

Published in final edited form as:

*J Cereb Blood Flow Metab.* 2009 June ; 29(6): 1166–1174. doi:10.1038/jcbfm.2009.41.

## Bone marrow stromal cells increase oligodendrogenesis after stroke

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### Abstract

Oligodendrocytes are sensitive to ischemic damage. The Sonic hedgehog (Shh) pathway is critical in oligodendrogenesis; Gli1 is the principal effector of Shh signaling. We investigated oligodendrogenesis and Shh/Gli1 pathway activation after bone marrow stromal cell (BMSC) treatment of stroke in rats. Rats were subjected to the middle cerebral artery occlusion (MCAo). BMSCs have been shown to promote functional recovery post stroke. A therapeutic dose of BMSC ( $3 \times 10^6$  cells) treatment was initiated 1 day after MCAo. Immunohistochemistry was carried out to measure the oligodendrocyte progenitor cells, oligodendrocytes, myelin, and expressions of Shh and Gli1 at 14 days after MCAo. Gene expression of Shh and Gli1 was tested at 2 days after MCAo. An *in vitro* study was used to investigate the effects of BMSC on a premature oligodendrocyte cell line (N20.1 cells). BMSC treatment significantly increased O4<sup>+</sup> oligodendrocytes, MBP<sup>+</sup> area, and bromodeoxyuridine (BrdU)<sup>+</sup>, NG2<sup>+</sup>, BrdU<sup>+</sup>-NG2<sup>+</sup> cells, and mRNA and protein expressions of Shh and Gli1 in the ipsilateral brain of the MCAo rats than that in phosphate buffered saline (PBS)-treated rats. BMSCs promoted N20.1 cell proliferation and Gli1 mRNA expression, and these effects were abolished by the Shh pathway inhibitor cyclopamine. These data indicate that the BMSC treatment stimulates oligodendrogenesis by activation of the Shh/Gli1 pathway post stroke.

### Keywords

bone marrow stromal cells; Gli1; MCAo; oligodendrocyte; oligodendrogenesis; sonic hedgehog

### Introduction

Oligodendrocytes generate the myelin sheaths that enwrap axons, thereby facilitating saltatory conduction (McTigue and Tripathi, 2008); they are vulnerable to damage in a variety of neurologic diseases, such as ischemic cerebrovascular diseases, and oligodendrocyte damage

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#### Conflict of interest

The authors declare no conflict of interest.

leads to demyelination, which contributes to neurologic functional deficits (Dewar *et al*, 2003; McTigue and Tripathi, 2008; Micu *et al*, 2006). Recruited oligodendrocytes can remyelinate axons, raising the possibility of therapeutic intervention (Chang *et al*, 2000). Mature oligodendrocytes in the adult mammalian central nervous system (CNS) were considered to be postmitotic and are unable to proliferate in response to injury (McTigue and Tripathi, 2008). However, abundant oligodendrocyte progenitor cells exist in the white and gray matter of normal CNS, and are present in the lesions (Komitova *et al*, 2006; McTigue and Tripathi, 2008; Tanaka *et al*, 2003), and proliferating oligodendrocyte progenitor cells contribute to remyelination (Carroll and Jennings, 1994; Gensert and Goldman, 1997; Jiang *et al*, 2008; Keirstead *et al*, 1998; Prineas *et al*, 1989).

Our previous *in vivo* studies found that bone marrow stromal cells (BMSCs) stimulate the production of restorative factors by parenchymal cells and evoke white matter remodeling in the injured brain (Chopp and Li, 2002; Chopp *et al*, 2009), which lead to improvement in neurologic function post stroke (Chen *et al*, 2000; Chen *et al*, 2001a; Shen *et al*, 2007; Zhang *et al*, 2004), and our *in vitro* results suggest that BMSCs protect oligodendrocytes subjected to oxygen–glucose deprivation (Zhang *et al*, 2008). These studies provide insight into white matter damage and the therapeutic benefits of BMSC cell based-remyelinating therapy after stroke. In this study, we investigated the effects of BMSC treatment of stroke on oligodendrogenesis.

To elucidate the underlying mechanisms of oligodendrocyte progenitor cell proliferation after treatment, we used N20.1 cells, which are premature oligodendrocytes (Paez *et al*, 2004). These cells are widely used to study the cellular and molecular mechanisms involved in the development, maturation, and formation of myelin by oligodendrocytes in the CNS (Garcia *et al*, 2007; Paez *et al*, 2004; Zhang *et al*, 2008).

