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Expression pattern of transcription factor SOX2 in reprogrammed oligodendrocyte precursor cells and microglias: Implications for glial neurogenesis

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Oligodendrocyte Precursor Cells (OPCs) can revert to multipotential Neural Stem-Like Cells (NSLCs) which can self-renew and give rise to neurons, astrocytes and oligodendrocytes when exposed to certain extracellular signals. This is a significant progress to understand developmental neurobiology, in particularly the possibility of converting glia to stem cells for the treatment of neurological disorders. Similarly, recent findings revealed that brain-resident microglias (MGs) can be converted to multipotential state through de-differentiation. In this study, we investigated the role of SRY (sex-determining region)-box 2 (SOX2), a high-mobility group DNA binding domain transcription factor, in the reprogramming of OPCs and MGs and molecular pathways involved in these process. Immunocytochemical analyses demonstrated that expression of SOX2 was upregulated in the reprogrammed MGs and OPCs as well as other neural stem cell markers such as CD15 and nestin. Western blot and double immunostaining analyses further confirmed that activation of bone morphogenetic proteins (BMPs) signaling partnering with SOX2 might be one of the molecular pathways involved in lineage reprogramming of OPCs which is also true in the reversion of MGs. Taken together, these results indicated that lineage reprogramming of OPCs and MGs are both controlled by the same signaling pathway and glia can be reprogrammed in culture by inducing expression of neurogenic transcription factors to transgress their lineage restriction and can stably acquire a neuronal identity. Our results suggested innovative perspectives for cell therapy with glia cells.

Key words: astrocyte, oligodendrocyte, microglia, SOX2, BMP

ABBREVIATIONS:

OPCs – Oligodendrocyte Precursor Cells
NSLCs – Neural Stem-Like Cells
SOX2 – SRY (sex-determining region)-box 2
MGs – microglias
BMP – bone morphogenetic protein
NSCs – neural stem cells
T2As – type-2 astrocytes
bFGF– basic fibroblast growth factor

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INTRODUCTION

For many decades, a common assumption of classical neuroscience was that neural stem cells (NSCs) were depleted in the postnatal brain and neurogenesis already ceased after birth. However, recent studies demonstrated that adult brain still contains stem cells that can produce neurons or glial cells throughout whole life of rodents. Surprisingly, these multipotent NSCs in some restricted regions of adult rodent brain like the subependymal zone and the subgranular zone of the dentate gyrus, often show astrocytic identity and retain distinct features of radial glial cells (Alvarez-Buylla et al. 2001, Doetsch 2003, Ma et al. 2005, Kriegstein and Alvarez-Buylla 2009, Robel et al. 2011, Morrens et al. 2012). NG2-glia/Oligodendrocyte Precursor Cells (OPCs),

another ubiquitous glial cell type during development of all vertebrate brains, also act as latent NSCs. It was demonstrated in a relatively recent study that lineage-committed OPCs can be converted to multipotent neural stem-like cells (NSLCs), capable of generating both neurons and glia after exposure to BMP4 (bone morphogenic protein 4) or fetal bovine serum (FBS), followed by basic fibroblast growth factor (bFGF) (Kondo and Raff 2000). This study suggests that OPCs are more plastic than previously believed. In comparison with OPCs which are considered neuroectodermal originally, microglias (MGs) are generally recognized as mesodermal cells. However, a recent study has even demonstrated that these ramified macrophages expressing CD11b have the propensity to transform into neurons, astrocytes or oligodendrocytes through microglioblasts and promicroglioblasts which are obtained by two-step culture in 10% and 70% serum-supplemented media (Yokoyama et al. 2004). Importantly, both reprogrammed oligodendrocyte lineage and MGs expressed similar cell-specific markers, which suggest that these two glial populations might show the same lineage property (Yokoyama et al. 2004).

What are the extrinsic/intrinsic signals endowing these glial cells with a neurogenic potential? It turns out to be a combination of four transcription factors (Oct4, SOX2, Klf4, and c-Myc) directly reprogramme fibroblasts into induced pluripotent stem cells, which raises one particularly perplexing question: Is there a core transcriptional network regulating the lineage conversion of these glial populations (Takahashi and Yamanaka 2006, Jaenisch and Young 2008)? Of all the molecules, the SRY (sex-determining region)-box 2 (SOX2) gene is of particular interest to us, it encodes a transcription factor required for maintenance of the pluripotent state of NSCs (Graham et al 2003, Cavallaro et al. 2008). SOX2 is expressed throughout developing cells in the neural tube as well as in proliferating CNS progenitors (Rex et al. 1997). Remakably, the conversion of OPCs to NSLCs was proved to be mediated partially through the progressive reactivation of SOX2. This core transcription factor plays a pivotal role in the identification and maintenance of the neural stem cell state while simultaneously silencing a large group of oligodendrocyte lineage-specific genes (Kondo and Raff 2004, Lyssiotis et al. 2007). Another target of this study is BMP-2, since a previous study revealed that BMPs are known to induce OPCs to reprogram into multipotential neural stem cells (Kondo and Raff 2000).

Here in this study, we investigated the possibility that SOX2 is involved in the lineage transgression of MGs and the generation of fully functional neurons eventually. We examined the cellular localization and expression level of SOX2 in these reprogrammed glial cells by immunocytochemical and western blot analyses. We also investigated whether BMP-2 is involved in the activation of SOX2.

METHODS

Primary OPCs and MGs culture

Experiments were conducted strictly in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, USA, 1996) and were approved by the Chinese National Committee to Use of Experimental Animals for Medical Purposes, Jiangsu Branch. Primary rat OPCs and MGs were isolated as described in the literature (Armstrong 1998). Briefly, postnatal (0–1 day) Sprague Dawley rat pups were sacrificed by decapitation and the cortices were dissected from the skull. The tissues were then enzymatically and mechanically dissociated to form a single-cell suspension. Cells were seeded onto Corning T75 cm^2 culture flasks precoated with 200 μg/ml poly-L-lysine (PLL, Sigma) at a density of 2×10^7 cells/flask. Cells were incubated in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (1:1 DMEM/F12, Invitrogen) medium [supplemented with 25 mM HEPES, with penicillin and streptomycin (final concentration of 100 U/ml penicillin and 100 μg/ml streptomycin)] with 10% fetal bovine serum (FBS, Invitrogen) at 37°C, 5% CO_2, and 95% air. After 7 to 10 days, the flasks were placed in a horizontal shaker and preshaken for 2 h at 180 rpm at 37°C. After 2 h, the supernatant, which containing MGs, was incubated in DMEM/F12 medium with 10% FBS for 2 h to 3 days. Then the remaining cells were incubated in DMEM/F12 medium with 10% FBS for 2–3 h, and the flasks were shaken for 18 h at 200 rpm again, the supernatant containing OPCs, was incubated for 5 days with DMEM/F12 medium containing platelet-derived growth factor (PDGF) (10 ng/ml, CST) and bFGF (10 ng/ml, CST).

