Smad Interacting Protein-1 is Essential for Oligodendrocyte Differentiation

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Abstract

Precursor/stem cell substitutive therapy to promote remyelination is an ideal strategy for central nervous system demyelinating diseases such as spinal cord injury (SCI). However, the microenvironment of the injured area is not conducive to the survival, differentiation, and functions of the transplanted cells. Identifying and regulating the key inhibitory factors might be an important target for the treatment of demyelinating diseases. Smad interacting protein-1 (Sip1) is a transcription factor that binds to phosphorylated R-Smad in the nucleus, which promotes remyelination by inducing the differentiation of oligodendrocytes. In this study, we show that the expression of Sip1 is up-regulated and peaks by 1 day and then returns to normal levels 7 days after SCI. Most Sip1 positive cells were oligodendrocytes. *In vitro*, Sip1 was weakly expressed in the cytoplasm of oligodendrocyte progenitor cells (OPCs), significantly up-regulated in immature oligodendrocytes, and showed significant nuclear transposition. In contrast, Sip1 expression levels in mature oligodendrocytes decreased to levels similar to those in OPCs. The RNA interference of Sip1 in OPCs reduced the level of myelin basic protein (a mature oligodendrocyte marker protein, MBP) and pERK1/2 (a key molecule of the ERK/MAPK pathway) in oligodendrocytes. These findings suggest that Sip1 is essential for oligodendrocyte differentiation and might affect the ERK/MAPK signal pathway. The results provide a theoretical basis for the treatment of demyelinating lesions such as spinal cord injury by regulating Sip1 expression in oligodendrocytes.

1. Introduction

Oligodendrocytes are unique cells that form myelin in the central nervous system. However, the degeneration and loss of oligodendrocytes lead to demyelinating disease in the central nervous system resulting in neurological dysfunction [1, 2]. Promoting remyelination and neuroprotection is an ideal strategy for the treatment of demyelinating diseases. Oligodendrocytes are separated from the cell cycle and do not possess myelin regeneration ability; therefore, remyelination of the central nervous system in adult vertebrates must be accomplished by newly-generated oligodendrocytes differentiated from stem cells or precursor cells OPCs [3, 4], glial progenitor cells, are ideal for the treatment of demyelinating diseases [5] such as SCI.

Multiple inhibitors disturb remyelination in the microenvironment after SCI [6, 7], which makes it difficult to achieve the expected effect of remyelination after the recruitment of OPCs. Therefore, discovering and regulating key inhibitors in the local microenvironment of injury is an important therapeutic target for SCI.

Sip1, an important transcription factor in neurodevelopment, regulates the expression of genes in all aspects of neurogenesis [8]. Sip1 is encoded by the Zeb2 gene that belongs to the small Zeb protein family. After binding to the MH2 domain of phosphorylated R-Smads that is transferred into the nucleus, it acts on target genes that are involved in the regulation of transforming growth factor (TGF-β) and bone morphogenetic protein (BMP) signaling [9]. Studies showed that the expressions of several BMPs in the microenvironment were significantly up-regulated in SCI [10]. BMPs are members of the TGF-β superfamily and play an important role in vertebrate neurodevelopment and nerve damage repair [11–13].

BMPs mediate their biological effects through a signaling pathway mediated by the Smad protein family in the cytoplasm. It was reported that Sip1 antagonizes phosphorylated Smad1 in the nucleus and promotes OPC differentiation [14]. However, it is unclear whether Sip1 is involved in the processes of injury and repair in SCI and whether this involves oligodendrocytes or other cell types.

In this study, we evaluated Sip1 expression in animals to determine its potential role in SCI. Sip1 was detected during OPC differentiation *in vitro*. In addition, RNA interference of Sip1 in OPCs was used to observe the role of Sip1 in the differentiation and maturation of oligodendrocytes and whether ERK/MAPK signaling is involved. Our data suggest there is an intrinsic relationship between Sip1, oligodendrocytes, and SCI. Future studies should investigate the regulation of Sip1 expression in oligodendrocytes as a treatment for SCI.

2. Materials And Methods

2.1 Animals

All experimental procedures and protocols were performed by the Central South University Ethics Committee and followed the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (eighth edition). Sprague-Dawley rats were purchased from Slac Corporation (Changsha, China).

2.2 Spinal Cord Injury

Adult female Sprague-Dawley rats (n=20; 200-220 g; 8-weeks-old) were anesthetized with intraperitoneal injections of 1% Pelltobarbitalum Natricum, shaved, and disinfected with 70% ethanol scrubs between the skin of the neck and hindlimbs for approximately 2 cm. The T8-T11 spinal column was exposed by incising the midline of skin, and bilateral muscles were dissected to make the lamina visible. A complete laminectomy was performed at T9, and rats were clamped using a stereotactic device to stabilize the position of the spine for contusion injury. After impact, the deep and superficial muscle layers and skin were sutured.

2.3 BBB score

Basso, Beattie, and Bresnahan (BBB) scales were used to evaluate the exercise, weight support, and coordination ability of animals after SCI, to evaluate the motor function recovery of rats after SCI. The BBB scoring scale includes 0-21 points. A score of 0 indicates no visible hind limb movement, and 21 indicates a sustainable stable movement of the hind limb. That is, the higher the exercise score of the rat, the stronger the motor function of the rat. This experiment was scored by a double-blind method.