A signaling pathway by which BMSCs induce oligodendrogenesis was investigated. Sonic hedgehog (Shh) is a member of the family of the hedgehog proteins; it plays a critical role in the induction, survival, proliferation, and migration of oligodendrocytes (Marti and Bovolenta, 2002; Seifert *et al*, 2005; Sussman *et al*, 2002). The Shh signaling pathway is well conserved (Marti and Bovolenta, 2002), Shh binds to the transmembrane receptor protein, patched, to activate another transmembrane receptor, smoothed (Ingham and McMahon, 2001), and induces intracellular reactions that target the Gli family of transcription factors (Ruiz i Altaba *et al*, 2002). Gli1 is the principal effector of Shh signaling in neural progenitor cells (Ahn and Joyner, 2005; Wang *et al*, 2007). In this article, we show that BMSC treatment, as an effective restorative treatment for stroke, enhances white matter remodeling and stimulates the Shh pathway.

## Materials and methods

All experimental procedures have been approved by the Institutional Animal Care and Use Committee of Henry Ford Health System.

### Bone Marrow Stromal Cell Preparation

Rat (r) and mouse (m) BMSCs were generously provided by Cognate Therapeutics Inc. (Baltimore, MD, USA). BMSCs were isolated, grown, and tested, as described previously (Zhang *et al*, 2004). Briefly, the bone marrow was obtained from adult donors and placed into media with Dulbecco's modified Eagle's medium-low glucose and 10% selected fetal bovine serum (FBS). The adherent cells were fed and generally lasted for about 2 weeks. When dense colonies of spindle-shaped cells covered greater than 80% of the dish, the cells were passaged into the secondary culture. When dose and purity levels were achieved, cells were harvested

and cryopreserved in appropriate dose-related aliquots in Plasma-Lyte (Baxter Healthcare Corporation, Deerfield, IL, USA) containing serum albumin and dimethyl sulfoxide.

### Animal Model

Adult male Wistar rats (270 to 300 g,  $n = 27$ ; Charles River Laboratories, Wilmington, MA, USA) were used in our experiments. Middle cerebral artery occlusion (MCAo) was induced by a method of intraluminal vascular occlusion modified in our laboratory (Zhang *et al*, 2004). Briefly, a length of 4-0 monofilament nylon suture (18.5 to 19.5 mm) was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the MCA. Rats were randomly divided into the following:

**Normal control group**—This control group consisted of naive rats ( $n = 9$ ) without surgery.

**MCAo control group**—Rats ( $n = 9$ ) were subjected to permanent MCAo and 1 mL phosphate buffered saline (PBS) was injected into a tail vein at 1 day after MCAo.

**BMSC treatment group**—After washing with PBS and subsequent centrifugation, rBMSCs (rat BMSC) ( $3 \times 10^6$ ) in 1 mL of PBS were injected into a tail vein at 1 day after MCAo ( $n = 9$ ). This dose of cells was shown earlier to be highly effective in improving functional recovery after stroke (Chen *et al*, 2001a, 2003; Zhang *et al*, 2004). Bromodeoxyuridine (BrdU, Sigma Chemical) was used for mitotic labeling. It (100 mg/kg) was intraperitoneally injected twice a day for 7 consecutive days into normal or ischemic rats starting 1 day after MCAo.

### Tissue Preparation

To measure the expressions of Shh and Gli1 mRNA, normal rats ( $n = 3$ ) and ischemic rats with BMSC or PBS treatment were killed at 2 days after MCAo ( $n=3$  per treatment group). The ischemic boundary zone (IBZ) was extracted from the brains of the rats subjected to stroke (Zhang *et al*, 2004). For immunohistochemical evaluation, normal rats ( $n = 6$ ) were killed at 7 days after BrdU injection. Rats treated with BMSCs or PBS were killed at 14 days ( $n = 6$  per treatment group) after MCAo. Brains were fixed in 4% of paraformaldehyde and embedded in paraffin. A coronal slide (6  $\mu$ m thick) from the center of the ischemic core (bregma 0.2 mm) (Zhang *et al*, 2004) was used for immunohistochemical staining.

### Real-Time Reverse Transcriptase-PCR Analysis

Quantitative PCR was carried out using the SYBR Green real-time PCR method. Total RNA was isolated from the IBZ using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA). Quantitative reverse transcriptase-PCR (RT-PCR) was carried out on an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA, USA) using three-stage program parameters provided by the manufacturer as follows: 2 mins at 50°C, 10 mins at 95°C, and then 40 cycles of 15 secs at 95°C and 1 min at 60°C. Specificity of the produced amplification product was confirmed by the examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during PCR. Polymerase chain reaction products were run on 2% agarose gels to confirm that correct molecular sizes were present. Each sample was tested in triplicate, and samples obtained from three independent experiments were used for analysis of relative gene expression using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

Primer sets used to detect Shh and Gli1 mRNAs were listed as follows: Shh (Fwd, ATAACCTTGCCTGCTGTTGC; Rev, GAGACCCAACCTCCGATGTGT), Gli1 (Fwd, CAGCTCAAAGCTCAGCTCCT; Rev, CTTGGGGCTCTGATATGGAA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fwd, AGAACATCATCCCTGCATCC; Rev, CACATTGGGGGTAGGAACAC). One-way

ANOVA (analysis of variance) followed by the Student–Newman–Keuls test was carried out. The data are presented as means  $\pm$  s.d. A value of  $P < 0.05$  is considered significant.