OPCs and MGs reprogrammed to become multipotential CNS stem cells

According to the method of Kondo and Raff (Kondo and Raff 2000), rat primary OPCs can be reprogrammed into pluripotent stem cells that can produce both neurons and glia. Briefly, purified OPCs on PLL-coated dishes were first treated with 15% FBS and PDGF (10 ng/ml) for 2~3 days to generate type-2 astrocytes (T2As), followed by growth in bFGF (10 ng/ml) for 5~10 days to generate free-floating spheres containing neural stem cells. Generated oligospheres were then dissociated and passaged when spheres started to detach from the bottom of the flask. The medium used for NSLCs culture was composed of neural basal media (NBM) (neurobasal-A freshly supplemented with 2% B-27 and 1% N2, Invitrogen) and growth factors (10 ng/ml bFGF, 10 ng/ml EGF, CST). To induce NSLCs differentiation, spheres were seeded onto PLL-coated coverslips at a density of 3×10^4 cells/coverslip, and the medium was replaced with NBM containing 1% FBS for subsequent 3~5 days.

In the meantime, as described previously (Yokoyama et al. 2004), we de-differentiated MGs and then induced them into neuronal phenotypes. For the induction of de-differentiation, the enriched MGs were first cultured for 3 days in DMEM containing 10% FBS, then for 2 days in DMEM containing 70% FBS. To induce neuroectodermal phenotype from MG-derived cells, the cells were scraped off, seeded onto PLL-coated culture dishes or glass coverslips and maintained in E2 medium (serum-free DMEM containing 10 mM HEPES, 4.5

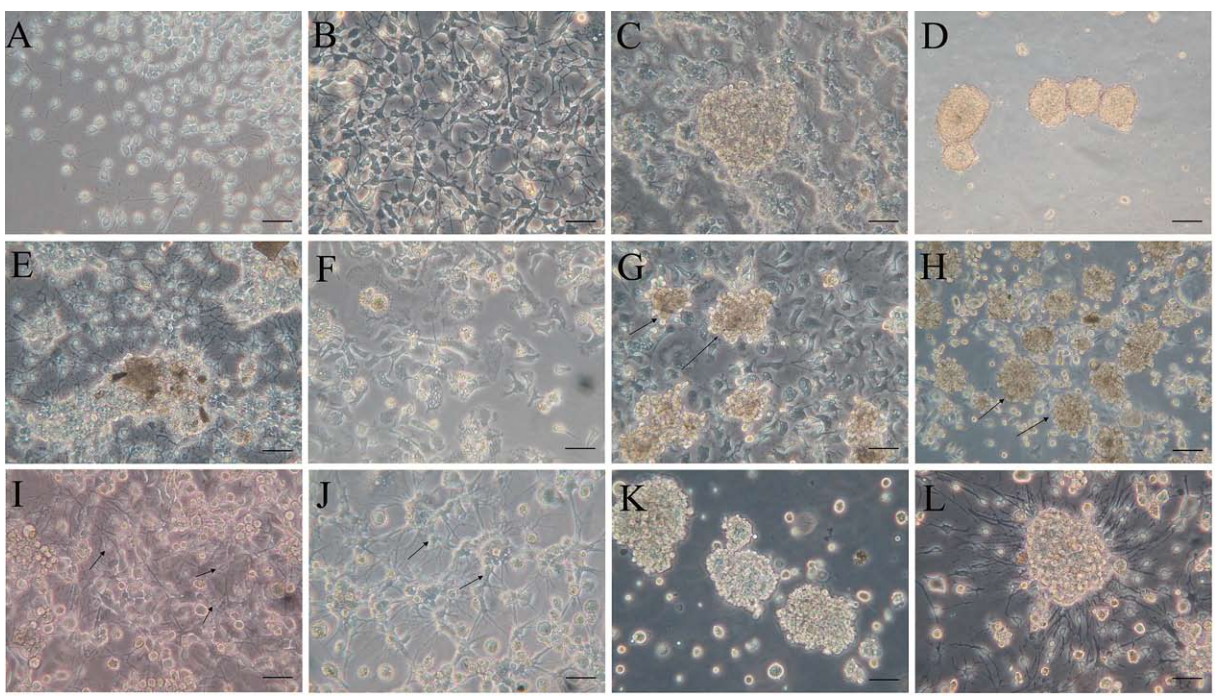
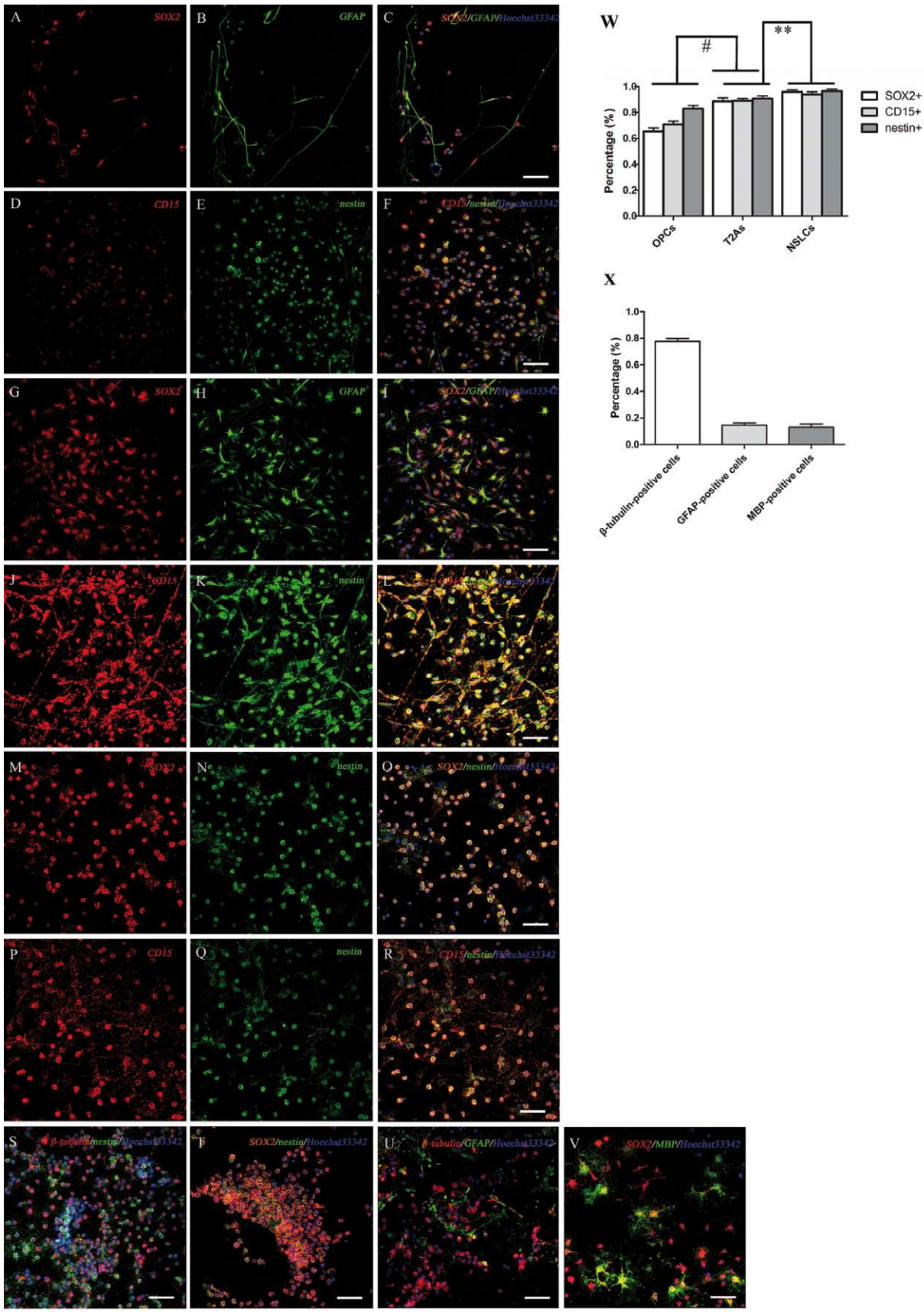