2.4 Cell culture

Primary OPC cultures were prepared from the cerebral cortex of 1-day old SD rats after refrigeration anesthesia. Primary OPCs were isolated by a protocol similar to that described by O'Meara [15] with some modifications. Briefly, the cortices of postnatal day 1 rats were dissected and placed in a Petri dish to

remove the meningeal layer under a microscope. The cortices were diced using ophthalmic scissors, transferred to a 15 ml centrifuge tube, and incubated for 5 min at 37℃ to soften the brain tissue. It was digested with DMEM containing papain (2 μg/ml) for 20 min at 37℃. The centrifuge tube was turned upside-down every 2 min to refloat the sunken tissue. The digestion was stopped by adding an equal volume of DMEM containing 10% fetal bovine serum. Tissues were pipetted up and down until homogenized and then filtered through a 70-μm nylon mesh. This was centrifuged at 1200 rpm for 5 min, then the supernatant was discarded and the cells were suspended. The cells were added to a poly-D-lysine (PDL,0.1 mg/mL) coated T25 flask and cultured in a 37°C tissue culture incubator at 5% CO_2. After 7-9 days of culture, type Ⅱ astrocytes were confluent and the topmost microglia were shaken in a horizontal shaker for 1 hour at 90 rpm, washed with PBS in the cold three times. Then, the media was refreshed with DMEM containing 10% fetal bovine serum and placed in an incubator for 2 hours. OPCs were removed from the astrocyte surface by shaking the flasks on a rotary shaker for 18 hours at 220 rpm. After centrifugation, they were inoculated into culture bottles and the medium was changed to serum-free neurobasal medium supplement 2% B27, with 10 ng/ml PDGF-AA (Sigma-Aldrich) and 20 ng/ml bFGF (Sigma-Aldrich). Cells were passaged using Accutase (Thermo Fisher, USA) when they reached 80-90% confluence. To induce oligodendrocyte differentiation, the cells were continuously cultured in medium with 40 ng/ml T3.

2.5 SiRNA

For transfection with siRNA, OPCs were passaged using Accutase (Thermo Fisher, USA) and plated at 50-60% confluence in 6-well plates. After 24 hours, cells were transfected with siRNA using Lipofectamine 3000 (Invitrogen, Shanghai, China) and the final siRNA concentration was 50 nmol/ml.

2.6 Western blotting

Cells were harvested in ice-cold lysis buffer containing RIPA (BCA kit; Cwbiotech). Protein concentration was determined by BCA kit (Cwbiotech, Beijing, China). The samples were boiled at 100℃ for 7 min in loading buffer and separated by SDS-PAGE. Depending on the molecular weight of the protein of interest, the extractions were performed on 10-12% gels. The proteins were then transferred to NC membranes and incubated with blocking buffer (0.01 M PBS with 0.05% Tween-20, 5% nonfat milk) for 2 hours at room temperature and then incubated with rabbit anti-SIP1 (Abcam, 1:800) or mouse anti-MBP (Abcam,1:800) overnight at 4℃ with gentle rocking. After thorough washing, the membranes were incubated with anti-rabbit lgG HRP (Cwbiotech 1:5000) or anti-mouse lgG HRP (Cwbiotech, 1:5000) for 2 hours at room temperature. After washing, immunostaining was detected by using chemiluminescence ECL reagents (Cwbiotech).

2.7 Immunofluorescence

Cells were fixed in 4% PFA in PBS for 20 min at room temperature, washed 3 times in PBS, and blocked with 1x PBS containing 0.1% Triton X-100 and 5% bovine serum albumin. After 1 hour, cells were incubated with a blocking solution containing primary antibody at 4°C overnight. After PBS washing 3 times, cells were incubated with the appropriate fluorescence-conjugated secondary antibody for 1 hour

at room temperature in the dark. Nuclei were stained with DAPI (Vector, United States). Antibodies used in this assay were as follows: rabbit anti-SIP1(1:200) and mouse anti-MBP (1:500).

2.8 Immunohistochemistry

At different time points post-injury, rats were perfused with 4% PFA in 0.1 M phosphate buffer (PB). Rats were divided into 6 groups: sham operated, 4 h post injury, 16 h post injury, 1 day post injury, 3 days post injury, and 7 days post injury. The spinal cord segments were cryoprotected in 15% sucrose buffer overnight at 4°C and then moved into 30% sucrose buffer overnight at 4°C. Segments were embedded in OCT compound, and 12 series of 20-μm thick sections were picked up on glass slides and quenched with 0.3% H_2O_2. They were blocked with 5% donkey serum in PBS containing 0.1% Triton X-100 for 2 hours at room temperature, then incubated in PBST containing 5% donkey serum, rabbit anti-Sip1 (Abcam, 1:200) overnight at 4°C. After 3 washes, sections were incubated with a secondary antibody for 2 hours at room temperature, and then a third antibody using the same protocol as for the secondary antibody. DAB was added to each section and monitored closely until sections provided an acceptable staining intensity.

2.9 Statistical analysis

All data were described as the mean ± standard deviation (SD). One/two-way analysis of variance (ANOVA) was applied for differences among the groups and the t-test for intra-group differences. The data were analyzed using GraphPad Prism 7 and $P < 0.05$ was set as the threshold of statistical significance.

3. Results

3.1 Expression of Sip1 in the injured spinal cord

The hindlimbs of the rats were paralyzed after SCI. On 1-day post-injury, the BBB score was 0, and there was no hindlimb movement, indicating successful modeling (Fig. 1).

Immunohistochemistry staining was used to compare the expression of Sip1 in the T9 spinal cord at 4h, 16h, 1d, 3d, and 7d after SCI in the sham operation group (Sham), where only the lamina was opened and rats did not suffer a stoke. At our observation time points, Sip1 was expressed in all groups. Increased Sip1-positive cells were observed at 16h, they were significantly increased at 1 dpi and 3 dpi, and peaked at 1-day post-injury; however, this was not statistically significant at 7d compared with the sham operation group (Fig. 2).

Immunofluorescence staining was used to analyze the colocalization of Sip1 and OL in the Sham group at 1 dpi. APC was the antibody most commonly used to label mature oligodendrocytes without labeling myelin. Results showed that most APC-positive cells were stained by Sip1 antibody (Fig. 3).