### Immunohistochemistry and Quantification

Oligodendrocytes were identified by antibodies O4 (1:100, Chemicon, Billerica, MA, USA) or myelin basic protein (MBP) (1:50, Abcam, Cambridge, MA, USA). The NG2 antibody (1:100, Chemicon) was used to mark oligodendrocyte progenitor cells. To test the protein expressions of Shh and Gli1 in the MCAo brains with or without BMSC treatment, immunohistochemistry stainings were carried out using a goat polyclonal antibody (pAb) against Shh (1:50, Santa Cruz Biotechnology) and a rabbit pAb against Gli1 (1:200, Santa Cruz Biotechnology).

Double immunostaining for NG2 with BrdU (a mouse monoclonal antibody, 1:100, Dako, Carpinteria, CA, USA) was used to identify oligodendrocyte progenitor cell proliferation. To clarify the relationship of the expressions of Shh and Gli1 in neural cells, double immunostaining for Shh with NeuN (marker of neurons, Chemicon), glial fibrillary acidic protein (GFAP, marker for astrocytes, Dako), and O4, as well as Gli1 with NG2 were used.

Immunostaining was carried out following standard protocols. Slides were treated first with the primary antibody and then with the antibody conjugated to fluorescein isothiocyanate (FITC, Jackson ImmunoResearch, West Grove, PA, USA). These slides were then treated with a second primary antibody and then incubated with an antibody conjugated to Cy3 (Jackson ImmunoResearch). Negative control slides for each animal received identical preparations for immunostaining, except that primary antibodies were omitted.

To measure immunoreactive cells, numbers of O4<sup>+</sup>, Shh<sup>+</sup>, Gli1<sup>+</sup>, NG2<sup>+</sup>, BrdU<sup>+</sup>, and NG2<sup>+</sup>–BrdU<sup>+</sup> cells were counted in eight fields of view from the IBZ of a standard section from the center of the ischemia core (bregma 0.2 mm) and in all the fields from the ipsilateral subventricular zone (SVZ) in the same slide. The fields were digitized under the light microscope (Olympus BX40, Tokyo, Japan) using a 3-CCD color video camera (Sony DXC-970 MD, Tokyo, Japan) interfaced with Micro Computer Imaging Device (MCID) analysis system (Imaging Research, Saint Catharines, ON, Canada). The immunopositive cells were calculated and divided by the measured areas, and presented as numbers per mm<sup>2</sup>. The density of the MBP<sup>+</sup> area was measured in eight fields of view in the IBZ and presented as a proportional area by dividing the measured MBP<sup>+</sup> area by the total scanned area. Double immunostaining images were taken using Zeiss (Oberkochen, Germany) laser-scanning confocal microscopy (LSM 510 META). 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei were visualized by two-photon microscopy using a Chameleon pulsed laser (Coherent Inc., Santa Clara, CA, USA) set at 720 nm excitation. These regions were scanned in 512  $\times$  512 pixel (279  $\times$  279  $\mu\text{m}^2$ ) format in the  $x$ – $y$  direction using a  $\times$  5 frame-scan average, and five optical sections along the  $z$ -axis with a 1- $\mu\text{m}$  step size were obtained under a  $\times$  63 objective.

Data are presented as mean  $\pm$  s.d. Significance between the two groups was examined using ANOVA analysis. A value of  $P < 0.05$  was considered significant.

### *In Vitro* Oligodendrocyte Proliferation

We used an immortalized mouse premature oligodendrocyte cell line (N20.1, generously provided by Dr Anthony Campagnoni, University of California at Los Angeles) to measure oligodendrocyte proliferation and differentiation. N20.1 cells were obtained from mouse primary cultures of oligodendrocytes conditionally immortalized by transformation with a temperature-sensitive large T-antigen (Paez *et al*, 2004). N20.1 cells were cultured in Dulbecco's modified Eagle's medium/F12 (Invitrogen, Carlsbad, CA, USA) with 3.6 g/L of

dextrose anhydrous, 3.38 g/L of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2.16 g/L of sodium bicarbonate, 90 mg/L of gentamicin, 1% of FBS, and G418 (100  $\mu\text{g}/\text{mL}$ ) at 39°C (Paez *et al*, 2004).

N20.1 cells were incubated in the following groups ( $n = 3$  per group, three wells were used in each group): (1) regular culture medium for control; (2) mouse BMSCs (mBMSCs) cocultured with normal N20.1 cells (oligodendrocytes: mBMSCs = 10:1), an insert (0.4  $\mu\text{m}$ , BD Biosciences, Franklin Lakes, NJ, USA) was used to contain mBMSCs. Oligodendrocytes were plated on the base of the culture wells and the upper transwell compartments were seeded with mBMSCs; (3) mBMSCs cocultured with 5  $\mu\text{mol}/\text{L}$  cyclopamine (Calbiochem, Gibbstown, NJ, USA)-treated N20.1 cells. Cyclopamine is a specific inhibitor of smoothened (Wang *et al*, 2007).