Fig. 1. Phase contrast micrographs of OPCs and MG lineage cells during their dedifferentiation process. (A) Purified OPCs grown in PDGF (10 ng/ml) and bFGF (20 ng/ml) for 5 days showed typical bipolar or tripolar morphology; (B) OPCs were induced into T2As by adding 15% FBS for 3 days without bFGF; (C) Differentiated T2As were converted to NSLCs in the presence of bFGF for a further 5 days without FBS and PDGF; (D), (E), (K), (L) Neurospheres derived from reprogrammed OPCs (D, E) or MGs (K, L) had the capacity to self-renew in neural basal media (neurobasal-A with 2% B-27 supplement and 1% N2 supplement, NBM) and growth factors (10 ng/ml bFGF and EGF), and finally differentiated into the three primary CNS phenotypes in NBM without growth factors containing 1%FBS. (F) Purified MGs grown in 10% FBS for 12 h displayed typical macrophage-like morphological properties; (G) The enlarged microglioblasts became very proliferative and sometimes formed aggregates (arrowheads) after 3-day culture in 10% FBS; (H) Microglioblasts were dedifferentiated into promicroglioblasts in 70% FBS for 2 days and formed more aggregates (arrowheads); (I), (J) Promicroglioblasts turned into neurons (arrowheads) 24 h (I) or 4d (J) after transferred into E2 medium. Scale bars are: 100 μm (D and H), 50 μm (other pictures).


A SOX2
B GFAP
C SOX2/GFAP/Hoechst33342
D CD15
E nestin
F CD15/nestin/Hoechst33342
G SOX2
H GFAP
I SOX2/GFAP/Hoechst33342
J CD15
K nestin
L CD15/nestin/Hoechst33342
M SOX2
N nestin
O SOX2/nestin/Hoechst33342
P CD15
Q nestin
R CD15/nestin/Hoechst33342
S β-tubulin/nestin/Hoechst33342
T SOX2/nestin/Hoechst33342
U β-tubulin/GFAP/Hoechst33342
V SOX2/MBP/Hoechst33342
W
#
**
Percentage (%)
1.0
0.8
0.6
0.4
0.2
0.0
OPCs
T2As
NSLCs
SOX2+
CD15+
nestin+
X
Percentage (%)
1.0
0.8
0.6
0.4
0.2
0.0
β-tubulin-positive cells
GFAP-positive cells
MBP-positive cells

mg/ml of glucose, 5 μg/ml insulin, 5 nM sodium selenite, 5 μg/ml transferrin Roche) with 0.2 mg/ml bovine serum albumin (Sigma) for 3~5 days. When microglia-derived de-differentiated aggregates (referred as promicroglioblasts) formed and detached from dishes, these aggregates were transferred into NBM containing 1% FBS to induce their differentiation.