3.2 Expression of Sip1 during the differentiation of oligodendrocytes

We analyzed the expression of Sip1 and MBP at undifferentiation (0h) and various time points (4h, 8h, 16h, 1d, 2d, 3d, 5d, 7d) during OPC differentiation to oligodendrocyte *in vitro*. MBP is a marker of mature oligodendrocytes, and the appearance of MBP expression indicates mature oligodendrocyte formation[28]. At our observation time points, western blot revealed that the expression of Sip1 was increased compared to that at 0h. Sip1 expression during differentiation gradually increased at 4h-2d and peaked at 2d, and then it was remarkably decreased at 3d, 5d, and 7d. The expression of Sip1 at 7d after differentiation was similar to that at undifferentiation. However, following differentiation, the expression of MBP was not detected until 3d, and then it increased remarkably at 5d and 7d (Fig. 4).

We next examined whether the expressed location of Sip1 in cultured OPCs changed after differentiation. Immunofluorescence staining showed that Sip1 was predominantly located in the cytoplasm at 0h and 4h initially; however, following differentiation, Sip1 was located in the nucleus, and Sip1 was predominantly located in the nucleus after 2 days (Fig. 5).

The results suggest that Sip1 functions at the early stage of oligodendrocyte differentiation, and may be involved in mature oligodendrocytes.

3.3 Effect of interfering with Sip1 expression on oligodendrocyte differentiation

To investigate the relationship between increased Sip1 expression and oligodendrocyte differentiation, we knocked down the expression of Sip1 using siRNA (Fig. 6).

We investigated which sequence was effective by western blotting. The results showed that there was no statistical difference between OPCs transfected with empty vector (Mock) and OPCs transfected with negative control siRNA (NC). The expression of Sip1 in OPCs transfected with #2 sequence siRNA was markedly decreased compared with NC (Fig. 6A, B).

Next, we investigated the effect of Sip1 on OL differentiation, and examined protein levels on day 3 of differentiation. Sip1 and MBP were detected simultaneously and at significantly higher levels compared with undifferentiation (0h). Western blot results showed that Sip1 and MBP protein levels were decreased in Sip1-knockdown OPCs compared with OPCs transfected with negative control siRNA (NC group) (Fig. 6C, D). Immunofluorescence staining showed that the fluorescence intensities of Sip1 and MBP were decreased in Sip1-knockdown OPCs compared with OPCs transfected with negative control siRNA (Fig. 6E).

These results suggest that the knockdown of Sip1 expression interfered with oligodendrocyte differentiation and maturation.

3.4 Effect of interfering with Sip1 expression on ERK/MAPK pathway

The ERK/MAPK pathway is an important way to regulates cell development. It was reported that ERK/MAPK has a positive regulating effect on oligodendrocyte differentiation, and mainly regulates the transition from OPCs to early oligodendrocytes [16, 17]. There is a similar effect between Sip1 and the ERK/MAPK pathway in regulating the differentiation of OPC, so we investigated a potential correlation.

We detected the expression of Sip1 in OPCs and the differentiation of intracellular Sip1 and pERK1/2 after 1 day, which is a key molecule for ERK/MAPK activation, and based on a previous study, we found that ERK/MAPK functions at an early stage of OPC differentiation [17]. These results showed that the expressions of Sip1 and p-ERK1/2 were decreased compared with the negative group and that the difference was significant (Fig. 7). The effect of Sip1 on oligodendrocyte differentiation may be related to the regulation of the ERK/MAPK pathway.

4. Discussion

Precursor/stem cell substitutive therapy to promote remyelination is a well-established effective strategy for the treatment of central nervous system injuries such as SCI. However, lesions are not conducive to the survival, differentiation, and function of transplanted cells. Identifying potential factors that impair cell survival in lesions will be important for the treatment of central nervous system diseases such as SCI.

After SCI, increased levels of proteins such as BMP4 in lesions might inhibit remyelination and repair [12]. After antagonizing BMPs with Noggin, the function of injured spinal cord was partially recovered [18, 19]. However, some experiments also showed that blocking BMP signaling was not conducive for repair [20, 21], whereas the activation of BMP signaling promoted nerve regeneration in the injured spinal cord [22]. These results suggest that the roles of BMPs in injured spinal cord are complex and contradictory. These different consequences may be due to the activation of different downstream target genes of the BMP signaling pathway. However, the above studies investigated the regulation of BMPs (ligand proteins), BMP receptors, and Smad levels in the cytoplasm and no interventions in BMP signaling were performed in the nucleus. Sip1 is a transcription factor that functions as a transcript target DNA in the nucleus and participates in regulating TGF-β signaling including BMP. The overexpression of Sip1 promoted oligodendrocyte differentiation [14]. However, the expression of Sip1 in SCI and the mechanism of promoting the maturation of oligodendrocytes is still unclear.

Our *in vivo* results revealed that the expression of Sip1 was increased in the early stages of SCI and peaked at 1 day. The increased expression of Sip1 was predominantly located in oligodendrocytes of the white matter, and then gradually decreased close to normal levels at 7 days post-injury. Previous studies suggested that mRNA levels of BMPs were up-regulated several hours after central nervous system injury [23]. After SCI, various molecules related to BMP signaling were significantly up-regulated and lasted until 1 month after injury. Oligodendrocytes also responded quickly. Newly-proliferating OPCs were detected in the remaining white matter of the spinal cord at 1 day post-injury, reached a peak at 3 days post-injury, and then gradually decreased [24]. Our results suggest that Sip1 may be involved in the response of

oligodendrocytes to BMPs after SCI. With reference to a previous study [14], we speculated that the up-regulation of Sip1 may be involved in the differentiation of recruited/generated OPCs after SCI.

Next, the specific timepoint where Sip1 participates in oligodendrocyte differentiation was investigated *in vitro*. The development of oligodendrocytes can be divided into three phases, OPCs, immature oligodendrocytes, and mature oligodendrocytes. MBP is a marker of mature oligodendrocytes [25, 26]. Our results showed that Sip1 was weakly expressed in OPCs and predominantly located in the cytoplasm. During the process of differentiation, the Sip1 expression gradually increased, and peaked approximately 2 days post-differentiation. The location of Sip1 transferred from the cytoplasm to the nucleus. At this timepoint, most cells were immature oligodendrocytes and only a small amount of MBP was detected. Then, the expression of Sip1 decreased close to the level of undifferentiated OPCs at 7 days post-differentiation, although Sip1 was in the nucleus. At this timepoint, most cells had differentiated into mature oligodendrocytes.