For investigation of oligodendrocyte proliferation, N20.1 cells were treated for 12 h and 50  $\mu\text{g}/\text{mL}$  BrdU (Sigma) was added to the cell cultures for 1 h. BrdU immunostaining of the N20.1 cells was measured. Numbers of BrdU<sup>+</sup> cells were calculated by counting 10 random fields in each well with six wells per group. The results are presented as a percentage (positive cells divided by total cells).

### ***In Vitro* Activation of the Shh/Gli Pathway in Oligodendrocytes**

An additional set of *in vitro* experimental groups was used for mRNA analyses ( $n = 3$  per group). Primer sets used to detect mouse NG2 and Gli1 mRNAs were listed as follows: Gli1 (Fwd, TGTGTGAGCAAGAAGGTTGC; Rev, TTGCACACGTATGGCTTCTC), NG2 (Fwd, CGGCCAACAGTGGTTTCAAGT; Rev, CTTCTGTGAAGGCTGTCGATG), GAPDH (Fwd, AG AACATCATCCCTGCATCC; Rev, CACATTGGGGGTAGGAACAC). One-way ANOVA followed by the Student–Newman–Keuls test was carried out. The data are presented as means  $\pm$  s.d. A value of  $P < 0.05$  is considered significant.

### **Statistical Analyses**

Data were evaluated by an investigator blinded to the treatment status of each animal. Data were evaluated for normality, and data transformation or nonparametric analysis was considered if data were not normal. Repeated analysis of covariance was used to test the BMSC effects on measurements. The analysis began testing for BMSC by time interaction, followed by testing the main effect of BMSCs if no interaction was observed at the 0.05 level.

## **Results**

### **BMSC Treatment Increases the Area of Myelin in the Brain of Rats Subjected to MCAo**

Given the significant improvement in neurologic outcome when rodents subjected to MCAo were treated with BMSCs (Chen *et al*, 2001a; Chopp *et al*, 2009; Shen *et al*, 2007; Zhang *et al*, 2004), we sought to address whether BMSC treatment affects myelin. As indicated by MBP immunostaining, myelin damage was obvious in the IBZ of MCAo rats at 14 days (Figure 1A). After BMSC treatment, the proportional area of myelin (MBP<sup>+</sup>) density was significantly increased in the IBZ of BMSC-treated rats than that of PBS-treated rats (73.7%  $\pm$  4.8% versus 64.9%  $\pm$  2.1%,  $P < 0.05$ , Figures 1B and 1C). These data indicate that BMSC treatment protects myelin and/or induces oligodendrogenesis and the remyelination in the white matter of MCAo rats.

### **BMSC Treatment Increases Oligodendrogenesis in the Brain of MCAo Rats**

Given the significant increase of the myelin area, we sought to address whether BMSC treatment affects oligodendrocyte proliferation and remyelination. After ischemic injury, O4<sup>+</sup> oligodendrocytes were significantly increased in the IBZ and the SVZ of BMSC-treated

rats than those of the PBS treatment group ( $350.7 \pm 44.9$  per  $\text{mm}^2$  versus  $195 \pm 25$  per  $\text{mm}^2$  in IBZ;  $107 \pm 23.1$  per  $\text{mm}^2$  versus  $90 \pm 7.5$  per  $\text{mm}^2$  in SVZ,  $P < 0.05$ , Figures 1D to 1F).

Using double BrdU and NG2 immunostaining, we found that BrdU<sup>+</sup> proliferating cells, NG2<sup>+</sup> oligodendrocyte progenitor cells, and BrdU<sup>+</sup>-NG2<sup>+</sup> proliferating oligodendrocyte progenitor cells were significantly increased in the ipsilateral brain of BMSC-treated MCAo rats than that of PBS-treated rats ( $125.9 \pm 11.5$  per  $\text{mm}^2$  versus  $65.8 \pm 17$  per  $\text{mm}^2$ ;  $186.1 \pm 20.7$  per  $\text{mm}^2$  versus  $97.2 \pm 6.3$  per  $\text{mm}^2$ ;  $31.3 \pm 5.1$  per  $\text{mm}^2$  versus  $15.2 \pm 3.3$  per  $\text{mm}^2$ , respectively,  $P < 0.05$ , Figures 1G to 1O). These data indicate that BMSC treatment stimulates oligodendrogenesis and evokes white matter remyelination in the injured brain.