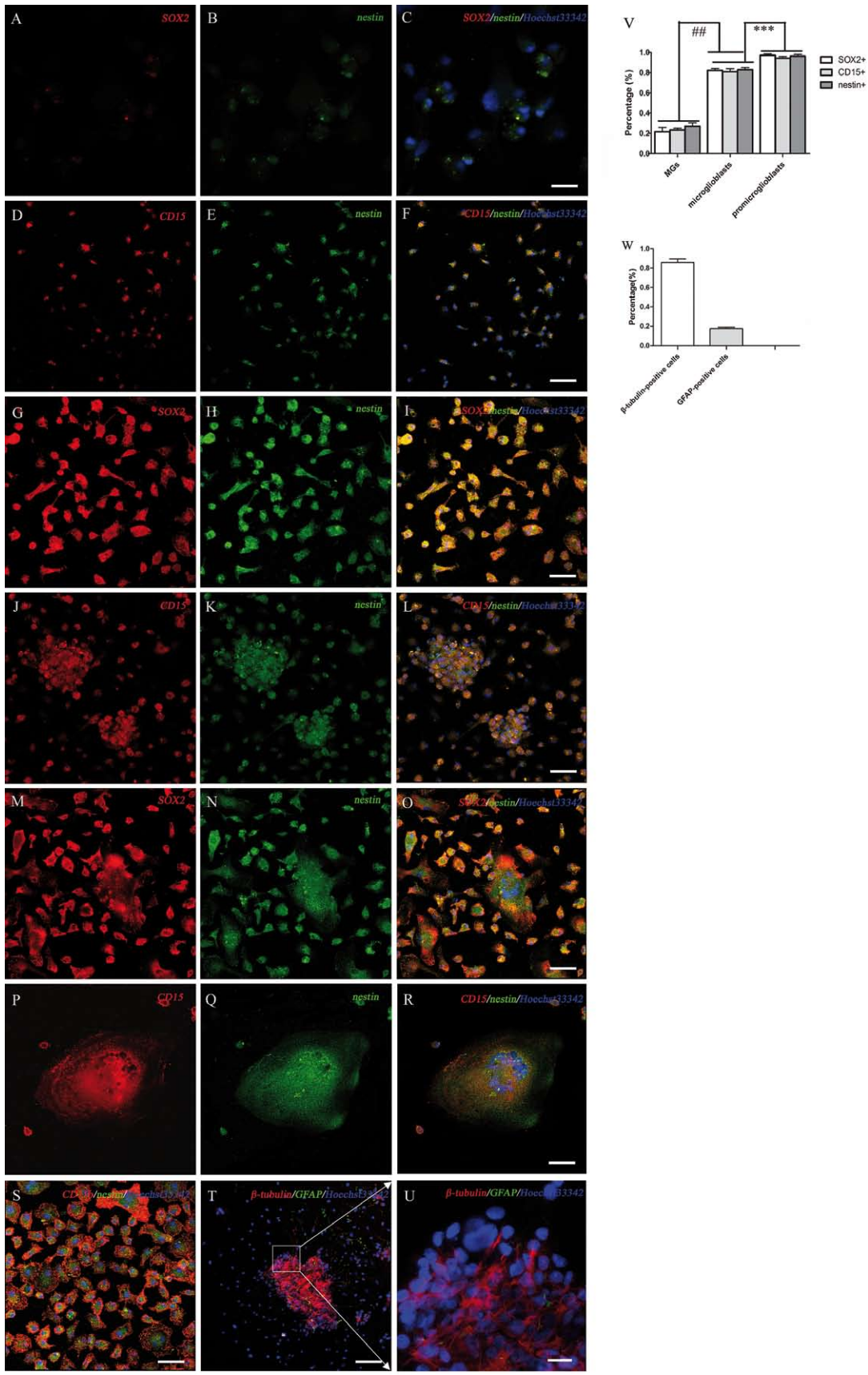
Immunocytochemical detection

Before being immunostained, cells cultured on PLL-coated coverslips were fixed with 4% paraformaldehyde in PBS (0.01 M, pH 7.4) for 10 min at room temperature followed by several rinses in PBS, and then blocked with 5% normal goat serum (NGS, Sigma) diluted in PBS (for cell surface markers) or with 5% NGS diluted in 0.1% Triton-X-100-PBS (for intracellular antigens) for 30 min, respectively. Cells were then incubated overnight at 4°C with primary antibodies. The following antibody markers were used to identify neural progenitors: monoclonal mouse anti-SOX2 (dilution 1:500, Millipore), monoclonal mouse anti-CD15 (dilution 1:200, Abcam), polyclonal rabbit anti-nestin (dilution 1:200, Abcam). Polyclonal rabbit anti-GFAP (dilution 1:1 000, Abcam) was used as a marker to identify oligodendrocyte lineage cells, monoclonal mouse anti-CD11b (dilution 1:100, Serotec) for MG lineage cells. To identify neuroectodermal phenotypes of differentiated cells from NSLCs or promicroglioblasts, the following primary antibodies were used: monoclonal mouse anti-β-tubulin (dilution 1:500, Sigma), polyclonal rabbit anti-GFAP and polyclonal rabbit anti-MBP (dilution 1:400, Millipore). To detect BMP2 expression level in above glial lineage cells, the related primary antibody was used: polyclonal rabbit anti-BMP2 (dilution 1:200, Abcam). After rinsing off the primary antibody, the coverslips were incubated with fluorescently labeled secondary antibodies (Dylight™ 488-conjugated goat anti-rabbit IgG and Dylight™ 649-conjugated goat anti-mouse IgG; dilution 1:400, KPL) for 1 h. Hoechst 33342 (dilution 1:100, Sigma) was used to stain the nucleus for 30 min at 37°C. Immunopositive cells were observed using a Leica TCS SP2 confocal laser scanning microscope.

Quantification of cells positive for SOX2, CD15, nestin and BMP2

Frequency of SOX2- (or CD15-, or nestin-, or BMP2-) positive cells were determined by taking a digital overview image of each Hoechst33342-stained coverslip at a low power magnification (10×2). In each of these images 4 areas in the region of the highest density of Hoechst33342-stained cells were manually marked, using image analysis software (Image Pro Plus, vs. 5.01, Media Cybernetics, Silver Spring, MD). Next, these selected areas were retraced at high power (10×20) magnification and in each set of SOX2, CD15, nestin and BMP2-stained coverslips digital images were captured of these spots. The numbers of positive cells in interrelated images were counted using the "manual tag" option of Image Pro Plus. By assigning total number of Hoechst33342-stained cells per group as 100%, SOX2- (or CD15-, or nestin-, or BMP2-) positive cells were calculated, normalized and expressed as mean ± SEM (n=3).