The above results suggest that Sip1 may play important roles in the early stage of OPC differentiation to oligodendrocytes or when oligodendrocytes respond to injury, but it has limited functions in matured or stable oligodendrocytes. At the same time, Sip1 was increased transiently in the early stage of SCI, which is not completely consistent with the trend of increased BMPs and OPC proliferation, especially the former, which suggests that Sip1 may become exhausted. This might explain new OPC differentiation disorders and remyelination difficulties.

Finally, we interfered with the expression of Sip1 in OPCs to observe the effect on the differentiation and maturation of oligodendrocytes. The results showed MBP levels in the OPCs were significantly decreased after intracellular interference with SIP1 siRNA. This indicated that the differentiation of oligodendrocytes was significantly restricted after the down-regulation of Sip1 levels.

Although Sip1 is indispensable for the differentiation of oligodendrocytes, the targets of Sip1 in the process of oligodendrocyte differentiation are still unclear. The MAPK/ERK signaling pathway is a recognized and classical pathway that regulates cell differentiation [27]. ERK1/2 is the ultimate factor of this pathway [27, 28]. In addition, previous articles reported a strong correlation between ERK activity and OPC differentiation [29, 30]. The number of OPCs differentiated into mature oligodendrocytes was reduced with MAPK/ERK signaling inhibitors. This indicated that the MAPK/ERK pathway plays an important role in OPC differentiation [31–33, 27, 34, 17, 35]. Our experimental results showed that when the expression of Sip1 was decreased, the expression of P-ERK1/2, the final executor of the MAPK/ERK signaling pathway, was also significantly decreased. We speculate that Sip1 may affect OPC differentiation by regulating the expression or activation of ERK, and this specific regulatory relationship and locations need further study.

Declarations

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Contributions

Jufang Huang and Chunling Fan designed the experiments and wrote the manuscript; Ziwei Chen and Yuanmei Wang conducted the in vivo experiments; Xiaobin Fan conducted the ex vivo experiments; Jufang Huang, Chunling Fan and Ziwei Chen contributed to data analysis;

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Consent for Publication All authors consent to publication.

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Not applicable.

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Figures

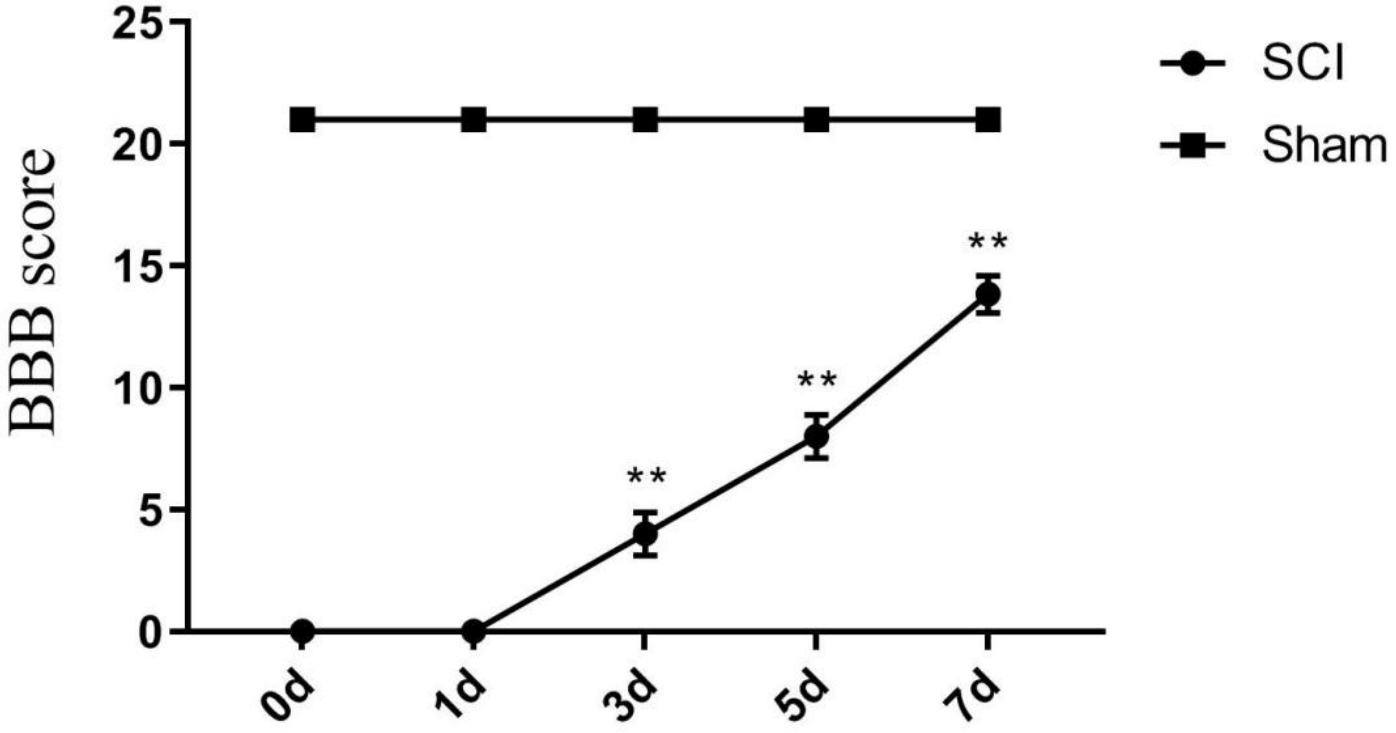

Figure 1

BBB scores BBB scores in the sham operated group and 0 d, 1 d, 3 d, 5 d, and 7 d after SCI. ** $p < 0.01$.