### **BMSC Treatment Activates the Shh/Gli1 Signaling Pathway**

The Shh/Gli1 signaling pathway regulates oligodendrogenesis in the adult rodent CNS (Wang *et al.*, 2007). To determine whether BMSCs induce Shh/Gli1 signaling activation, we examined the expression of Shh and its effector Gli1. Immunostaining showed that Shh<sup>+</sup> and Gli1<sup>+</sup> cells are present in the ipsilateral brain at 14 days after MCAo, and significantly increased after the BMSC treatment compared with the PBS treatment (Figures 1P to 1T). Real-time RT-PCR analysis showed that Shh and Gli1 mRNA expressions are present in the IBZ at 2 days after MCAo. After BMSC treatment, gene expressions of Shh and Gli1 were significantly increased in the IBZ of BMSC-treated rats than that of the PBS-treated controls (Figure 1U).

Using double immunostaining of Shh with NeuN, GFAP, and O4, respectively, we found that Shh<sup>+</sup> cells colocalized with NeuN<sup>+</sup> neurons (Figures 2A to 2D), GFAP<sup>+</sup> astrocytes (Figures 2E to 2H), and O4<sup>+</sup> oligodendrocytes (Figures 2I to 2L). Thus, Shh protein may be released from neurons, astrocytes, and oligodendrocytes (Jiao and Chen, 2008; Paintlia *et al.*, 2005; Seifert *et al.*, 2005; Wallace, 1999; Wallace and Raff, 1999; Wechsler-Reya and Scott, 1999). Double immunostaining data showed that Gli1<sup>+</sup> cells colocalized with NG2<sup>+</sup> oligodendrocyte progenitor cells (Figures 2M and 2N), implying that Gli1 activity was involved in oligodendrogenesis after BMSC treatment. These data suggest that BMSCs stimulate the production of Shh by parenchymal cells, evoke oligodendrogenesis, and lead to white matter remodeling in the injured brain.

### **BMSC Treatment Promotes Oligodendrocyte Proliferation in Cell Culture**

As *in vivo* data suggest that activation of the Shh/Gli1 pathway induces oligodendrogenesis, and BMSC treatment after MCAo promotes Shh/Gli1 gene and protein expression leading to remyelination, we carried out an *in vitro* study to clarify the relationships among proliferation of N20.1 cells, the Shh/Gli1 pathway, and BMSC treatment. N20.1 cells are premature oligodendrocytes (Paez *et al.*, 2004). Under 1% FBS and 39°C conditions, most of N20.1 cells exhibited a significantly decreased growth rate. BMSC treatment significantly increased the N20.1 cell proliferation, as indicated by BrdU immunostaining, compared with the normal medium group ( $P < 0.01$ , Figure 2O). BMSC-induced N20.1 cell proliferation was significantly reduced by cyclopamine, a pharmacological inhibitor of the Shh signaling pathway ( $P < 0.05$ ). These data indicate that BMSCs induce N20.1 cell proliferation through the Shh pathway.

### **BMSC Treatment Activates the Shh/Gli1 Signaling Pathway in Oligodendrocyte Cell Culture**

To test the hypothesis that the effect of BMSCs on oligodendrogenesis is mediated through the Shh/Gli1 pathways, we used RT-PCR to measure NG2 and Gli1 gene expressions. RT-PCR data showed that the NG2 mRNA level was increased significantly after 12 h of BMSC coculture, implying an increase in the proliferation of N20.1 cell. Gli1 mRNA expression was increased significantly in N20.1 cells after coculture with BMSCs. All these BMSC effects were blocked by the Shh/Gli1 pathway inhibitor, cyclopamine (Figure 2P). These data suggest

that activation of the Shh/Gli1 pathway is involved in oligodendrogenesis, and BMSC treatment promotes this activation.

## Discussion

BMSCs, including multipotential mesenchymal stem and precursor cells, have broad therapeutic applications to the treatment of neurologic diseases (Chopp and Li, 2002; Chopp *et al.*, 2009). Functional recovery is evident after BMSC treatment of rodents with stroke from 14 days (Chen *et al.*, 2001a, 2003; Shen *et al.*, 2007; Zhang *et al.*, 2004) and up to 1 year (Shen *et al.*, 2007) after ischemic injury. BMSCs stimulate the production of restorative factors by parenchymal cells, evoke white matter remodeling in the injured brain, and lead to improvement in neurologic function post stroke (Chopp and Li, 2002; Chopp *et al.*, 2009). Similar restorative benefits were also obtained using other cell sources for transplantation in stroke (Borlongan, 2009; Chen *et al.*, 2001b). In this study, we show that BMSC treatment of rats subjected to MCAo induces oligodendrogenesis and activates the Shh/Gli1 pathway, which may contribute to functional recovery. We note, however, that our data do not show a causal relationship between oligodendrocyte proliferation and functional benefit. There are likely many factors that contribute to the BMSC-mediated functional improvement post stroke (Chen *et al.*, 2002; Chopp and Li, 2002; Chopp *et al.*, 2009; Qu *et al.*, 2007), among which are angiogenesis, neurogenesis, and synaptogenesis (Chopp and Li, 2002; Chopp *et al.*, 2009). As axonal integrity and myelination are necessary for brain function, and stroke often disrupts and damages white matter, it is reasonable to consider the BMSC-mediated induction of oligodendrogenesis as a potential contributing factor in promoting recovery post stroke. To our knowledge, this is the first study to show that a cell-based therapy stimulates oligodendrogenesis and that the Shh pathway may mediate this restorative process.