Fig. 2. Immunocytochemical analyses of OPCs reprogrammed by reactivation of SOX2 associated signaling pathway. Purified OPCs were cultured in PDGF (10 ng/ml) and bFGF (10 ng/ml) for 5 days (A–F), in 15% FBS and PDGF for 3 days to differentiate into T2As (G–L), and then in bFGF (10 ng/ml) for 5 days to revert to NSLCs (M–S). Sometimes these NSLCs formed free-floating oligospheres (T), keeping self-renewal and pluripotency properties (U, V). Immunostaining showed that most of the GFAP$^+$-T2As displayed were SOX2$^+$ (G), CD15$^+$ (J) and nestin$^+$ (K) whereas GFAP$^+$-OPCs exhibiting less immunoreactivity of these NSC-characteristic molecules (SOX2, A; CD15, D; nestin, E). During the conversion of OPCs to NSLCs, most cell body became increasing spherical and retractile, exhibiting a typical neuronal appearance as well as showing a strong upregulation of SOX2 (M), CD15 (P) and nestin (N, Q). Neurospheres formed by these β-tubulin$^+$ -NSLCs (S), displaying strong SOX2 and nestin (T) immunoreactivity. On 5 days after induction by NBM and 1% FBS, three phenotypes of cell (neuron, astrocyte or oligodendrocyte) were present in differentiated neurospheres from NSLCs (β-tubulin/GFAP, U; MBP, V). Cells were counterstained with Hoechst 33342 (blue), a nuclear dye. Scale bars are 50 μm. The statistical graphs showed quantification of SOX2- (A, G, M), CD15- (D, J, P) and nestin- (E, K, N, Q) positive cells (expressed as a percentage of Hoechst33342-positive cells) (W), and the phenotype analysis of differentiated neurospheres from NSLCs under differentiation condition (X). Data are given as Mean ± SD, n=3. $^{\#}P$<0.01 compared with OPCs or $^{**}P$<0.05 compared with T2As.


A SOX2
B nestin
C SOX2/nestin/Hoechst33342
D CD15
E nestin
F CD15/nestin/Hoechst33342
G SOX2
H nestin
I SOX2/nestin/Hoechst33342
J CD15
K nestin
L CD15/nestin/Hoechst33342
M SOX2
N nestin
O SOX2/nestin/Hoechst33342
P CD15
Q nestin
R CD15/nestin/Hoechst33342
S CD15/nestin/Hoechst33342
T β-tubulin/GFAP/Hoechst33342
U β-tubulin/GFAP/Hoechst33342
V
##
***
Percentage (%)
SOX2+
CD15+
nestin+
MGs
microglioblasts
promicroglioblasts
W
Percentage(%)
β-tubulin-positive cells
GFAP-positive cells

Western blot analysis

Proteins from the harvested cells were extracted and homogenized in a lysate buffer (1 M Tris-HCL pH 7.5, 1% TritonX-100, 1% Nonidet P-40, 10% SDS, 0.5% sodium deoxycholate, 0.5 M EDTA, 10 μg/ml leupeptin, 10μg/ml aprotinin, and 1 mM PMSF), then centrifuged at 12 000 g for 8 min at 4°C. The supernatant was collected and stored at −80°C. Protein concentrations were determined with a BCA protein assay kit (TIANGEN). Samples (40 μg of total protein) were boiled in a loading buffer (recipe or resource), and then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine diflouride filter (PVDF) membranes (Millipore). The membranes were blocked with 5% skimmed milk in TBST. After 2 h at room temperature, the membrane was washed with TBST and incubated overnight at 4°C with the following primary antibodies: monoclonal mouse anti-SOX2 (dilution 1:500, Millipore), polyclonal rabbit anti-BMP2 (dilution 1:800, Abcam) and β-actin (anti-mouse, 1:1 000, Abcam). After being rinsed with TBST, goat-anti-rabbit or goat-anti-mouse IgG conjugated to horseradish peroxidase (1:5 000, Southern-Biotech) was added for additional 2 h and the bolts were visualized using an enhanced chemiluminescence system (ECL, CST). Densitometric scanning of the protein bands was performed using the ImageJ software provided by NIH.

Statistical Analysis

There were at least three replicates for each treatment. All data are given in relative values and expressed as means ± SD. Statistical analysis of the data was performed using one-way ANOVA with GraphPad Prism software. P value <0.05 was considered as statistically significant.

RESULTS

NSLCs proliferation and maintenance depends on SOX2 when OPCs convert to T2As and NSLCs

Purified OPCs were isolated from newborn rat cortex according to their difference in adhesive potential, and then expanded for 5 days in PDGF (Fig. 1A). Here we showed that exposure to PDGF plus 15% FBS induced T2As differentiation in a 3 day culture (Fig. 1B), while culturing in bFGF caused many purified OPCs to revert to a state that resembled that of multipotential CNS stem cells (Fig. 1C). These NSLCs can self-renew and produce primary CNS phenotypes when transferred into NBM-containing serum (Fig. 1D, 1E). Immunocytochemical staining showed that the conversion of OPCs to T2As and NSLCs markedly elevated the expression levels of SOX2 or NSC markers such as CD15 and nestin (Fig. 2A–2R), and there was significance difference in the levels of these markers when compared to previous adjacent stage groups (Fig. 2W). These $SOX2^+$/$nestin^+$ NSLCs became increasing spherical and retractile, exhibiting a typical neuronal appearance and β-tubulin immunoreactivity (Fig. 2S, 2T). After induction with NBM and 1% FBS for 5 days, NSLCs-derived spheres on coated coverslips differentiated into the three primary CNS phenotypes [β-$tubulin^+$ neurons (72.7±12.6%, n=3), $GFAP^+$ astrocytes (15.5±3.2%, n=3) and MBP^+ oligodendrocytes (11.1±2.9%, n=3)] (Fig. 2U, 2V, 2X).

MG lineage cells acquire a multipotent property to give rise to neuroectodermal cells by progressive activation of SOX2 expression

Purified microglial cells were reprogrammed to multipotential stem cells through two-step culture in 10% and 70% serum-supplemented media for 5 days.