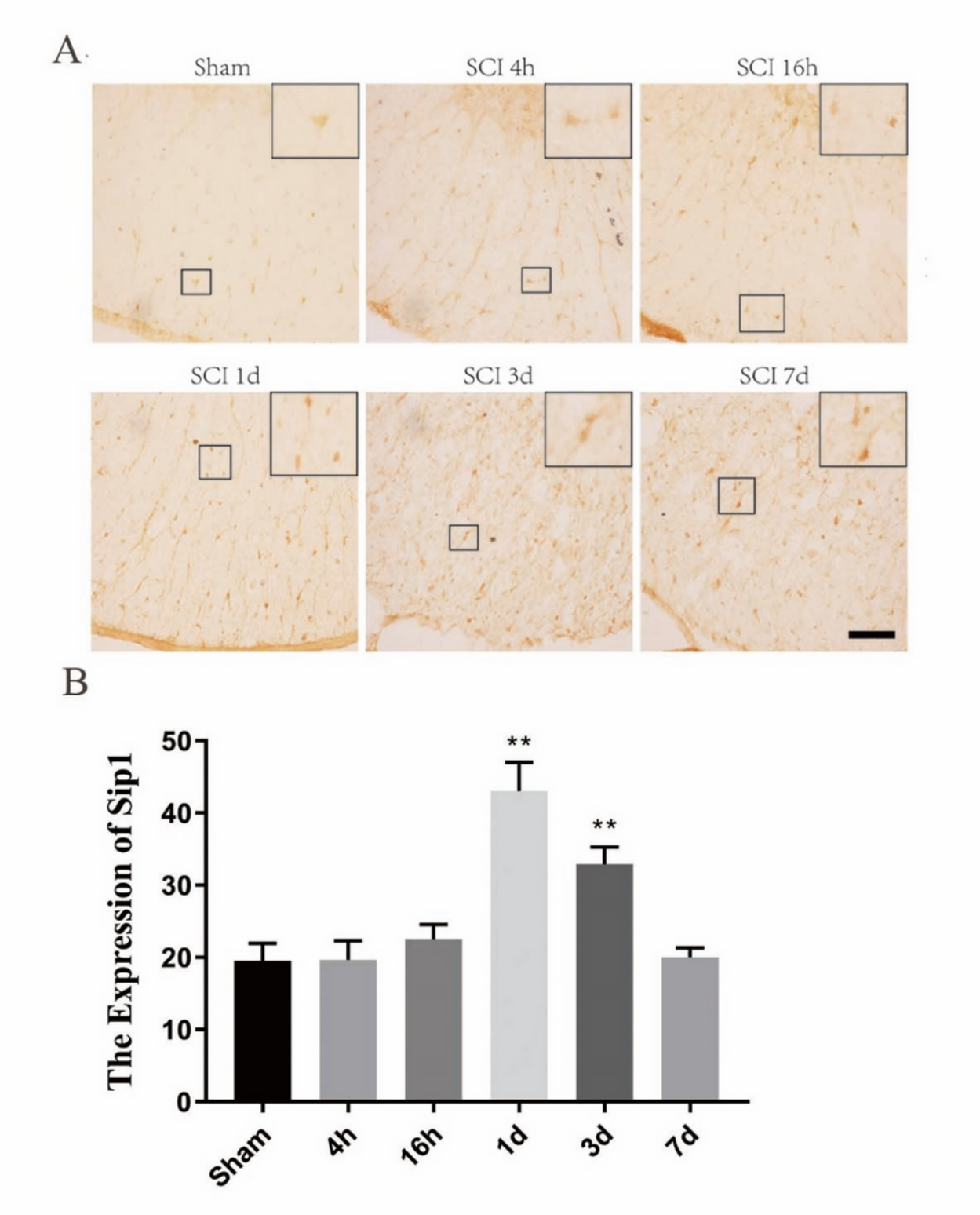

Figure 2

Expression of Sip1 after SCI. (A) Immunohistochemistry staining of Sip1 in the white matter of the spinal cord in the sham operated group and at different time points after SCI (4h, 16h, 1d, 3d, 7d) (×200). (B) Quantitative analyses of Sip1 in the spinal cord in the sham operated group and at different time points after SCI. ** $p < 0.01$ vs sham group. Error bars represent the mean ± SD. Scale bar = 100 μm.