Oligodendrocytes are the only myelin-producing cells in the CNS that enable rapid electrical conduction of impulses, and they are vulnerable to the ischemic injury (Dewar *et al.*, 2003; Domercq and Matute, 2004). The lack of oxygen and glucose, as well as free radicals, contributes to oligodendrocyte damage after stroke (Shibata *et al.*, 2000). The death of oligodendrocytes after MCAo leads to demyelination, and axonal conduction will be subsequently impaired or lost (Dewar *et al.*, 2003; McTigue and Tripathi, 2008; Micu *et al.*, 2006).

In this study, we focus on the effects of BMSCs on oligodendrogenesis and myelin remyelination. Abundant oligodendrocyte progenitor cells (NG2<sup>+</sup>) are present in the adult CNS (McTigue and Tripathi, 2008). BrdU and NG2 double immunostaining showed oligodendrocyte progenitor cell proliferation in the CNS after MCAo, and BMSC treatment significantly enhanced this proliferation. This proliferation may contribute to the remyelination, which is suggested by the significantly increased O4<sup>+</sup> cells and MBP<sup>+</sup> area. These data suggests that BMSC treatment promotes oligodendrocyte progenitor cell proliferation and differentiation into myelinated oligodendrocytes. Remyelination is likely a requisite for functional recovery, and recruited oligodendrocytes can replace the damaged oligodendrocytes, remyelinate axons, and reduce axonal loss.

Shh plays a critical regulatory role in oligodendrogenesis and facilitates remyelination (Marti and Bovolenta, 2002; Murray *et al.*, 2002; Nery *et al.*, 2001; Orentas *et al.*, 1999; Seifert *et al.*, 2005; Sussman *et al.*, 2002), and Gli1 is the principal effector of Shh (Ahn and Joyner, 2005; Wang *et al.*, 2007). Our data show Shh and Gli1 mRNAs are present in the IBZ at 2 days, and protein expressions of Shh and Gli1 were present in the ipsilateral brain at 14 days after MCAo, and BMSC treatment significantly enhanced these expressions. Double immunostaining data suggest that the Shh protein expressed in neurons, astrocytes, and oligodendrocytes and in the Gli1 activity are involved in oligodendrogenesis. These data suggest that BMSC treatment-

mediated enhancement of oligodendrogenesis may occur by promoting the production of Shh by parenchymal cells and activation of the Shh/Gli1 pathway in the ischemic brain. Also, our *in vitro* study shows that BMSCs stimulate N20.1 cell proliferation and Gli1 mRNA expression. Blockage of the Shh pathway with the Shh antagonist, cyclopamine, abolished the BMSC-induced proliferation of N20.1 cells, and also decreased Gli1 mRNA expression in N20.1 cells, implying that BMSCs promote oligodendrocyte proliferation by activation of the Shh/Gli1 pathway. These data suggest that activation of the Shh/Gli1 pathway underlies BMSC-induced oligodendrogenesis.

In summary, our data indicate that BMSC treatment promotes oligodendrogenesis and remyelination. Activation of the Shh/Gli1 pathway may underlie the restorative effects of BMSCs after MCAo in rats.

## Acknowledgments

The authors thank Mr. Siamak Pourabdollah, Dr Lei Wang, Cynthia Roberts, Supata Santra and Qinge Lu for their technical assistance.

This work was supported by the NIH Grants P01 NS42345 and P01 NS23393 and by the Benson Ford Foundation.