Fig. 3. Immunocytochemical characteristics of MGs as multipotential stem cells *in vitro*. Dissociated MGs were seeded onto PLL-coated coverslips in DMEM plus 10% FBS for 12 hours (A–F) and 3 days (G–L) successively, followed by 2-day culture in DMEM containing 70% serum (M–R) through which MGs acquire a multipotent property to give rise to neuroectodermal cells (T, U). Immunostaining of MG lineage cells demonstrated that SOX2 (A, G, M), CD15 (D, J, P) and nestin (B, E, H, K, N, Q) were significantly upregulated during de-differentiation process. Then these $CD11b^+$/$nestin^+$- promicroglioblasts aggregate (S), could generate β-$tubulin^+$ neurons and $GFAP^+$ astrocytes after transferred into NBM plus 1% FBS (T, U). Scale bars are 100 μm (T), 15 μm (U); 50 μm (other pictures). The statistical graphs showed quantification of SOX2- (A, G, M), CD15- (D, J, P) and nestin- (B, E, H, K, N, Q) positive cells (expressed as a percentage of Hoechst33342-positive cells) (V), and the phenotype analysis of differentiated neurospheres from promicroglioblasts under differentiation condition (W). Data are given as Mean ± SD, n=3. $^{\#\#}P$<0.01 compared with MGs or $^{***}P$<0.05 compared with microglioblasts.

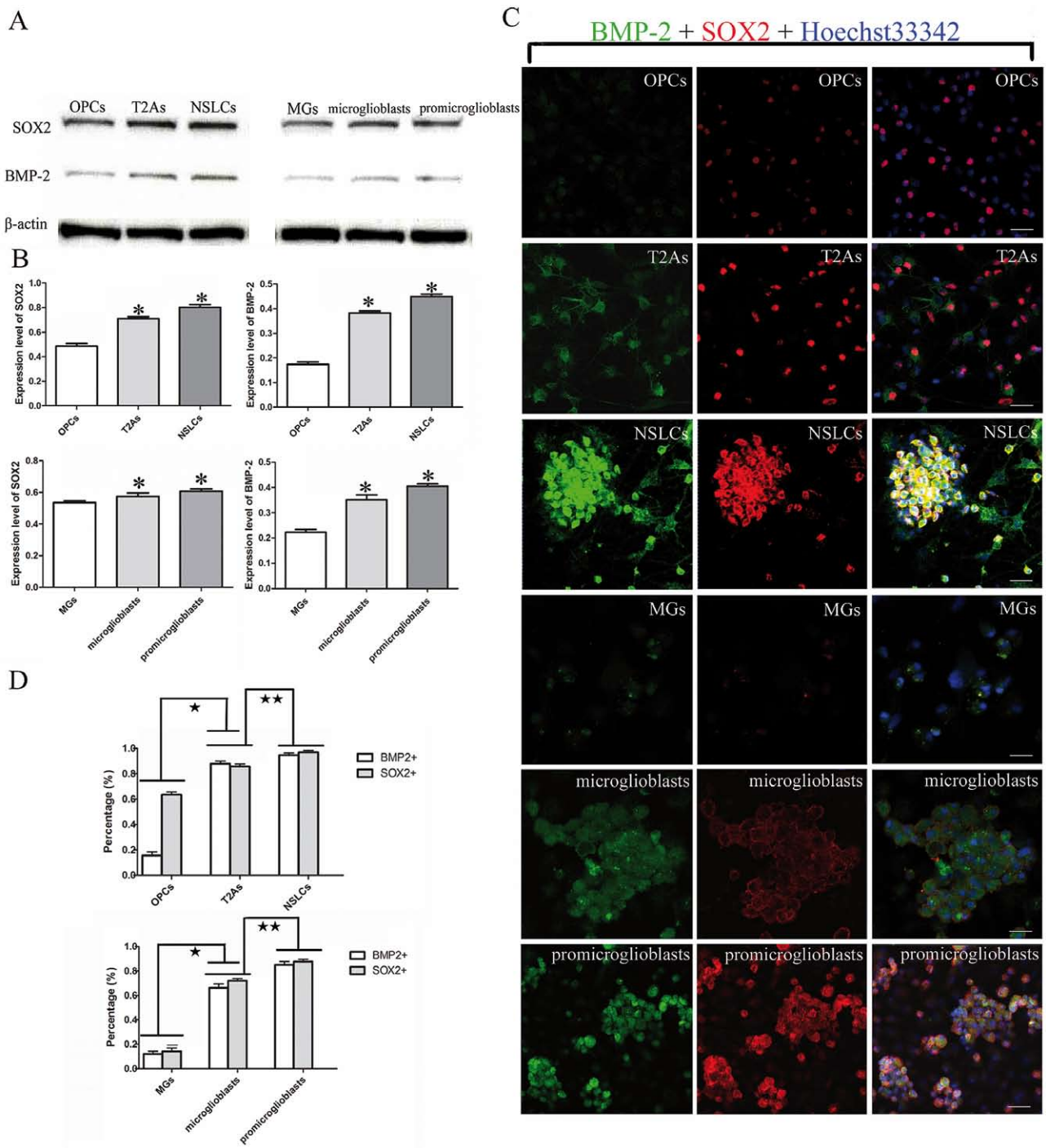


Fig. 4. Western blot and double-label immunofluorescence analyses of SOX2 and BMP-2 expression in reprogrammed oligodendrocyte lineage cells and microglia lineage cells. (A) SOX2 protein levels were found progressively increased. Similarly, expression of BMP-2 protein was also observed the enhancing tendency same as SOX2 protein. β-actin was used as the internal control. (B) Quantitative analysis of western blotting results. Each bar represents the mean ± SD (n=3). Differences *versus* OPCs or MGs (*P<0.05). (C) Confocal analyses in each glial group during their de-differentiation showed the same upregulation tendency of BMP-2 and SOX2 protein in these glial lineage cells (green for BMP-2, red for SOX2, blue for Hoechst33342, scale bar is 20 μm). (D) The statistical graphs showed quantification of BMP2- and SOX2-positive cells (expressed as a percentage of Hoechst33342-positive cells) according to confocal data. Data are given as Mean ± SD, n=3. *P<0.01 or **P<0.05 was used as criterion for the significance of difference.