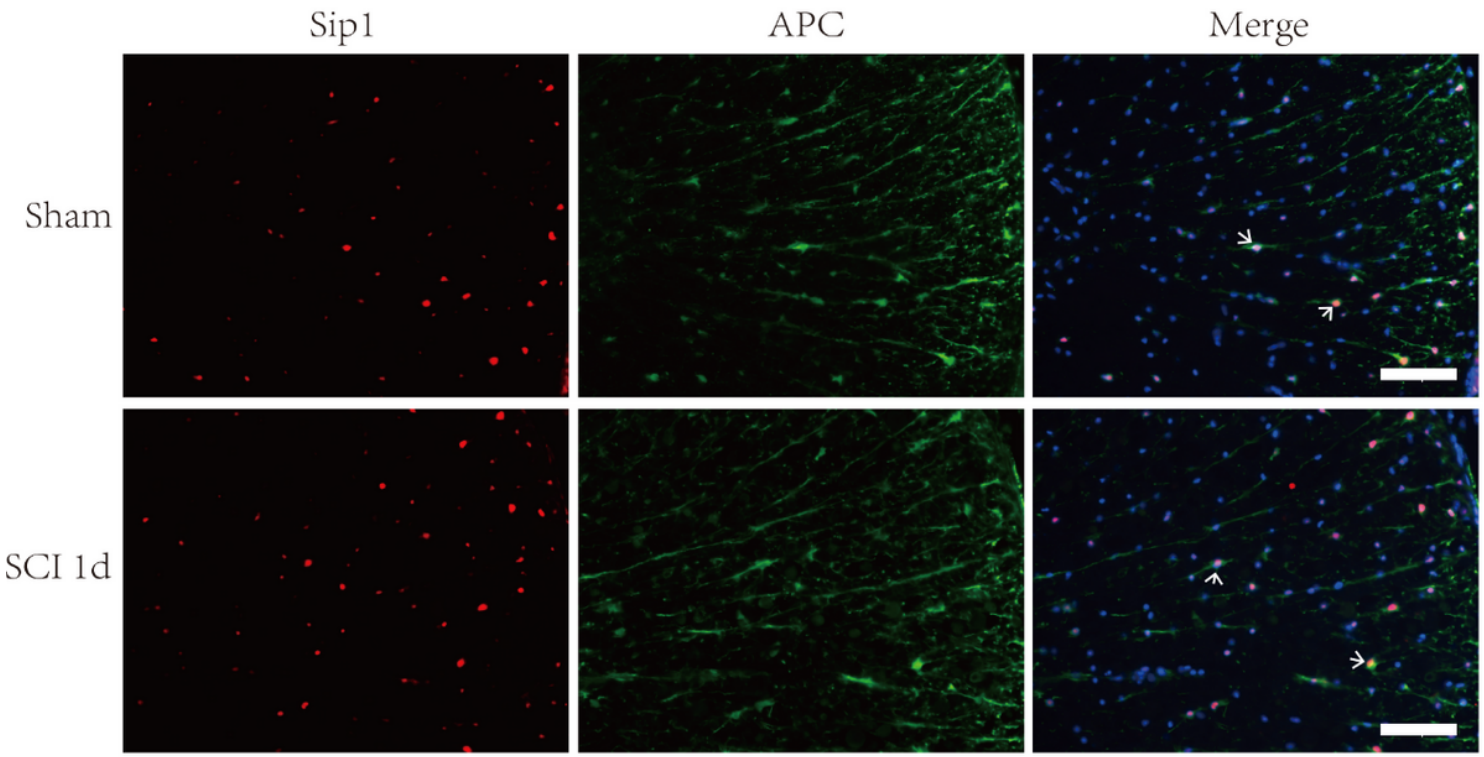

Figure 3

Double immunofluorescence staining for Sip1 (red), MBP (green), and nuclei (blue) in the spinal cord at 1d after SCI. Scale bar = 100 μm.

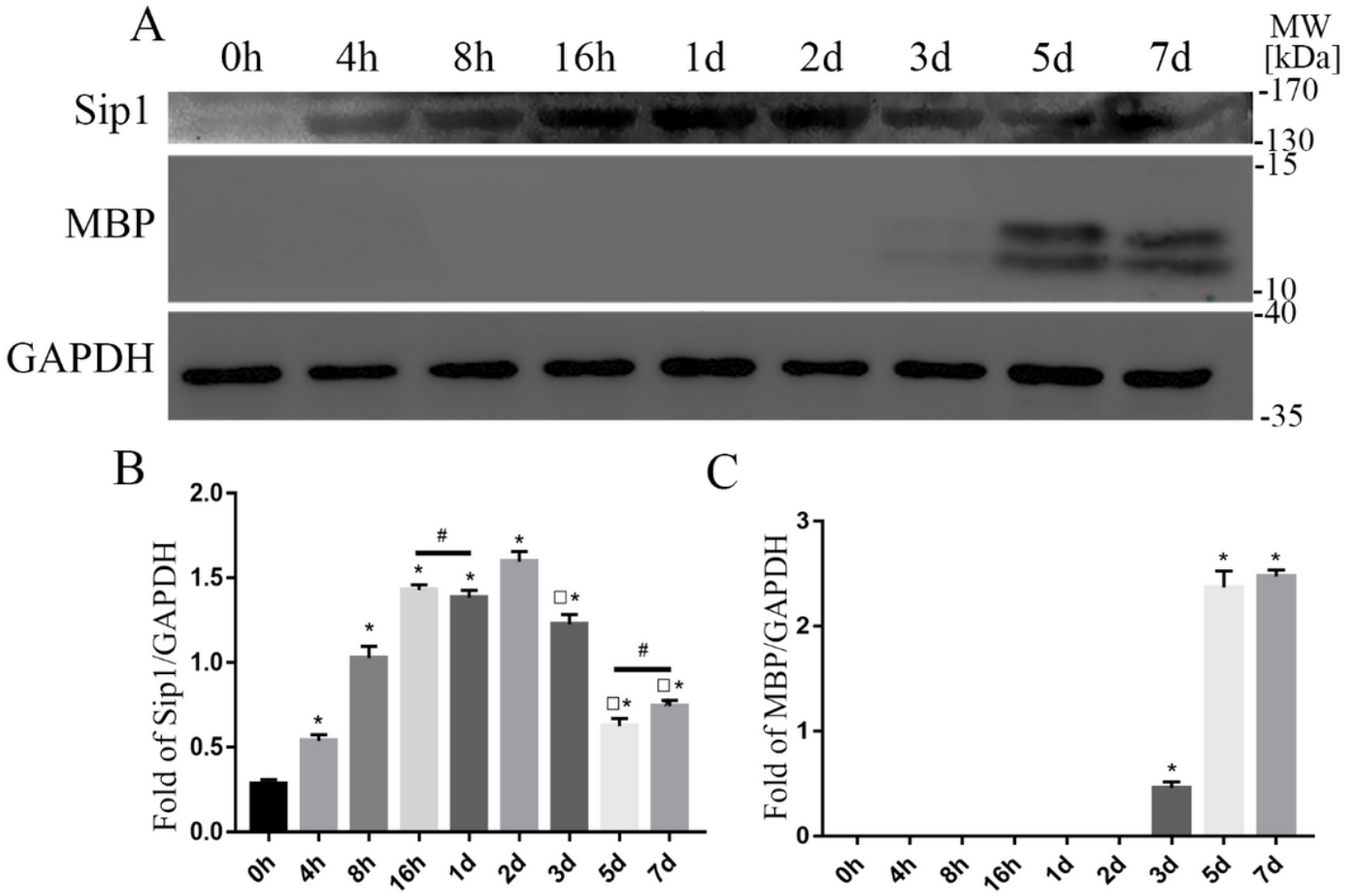

Figure 4

Expression of Sip1 and MBP during the differentiation of OPCs. (A) Representative western blot bands of Sip1 and MBP at undifferentiation (0h) and various time points during the differentiation of OPCs. (B) Quantitative analyses of Sip1 time-dependent expression levels during the differentiation of OPCs. (C) Quantitative analyses of MBP time-dependent expression levels during the differentiation of OPCs. * $p<0.05$ vs 0h, # $p>0.05$, $p<0.05$ vs 2d. Error bars represent the mean ± SD.

