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Journal of Cellular Biochemistry 116:310–319 (2015)

Journal of Cellular Biochemistry

Priming Wharton's Jelly-Derived Mesenchymal Stromal/Stem Cells With ROCK Inhibitor Improves Recovery in an Intracerebral Hemorrhage Model

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ABSTRACT

Mesenchymal stromal/stem cells (MSCs) have the potential to differentiate into neuron-like cells under specific conditions and to secrete paracrine factors for neuroprotection and regeneration. Previously, Rho-kinase inhibitors have been reported to potentiate differentiation of rodent bone marrow MSCs into neuron-like cells induced by $CoCl_2$ (HIF-1α activation-mimicking agent). Here, a strategy of priming MSCs with fasudil, a Rho-kinase inhibitor, was investigated using Wharton's jelly-derived MSCs (WJ-MSCs) to improve recovery in a rat model of intracranial hemorrhage (ICH). In vitro culture of WJ-MSCs by co-treatment with fasudil (30 μM) and $CoCl_2$ provoked morphological changes of WJ-MSCs into neuron-like cells and increased the expression of neuronal markers. Assessment of the secretion profiles showed that fasudil (30 μM) specifically increased glial cell line-derived neurotrophic factor (GDNF) among the secreted proteins at the transcription and secretion levels. For in vivo experiments, WJ-MSCs primed with fasudil (10 μM, exposure for 6 h) were transplanted into ICH rats with HIF-1α upregulation 1 week after injury, and neurological function was assessed via rotarod and limb placement tests for 7 weeks after transplantation. The group with WJ-MSCs primed with fasudil showed improved functional performance compared with the non-primed group. Accordingly, the primed group showed stronger expression of GDNF and higher levels of microtubule-associated protein 2 and neurofilament-H positive-grafted cells in the ICH lesion 3 weeks after transplantation compared with the non-primed group. Therefore, this work suggests that priming WJ-MSCs with fasudil is a possible application for enhanced cell therapy in stroke, with additional beneficial effect of up-regulation of GDNF. J. Cell. Biochem. 116: 310–319, 2015.

KEY WORDS: MESENCHYMAL STROMAL/STEM CELL; FASUDIL; ROCK INHIBITOR; GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR; INTRACEREBRAL HEMORRHAGE MODEL; TRANSPLANTATION

Mesenchymal stromal/stem cells (MSCs) have been investigated for their efficacy in cell therapy for conditions ranging from ischemic injury to neurodegenerative disorders because of their capacity for multipotential differentiation [Low et al., 2008]. From preclinical studies, the major potential therapeutic mechanisms of MSCs in neurorestoration have been proposed to be indirect paracrine effects by secreted factors, which may promote endogenous neurogenesis and angiogenesis, encourage synaptic connections and remyelination of damaged axons, decrease apoptosis, and regulate inflammation [Seo and Cho, 2012]. In a recent clinical trial in ischemic stroke patients, intravenous transplantation of autologous bone marrow (BM) MSCs improved functional recovery without adverse effects [Bang et al., 2005; Lee et al., 2010b]. However, the efficacy of MSC transplantation should be improved to provide appropriately feasible and efficient cell therapy.

MSCs can be found in a variety of tissues, including skin, adipose tissue, bone marrow, and umbilical cord, and MSCs have been shown

Hyun-Sun Lee, Kwang S. Kim, and Hee-Suk Lim contributed equally to this work.

Grant sponsor: The National Research Foundation of Korea (NRF); Grant numbers: NRF-2009-0079656, NRF-2012R1A5A2047939.

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Manuscript Received: 23 October 2013; Manuscript Accepted: 29 August 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 4 September 2014

DOI 10.1002/jcb.24969 • © 2014 Wiley Periodicals, Inc.

to differentiate into neuron-like cells [Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Sanchez-Ramos, 2002]. Wharton's jelly, the matrix of the umbilical cord, has also been identified as a readily attainable source of primitive, multipotent MSCs [Mitchell et al., 2003; Troyer and Weiss, 2008]. Wharton's jelly-derived MSCs (WJ-MSCs) have also been induced to express neural phenotypes by treatment with basic fibroblast growth factor (bFGF) and then with low-serum medium plus butylated hydroxyanisole and dimethylsulfoxide [Mitchell et al., 2003]. WJ-MSCs were also converted to dopaminergic neurons by step-wise culturing in neuron-conditioned medium, sonic hedgehog, and FGF8 [Fu et al., 2006], and transplantation of WJ-MSCs induced a therapeutic effect in a rodent model of Parkinson's disease [Weiss et al., 2006]. WJ-MSCs show low expression of class I and II major histocompatibility complex and possess immune properties for tolerance in allogeneic transplantation [Weiss et al., 2008], suggesting that WJ-MSCs may be a valuable alternative source for regenerative cell therapy in stroke.