After 3 days culture in 10% FBS, resting ramified MGs showed a highly proliferative and enlarged-morphology (termed microglioblasts) (Fig. 1F, 1G). During the following 2 days culture in 70% FBS, microglioblasts rapidly proliferated and became round. These cells were termed promicroglioblasts which often form many aggregates (Fig. 1H). When promicroglioblasts were maintained in E2 medium, they rapidly turned into neurons (Fig. 1I, 1J). These promicroglioblasts-derived aggregates often detached from dishes or coverslips, and then generated neuronal or glial-like cells after being transferred into NBM-containing 1%FBS (Fig. 1K, 1L). Confocal microscopy analysis of reprogrammed microglia lineage cells showed clearly detectable levels of SOX2 coexpressed with typical stem cell markers (CD15, nestin) similar to that of reprogrammed oligodendrocyte lineage cells (Fig. 3A–3R), and there was significance difference in the levels of these markers when compared to previous adjacent stage groups (Fig. 3V). These $CD11b^+$-promicroglioblasts aggregates could generate β-$tubulin^+$ neurons (82.3±4.5%, n=3) and $GFAP^+$ astrocytes (17.5±2.6%, n=3) after transferred into NBM plus 1%FBS (Fig. 3T, 3U, 3W).

Upregulation of SOX2 protein and BMP-2 protein in reprogrammed OPCs and MGs

Western blot and double-label immunofluorescence analysis were carried out to evaluate the SOX2 and BMP-2 protein levels in reprogrammed oligodendrocyte lineage cells and microglia lineage cells. Consistent with the confocal data, western blot results showed that the expression levels of SOX2 and BMP-2 proteins were both progressively elevated during the dedifferentiation process of these glial populations (Fig. 4A, 4C). Optical densitometry analysis and quantification of positive cells further indicated that BMP signaling might be activated when exposed to defined serum-containing medium, and meanwhile key transcription factor SOX2 might be also upregulated (Fig. 4B, 4D).

DISCUSSION

Direct reprogramming of somatic cells offers new avenues toward the restoration of damaged or degenerating tissues (Nicholas and Kriegstein 2010). Similarly, direct reprogramming of endogenous glia residing in the damaged brain tissue, may allow for the replacement of degenerated neurons without the need of transplantation and its associated complications (Robel et al. 2011). Previous studies have shown that early postnatal cortical astroglia in culture can be reprogrammed to adopt a neuronal phenotype after forced expression of some transcription factors such as Pax6, neurogenin-2 and Mash1 (Heins et al. 2002, Berninger et al. 2007). Moreover, there is an ever-growing list of evidences that NG2-positive cells including $NG2^+$ OPCs and $NG2^+$ MGs actually possess an intrinsic neurogenic potential and they are capable of neuronal differentiation in response to environmental stimuli (Yokoyama et al. 2006, Sypecka et al. 2013).

In this study, SOX2 upregulation was found during the generation of β-$tubulin^+$ and $GFAP^+$ cells from reprogrammed MGs, similar to that in OPCs. Importantly, appropriate concentration of serum treatment seems to be a necessary first step in reprogramming OPCs to become NSLCs or MGs to become promicroglioblasts, which suggests that lineage reprogramming of OPCs and MGs are controlled by the same signalling pathway associated with SOX2 reactivation. This notion is based on the following results: (1) SOX2 expression level was upregulated in FBS-treated OPCs and MGs; (2) During the progressive activation of SOX2, OPCs and MGs transgress their lineages and convert to multipotential neurospheres; (3) If OPCs or MGs are freshly purified from primary mixed cortical glia and cultured directly in bFGF or E2 medium without an initial exposure to serum-containing medium, they do not generate neuroectodermal cells eventually (data not shown). Previous studies indicated that serum enhances expression of inhibitor of DNA binding (Id) genes, whose products act as inhibitors of cellular differentiation. Microglia-derived dedifferentiated cells through two-step culture in 10 and 70% serum-supplemented media were shown to express early OPCs marker PDGFα, A2B5 and O4, which suggested that MGs and OPCs belong to the same lineage (Yokoyama et al. 2004).

Interestingly, we also observed that activation of BMP signaling in response to the lineage reprogramming of these glial populations and the change in BMP-2 expression level is concident with that of SOX2 expression level during the reprogramming process. Similar to this phenomenon, recent reports also found that when exposed to differentiation medium containing fetal bovine serum *in vitro*, BMP2 and BMP4 were highly expressed in both types-1 and type-2 astrocytes

differentiated from NSCs, while no detectable expression was observed in NSCs, neurons and oligodendrocytes (Hu et al. 2012). BMPs, a subgroup of the TGF-β superfamily, play pivotal roles in mediating the cell fate choices in neural stem cells (Mehler et al. 2000). Alternatively, it is well-established that BMPs mediate the switch of neural progenitor cells from neurogenesis to astrocytogenesis via the upregulation of bHLH transcription factor Id2 (Nakashima et al. 2001). Notably, Id gene is involved not only in the regulation of differentiation but also in transdifferentiation (Kondo et al. 2004). Recent studies also reported that activation of BMP signaling *via* Smad (distinct downstream molecules of BMP) and Id2 proteins is one of the main molecular pathways involved in the generation of microglia-derived MAP2-positive and GFAP-positive cells (Niidome et al. 2008). The reprogramming from OPCs to NSLCs is a multistep process, with BMP treatment and transition through the T2As being a necessary intermediate for responsiveness to the bFGF signal. A key event is the BMP-dependent re-expression of the SoxB1 gene Sox2 in the type-2 astrocyte, as there is increasing evidence for an essential role of SoxB1genes in the maintenance of neural stem cells (Zappone et al. 2000, Bylund et al. 2003, Graham et al. 2003). Sox2 expression is repressed in OPCs, with H3 histones at the enhancer region being in an inactive form, H3K9 being methylated and unacetylated and H3K4 being unmethylated, while in the T2As, the histone H3 modification pattern is switched to an 'active' pattern. The mechanism by which BMP2 initiates this reprogramming is not known; however, it does involve chromatin remodelling by recruitment to the Sox2 promoter/enhancer region of the Brca1- and Brm-containing SWI/SNF chromatin-remodelling complexes to reactivate the locus (Kondo and Raff 2004).

On the basis of these reports in combination with our findings, it is more likely that lineage reprogramming of OPCs and MGs were both mediated by BMP pathway, which concomitantly induce Smad-independent pathways including Erk, SAPK/JNK, or p38 MAPK pathways. Activated Smads upregulate master transcription factors such as SOX2 *via* chromatin remodelling to control glial cell fate transitions. To determine the correlation between BMP2 and SOX2 and their epigenetic control of these glial reprogrammed process, we should first investigate whether the depletion of SOX2 or BMP2 by RNAi would influence the proliferation and/or differentiation of these reprogrammed MGs or OPCs. Then we further detect the associated expression changes of BMP2 (or SOX2), Id2, Smad and Brca1 by using chromatin immunoprecipitation (ChIP) analysis after the knock-down of SOX2 (or BMP2) by RNAi. Further studies are needed to clarify these issues.