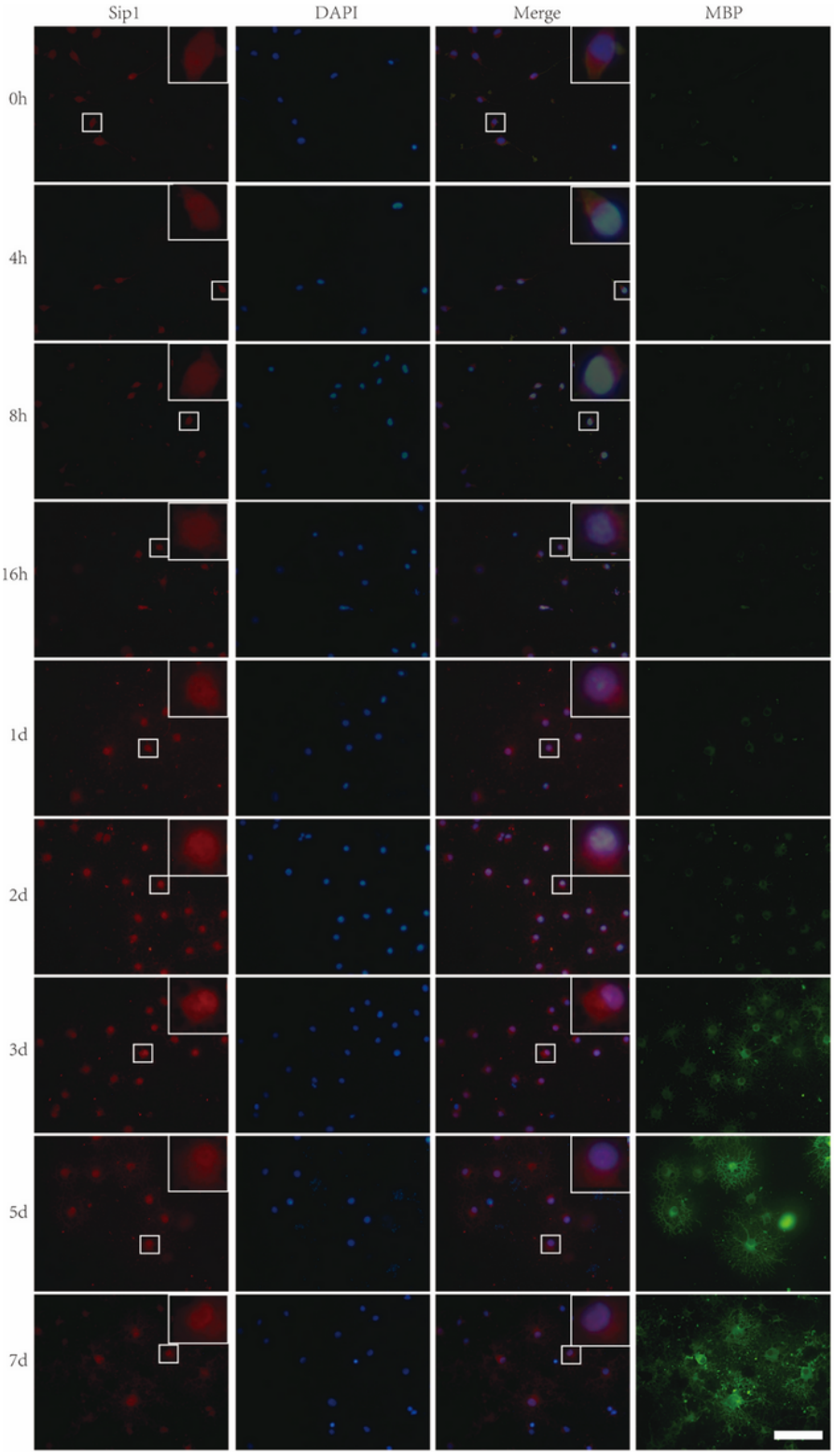

Figure 5

Temporal expression and cellular localization of Sip1 and MBP. Double immunofluorescence staining for Sip1 (red), MBP (green), and nuclei (blue) at undifferentiation (0h) and different timepoints during OPC differentiation (4h, 8h, 16h, 1d, 2d, 3d, 5d, 7d). Scale bar = 50 μm.