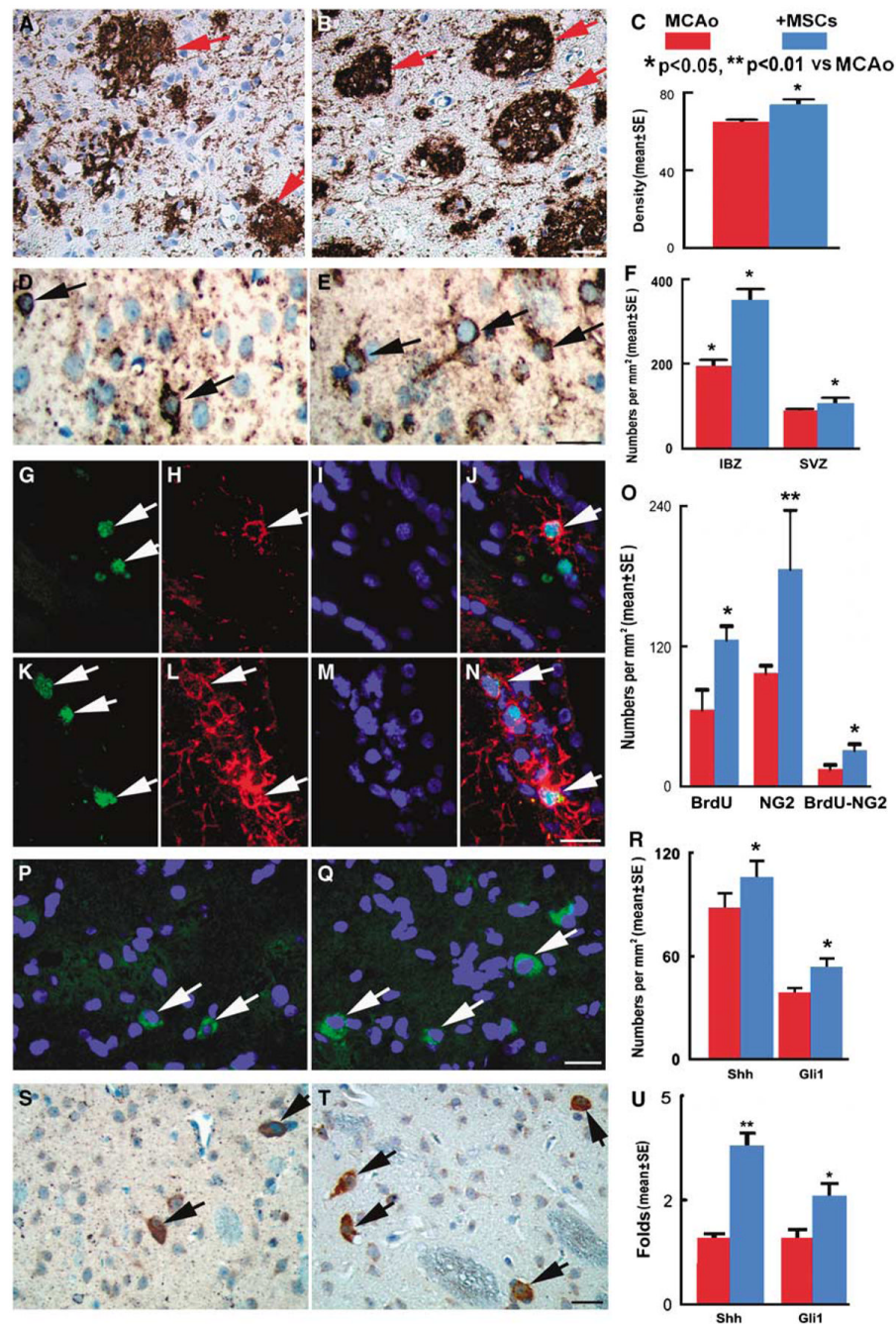
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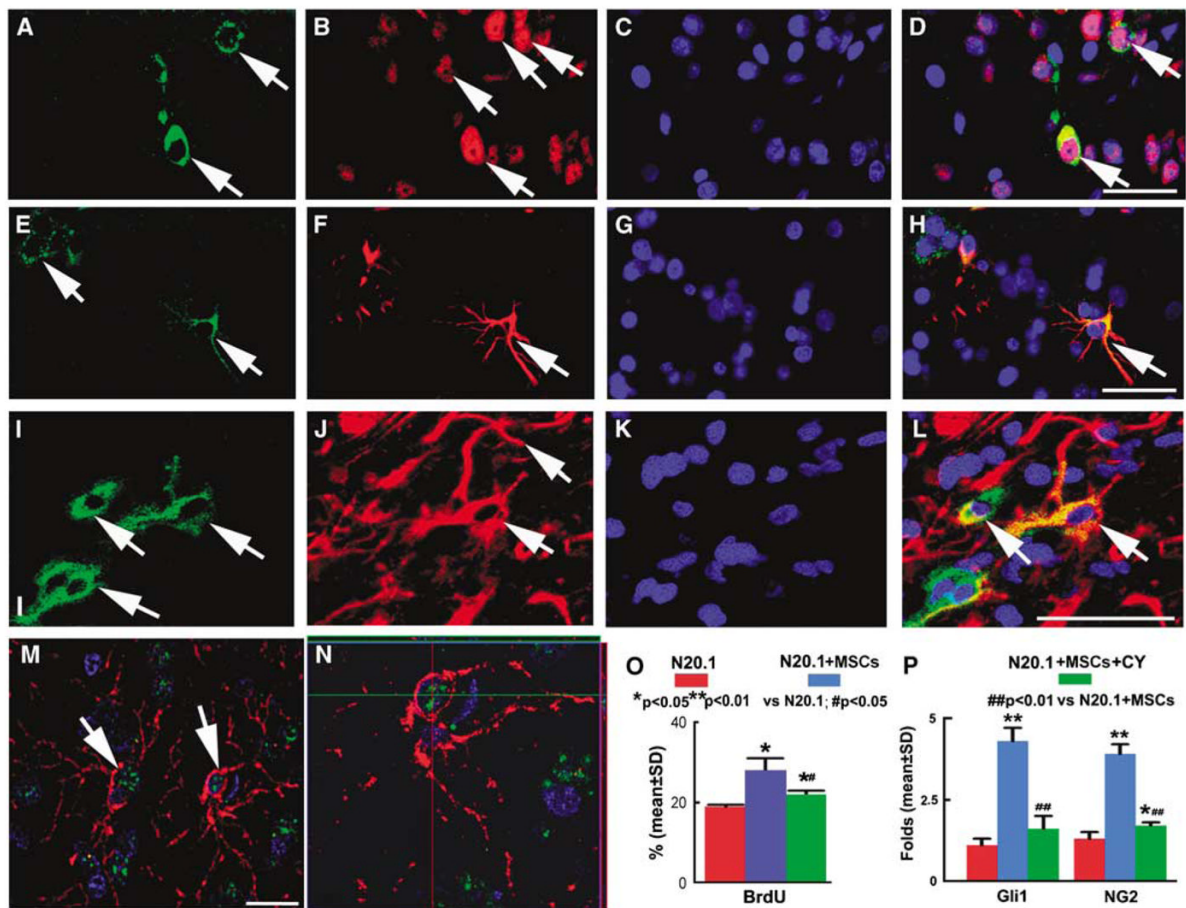
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**Figure 1.**