CONCLUSION

The present study demonstrates that purified OPCs and MGs can be reprogrammed *in vitro* and reacquire some of the hallmarks of neural stem cells or progenitors, through progressive activation of BMP signaling accompanied by SOX2 upregulation. Dissecting the regulatory pathways involved in these processes may help to gain control over glial cell fate decisions. These results also suggest a valuable alternative way for substitution of the neurons lost in neurological diseases.

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REFERENCES

Alvarez-Buylla A, García-Verdugo JM, Tramontin AD (2001) A unified hypothesis on the lineage of neural stem cells. Nat Rev Neurosci 2: 287–293.

Armstrong RC (1998) Isolation and characterization of immature oligodendrocyte lineage cells. Methods 16: 282–292.

Berninger B, Costa MR, Koch U, Schroeder T, Sutor B, Grothe B, Götz M (2007) Functional properties of neurons derived from in vitro reprogrammed postnatal astroglia. J Neurosci 27: 8654–8664.

Bylund M, Andersson E, Novitch BG, Muhr J (2003) Vertebrate neurogenesis is counteracted by Sox1–3 activity. Nat Neurosci 6:1162–1168.

Cavallaro M, Mariani J, Lancini C, Latorre E, Caccia R, Gullo F, Valotta M, DeBiasi S, Spinardi L, Ronchi A, Wanke E, Brunelli S, Favaro R, Ottolenghi S, Nicolis SK (2008) Impaired generation of mature neurons by neural stem cells from hypomorphic SOX2 mutants. Development 135: 541–557.

Doetsch F (2003) The glial identity of neural stem cells. Nat Neurosci 6: 1127–1134.

Graham V, Khudyakov J, Ellis P, Pevny L (2003) SOX2 functions to maintain neural progenitor identity. Neuron 39: 749–765.

Heins N, Malatesta P, Cecconi F, Nakafuku M, Tucker KL, Hack MA, Chapouton P, Barde YA, Götz M (2002) Glial cells generate neurons: the role of the transcription factor Pax6. Nat Neurosci 5: 308–315.

Hu JG, Zhang YX, Qi Q, Wang R, Shen L, Zhang C, Xi J, Zhou JS, Lu HZ (2012) Expression of BMP-2 and BMP-4 proteins by type-1 and type-2 astrocytes induced from neural stem cells under different differentiation conditions. Acta Neurobiol Exp (Wars) 72: 95–101.

Jaenisch R, Young R (2008) Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell 132: 567–582.

Kondo T, Raff M (2000) Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. Science 289: 1754–1757.

Kondo T, Raff M (2004) Chromatin remodeling and histone modification in the conversion of oligodendrocyte precursors to neural stem cells. Genes Dev 18: 2963–2972.

Kondo M, Cubillo E, Tobiume K, Shirakihara T, Fukuda N, Suzuki H, Shimizu K, Takehara K, Cano A, Saitoh M, Miyazono K (2004) A role for Id in the regulation of TGF-beta-induced epithelial-mesenchymal transdifferentiation. Cell Death Differ 11: 1092–1101.

Kriegstein A, and Alvarez-Buylla A (2009) The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32: 149–184.

Lyssiotis CA, Walker J, Wu C, Kondo T, Schultz PG, Wu X (2007) Inhibition of histone deacetylase activity induces developmental plasticity in oligodendrocyte precursor cells. Proc Natl Acad Sci U S A 104: 14982–14987.

Ma DK, Ming GL, Song H (2005) Glial influences on neural stem cell development: cellular niches for adult neurogenesis. Curr Opin Neurobiol 15: 514–520.

Mehler MF, Mabie PC, Zhu G, Gokhan S, Kessler JA (2000) Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineage fate. Dev Neurosci 22: 74–85.

Morrens J, Van-Den-Broeck W, Kempermann G (2012) Glial cells in adult neurogenesis. Glia 60: 159–174.

Nakashima K, Takizawa T, Ochiai W, Yanagisawa M, Hisatsune T, Nakafuku M, Miyazono K, Kishimoto T, Kageyama R, Taga T (2001) BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis, Proc Natl Acad Sci U S A 98: 5868–5873.

Nicholas CR, Kriegstein AR (2010) Regenerative medicine: Cell reprogramming gets direct. Nature 463: 1031–1032.

Niidome T, Matsuda S, Nonaka H, Akaike A, Kihara T, Sugimoto H (2008) A molecular pathway involved in the generation of microtubule-associated protein 2-positive cells from microglia. Biochem Biophys Res Commun 370: 184–188.

Rex M, Orme A, Uwanogho D, Tointon K, Wigmore PM, Sharpe PT, Scotting PJ (1997) Dynamic expression of chicken Sox2 and Sox3 genes in ectoderm induced to form neural tissue. Dev Dyn 209: 323–332.

Robel S, Berninger B, Götz M (2011) The stem cell potential of glia: lessons from reactive gliosis. Nat Rev Neurosci 12: 88–104.

Sypecka J, Sarnowska A, Gadomska-Szabłowska I, Lukomska B, Domańska-Janik K (2013) Differentiation of glia-committed NG2 cells:the role of factors released from hippocampus and spinal cord. Acta Neurobiol Exp (Wars) 73: 116–129.

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663–676.

Yokoyama A, Yang L, Itoh S, Mori K, Tanaka J (2004) Microglia, a potential source of neurons, astrocytes, and oligodendrocytes. Glia 45: 96–104.

Yokoyama A, Sakamoto A, Kameda K, Imai Y, Tanaka J (2006) NG2 proteoglycan- expressing microglia as multipotent neural progenitors in normal and pathologic brains. Glia 53: 754–768.

Zappone MV, Galli R, Catena R, Meani N, De Biasi S, Mattei E, Tiveron C, Vescovi AL, Lovell-Badge R, Ottolenghi S, Nicolis SK (2000) Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127: 2367–2382.