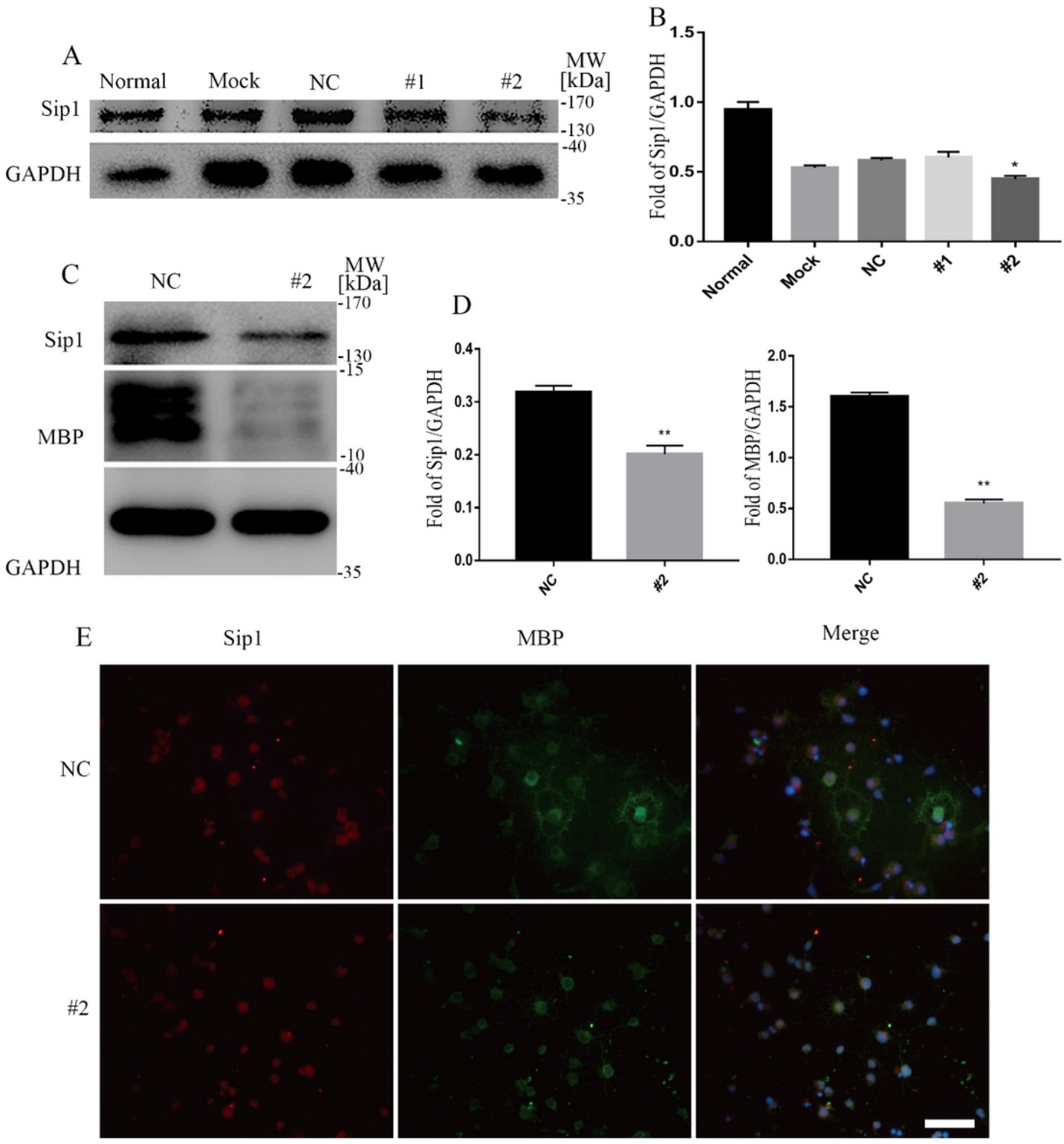

Figure 6

Expression of Sip1 and MBP after siRNA-Sip1 in OPCs. (A) Western blot of Sip1 protein after transfection. Mock: empty vector group; NC: negative control siRNA; #1 and #2: Sip1-knockdown group. (B) Quantitative analyses of Sip1 after Sip1-siRNA in OPC. (C) Western blot of Sip1 and MBP on day 3 of differentiation after transfection. (D) Quantitative analyses of Sip1 and MBP on day 3 of differentiation after transfection. (E) Double immunofluorescence staining for Sip1 (red), MBP (green), and nuclei (blue)

on day 3 of differentiation after transfection. * p < 0.05 vs Mock. ** p < 0.01 vs NC. Error bars represent the mean ± SD. Scale bar = 50 μm. NC = normal control.

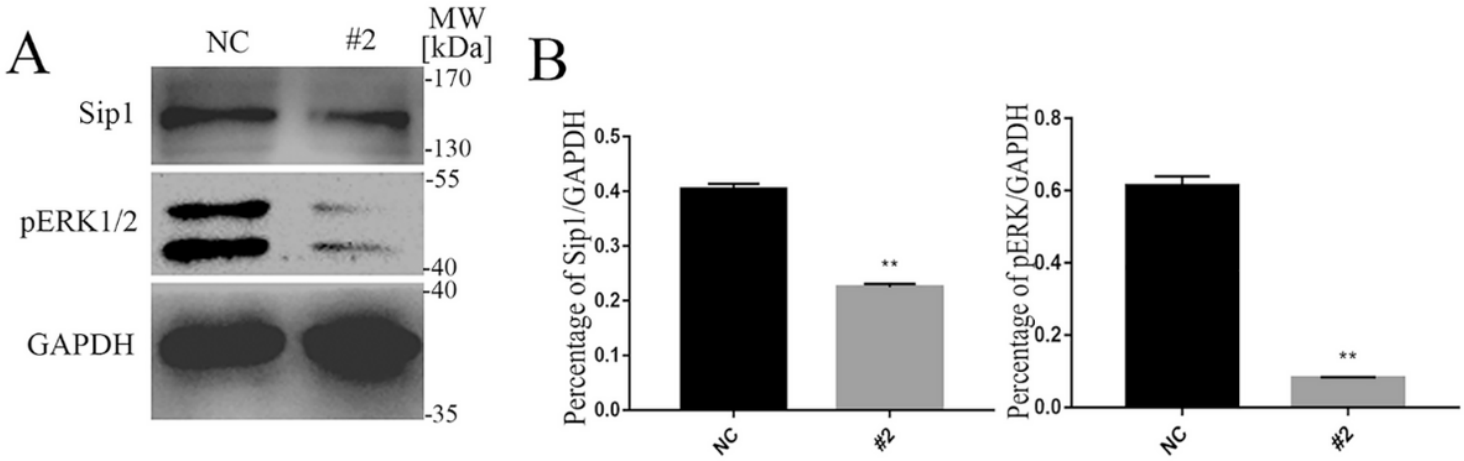

Figure 7

Expressions of Sip1 and pERK1/2 after siRNA-Sip1 in OPC. (A) Western blot of Sip1 and pERK1/2 on day 1 of differentiation after transfection. (B) Quantitative analyses of Sip1 on day 1 of differentiation after Sip1-siRNA of OPC, and quantitative analyses of pERK1/2 on day 1 of differentiation after Sip1-siRNA of OPC. ** p < 0.01 vs NC. Error bars represent the mean ± SD. NC = negative control.