, immunostained for microglial marker (CD11b, green), a neuron marker (MAP2, red; C,D), and an astrocyte marker (GFAP, red; E,F), and counterstained with DAPI (blue). Scale bar for all: (in C) 100 μm. Quantification of CD11b- (G), MAP2- (H), and GFAP- (I) positive cells (expressed as a percentage of DAPI-positive cells). Data represent means ± SEM from eight independent experiments. The numbers of cells transfected with control and Smad4 siRNA counted per experiment were 141 ± 18 and 159 ± 10, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with cells transfected with control siRNA. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

cells as compared to transfection with control siRNA (Fig. 3B–H). Next, we investigated the possibility that SMAD4 regulates the expression level of SOX2 because the expression level of SMAD4 was also increased after 70% FBS treatment [13]. Western blot analyses revealed that transfection with *Smad4* siRNA significantly decreased the expression level of SMAD4 to 12.8 ± 3.2% of the level observed following transfection of control siRNA within 1 h after 70% FBS treatment (Fig. 4A). Transfection with *Smad4* siRNA also significantly decreased the expression of SOX2 at 48 h after 70% FBS treatment to 32.1 ± 8.4% of the control level (Fig. 4B). Furthermore, transfection with *Smad4* siRNA significantly increased the percentage of CD11b-positive cells and decreased the percentage of both MAP2-positive and GFAP-positive cells relative to the total number of DAPI-positive cells (Fig. 4C–I) two days after 70% FBS treatment. Taken together, these results suggest that SMAD4 upregulates SOX2, which is essential for the generation of microglia-derived MAP2-positive and GFAP-positive cells.

Discussion

In this study, we found that SOX2 upregulation was essential for the generation of MAP2-positive and GFAP-positive cells from microglia. This conclusion is based on the following results: (i) SOX2 expression level was upregulated by 70% FBS treatment; (ii) SOX2 expression was evident in the nuclei of microglia-derived MAP2-positive and GFAP-positive cells, whereas it was not present in the nuclei of microglia; and (iii) *Sox2* siRNA inhibited the generation of MAP2-positive and GFAP-positive cells from microglia.

We recently demonstrated that upregulation of Id2, an inhibitory basic helix-loop-helix (bHLH) transcription factor of the inhibitor of differentiation and DNA binding family is essential for the generation of MAP2-positive and GFAP-positive cells from microglia [13]. Id proteins inhibit differentiation of a variety of cell types by acting as dominant-negative blockers of bHLH proteins. These findings led to the prediction that additional factors that determine the direction of differentiation into neural lineages (e.g., formation of neural stem/progenitor cell) are required in the generation of MAP2-positive and GFAP-positive cells from microglia. Interestingly, high SOX2 protein levels were observed on and after 36 h of 70% FBS treatment, while the initially upregulated Id2 returned to the baseline level [13]. Because the generation of MAP2-positive and GFAP-positive cells from microglia was inhibited by either Id2 siRNA or Sox2 siRNA, it is possible that Id2 initially inhibits the differentiation of microglia, but SOX2 later determines the direction of differentiation into neural lineages. SOX2 has various functions due to cell-specific partners. For example, SOX2 regulates a different set of genes in ES and lens cells in collaboration with Oct3/4 and δEF3, respectively [15]. It will be interesting to investigate cell-specific SOX2 partners during the generation of microglia-derived MAP2-positive and GFAP-positive cells. Oligodendrocyte precursor cells as well as microglia differentiate into MAP2-positive and GFAP-positive cells after FBS or bone morphogenetic protein (BMP) treatment [16]. SOX2 was also upregulated in oligodendrocyte precursor cells treated with BMP2 [17]. It is possible that bHLH transcription factors Olig1 and Olig2 might be specific partners of SOX2 because they are expressed in both microglia and oligodendrocyte precursor cells. This hypothesis is supported by the fact that Olig2 interacts with the HMG domains of SOX family proteins [18]. Interestingly, real-time quantitative PCR analyses revealed that both Olig1 and Olig2 mRNAs were upregulated in microglia treated with 70% FBS (data not shown). It is also possible that class III POU transcription factors Brn1 and Brn2 might be specific partners of SOX2 because SOX2 interacts with Brn2 and activates the *Nestin* neural enhancer [19].

We recently demonstrated that activation of BMP signaling is one of the molecular pathways involved in the generation of microglia-derived MAP2-positive and GFAP-positive cells [13]. In addition, the existence of molecular pathways other than BMP that promote the generation of MAP2-positive cells from microglia has been proposed because the increase in MAP2-positive cells generated by 70% FBS treatment was not completely inhibited by BMP antagonist noggin. In this study, the increase in MAP2-positive cells generated by 70% FBS treatment was largely inhibited by *Smad4* siRNA. It is possible that TGFβ signaling may be another molecular pathway involved in the generation of MAP2-positive cells from microglia because TGFβ signaling regulates SOX2 expression via SMAD4 in human ES cells [20].

In conclusion, this is the first report showing that SOX2 is essential for the generation of MAP2-positive and GFAP-positive cells from microglia. Our results provide fundamental insights that may be useful for therapeutic interventions for central nervous system disorders.

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