(A to C) The staining by MBP shows the myelin (red arrows) in the IBZ of MCAo rats treated by PBS (A) and BMSCs (B). Quantitative data show that MBP<sup>+</sup> areas (C) were significantly increased at 14 days after MCAo in the BMSC treatment group compared with those in the PBS group. (D to F) The staining by O4 shows the oligodendrocytes in the white matter of MCAo rats treated by PBS (D) and BMSCs (E). Quantitative data (F) show that O4<sup>+</sup> cells were significantly increased at 14 days after MCAo in the BMSC treatment group compared with those in the PBS group. (G to O) Double immunofluorescence staining (nuclei, DAPI, blue, I, M) indicated that BrdU<sup>+</sup> cells (FITC, green, G, K) were colocalized with NG2<sup>+</sup> oligodendrocyte progenitor cells (CY3, red, H, L). Quantitative data (O) show that BrdU<sup>+</sup>,

NG2<sup>+</sup>, and BrdU<sup>+</sup>-NG2<sup>+</sup> cells were significantly increased at 14 days after MCAo in the BMSC treatment group (**K to N**) compared with those in the PBS group (**G to J**). (**P to T**) The immunostaining data show that the Shh<sup>+</sup> (merged, green; nuclei, blue) in the white matter of MCAo rats treated by PBS (**P**) and BMSCs (**Q**), and Gli1<sup>+</sup> cells in the PBS group (**S**) and BMSC group (**T**). Quantitative data (**R**) show that Shh<sup>+</sup> and Gli1<sup>+</sup> cells were increased significantly at 14 days after MCAo in the BMSC treatment group compared with those in the PBS group. RT-PCR (**U**) analysis shows the mRNA expressions of Shh and Gli1 were significantly increased at 2 days in the MCAo rats treated by BMSCs compared with those in the PBS-treated rats. Bars=25  $\mu$ m (**A, B, D, E, G to N, P, Q, S, and T**). \**P* < 0.05, \*\**P* < 0.01 versus MCAo.



**Figure 2.**

Double immunofluorescence staining (nuclei, DAPI, blue) showed that  $Shh^+$  cells (FITC, green) were reactive to  $NeuN^+$  (CY3, red) (A to D),  $GFAP^+$  (CY3, red) (E to H), and  $O4^+$  cells (CY3, red) (I to L). Confocal images show that the  $Gli1^+$  cells (merged, green) were  $NG2^+$  (merged, red) (M). A  $\times 3$  zoom (with orthogonal views shown above and to the right in highlighted boxes) of panel M reveals colocalization of NG2, Gli1, and DAPI (N). Bars=50  $\mu m$  (A to D, E to H, and I to L); Bar=25  $\mu m$  (M). Quantitative data (O) of BrdU immunostaining in N20.1 cells show that the proliferation rate of N20.1 cells cocultured with BMSCs significantly increased compared with normal cells ( $P < 0.05$ ). The N20.1 cell proliferation significantly decreased after BMSC coculture plus cyclophamide treatment compared with BMSC coculture. RT-PCR analysis (P) shows the mRNA expression in N20.1 cells. mRNA expressions of NG2 and Gli1 were significantly increased after BMSC coculture compared with the normal N20.1 cells ( $P < 0.01$ ), and cyclophamide treatment significantly reduces these increases induced by BMSC treatment. \* $P < 0.05$ , \*\* $P < 0.01$  versus N20.1 cells; # $P < 0.05$ , ## $P < 0.01$  versus N20.1 + MSCs.