Rho kinase (ROCK) is a serine/threonine kinase and one of the major downstream effectors of the small GTPase Rho. The Rho-ROCK pathway is involved in neuronal functions including neurite outgrowth and retraction [Kubo et al., 2008]. This pathway is also closely related to the pathogenesis of several central nervous system (CNS) disorders including spinal cord injuries, stroke, and Alzheimer's disease. During the regeneration of injured axons in the adult CNS, myelin-associated neurite outgrowth inhibitors and other inhibitors including chondroitin sulfate proteoglycan (CSPG) mediate the inhibitory signals such as growth cone collapse and neurite outgrowth inhibition via action of Rho and ROCK.

Inhibitors of Rho kinase (ROCK) have been shown to have therapeutic effects in cardiovascular diseases and CNS disorders [Liao et al., 2007; Kubo et al., 2008]. Treatment with fasudil, a ROCK inhibitor, within 48 h of acute ischemic stroke onset significantly improves patient clinical outcomes [Shibuya et al., 2005], and administration of fasudil in ischemic brain was reported to lead to improved neuronal function and reduced infarct size in a rat model [Satoh et al., 2008]. Y27632, a ROCK inhibitor, has also been shown to promote axonal regeneration and functional recovery in the injured CNS in vivo [Fournier et al., 2003; Chan et al., 2005]. In MSC differentiation into neuron-like cells, synergistic effects of ROCK inhibition and hypoxia inducible factor (HIF-1) activation by $CoCl_2$ (hypoxia-mimicking agent) have been reported in rodent BM-MSCs [Pacary et al., 2006]. ROCK inhibition by Y27632 potentiated $CoCl_2$-induced MSC differentiation into dopaminergic neuron-like cells. The combination of Y27632 with deferoxamine, another HIF inducer, also showed a similar effect on MSC differentiation into neuron-like cells [Pacary et al., 2007]. However, the in vivo effects of this combination have not yet been studied.

Recently, we investigated the effect of several ROCK inhibitors on human BM-MSCs differentiation into neuron-like cells and found that the ROCK inhibitors, Y27632 and fasudil, with high specificity were more effective in inducing MSCs differentiation into neuron-like cells [Lee et al., 2010a]. Fasudil is the only clinically available ROCK inhibitor. Thus, we tested hypothesis that fasudil could effectively promote the neuronal differentiation of WJ- MSCs. In this study, we employed fasudil to promote WJ-MSCs differentiation into neuron-like cells and investigated whether priming WJ-MSCs with fasudil (a short time exposure to fasudil) could promote the regenerative capacity of WJ-MSCs in a rat intracerebral hemorrhage (ICH) model.

MATERIALS AND METHODS

PREPARATION OF WJ-MSCS

Isolation and expansion of MSCs from Wharton's jelly was performed as described previously [Choi et al., 2013]. Use of umbilical cords was approved by the Institutional Review Board at the Catholic University of Korea College of Medicine (CUMC09U158). The umbilical cords were obtained from donors with written informed consent.

IN VITRO DIFFERENTIATION OF WJ-MSCS INTO NEURON-LIKE CELLS

WJ-MSCs were seeded (40 cell/mm^2) and cultured in DMEM supplemented with 10% FBS overnight. After being washed with PBS, the cells were treated with 100 μM of $CoCl_2$ and/or 30 μM of fasudil (Calbiochem-Merck Bioscience, San Diego, CA) in endothelial cell growth medium (EGM) 2 (Lonza, Walkersville, MD) at 37 °C. During the ensuing 72 h, the cells were photographed in four representative fields (Leica, Inverted Microscope, Germany), and the images were analyzed using ImageJ (http://rsb.info.nih.gov/ij/) to determine the length of the cellular processes. The total length of the cellular processes was measured and divided by the number of cells to give an average total length of the cellular processes per cell. In total, 70–150 cells within four fields were assessed for each well.

IMMUNOFLUORESCENCE (IF)

Following the induction of in vitro differentiation into neuron-like cells for 72 h, the cells were washed with PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min at RT. The cells were permeabilized by treatment with 0.1% Triton-X 100 in PBS for 5–10 min at RT, followed by PBS containing 5% bovine serum albumin (BSA, Gibco BRL) and human IgG (I4506, Sigma) for 1 h at RT, and the cells were then incubated overnight at 4 °C with antibodies against nestin (1:500, MAB5326, Chemicon, Temecula, CA), neuron-specific class β-tubulin III (Tuj-1, 1: 500, MMS-435P, Covance, Richmond, CA), neuron-specific enolase (NSE, 1: 200, AB951, Chemicon), neurofilament-H (NF-H, 1:500, MAB5262, Chemicon), microtubule-associated protein (MAP2, 1: 200, MAB3418, Chemicon), or glial fibrillary acidic protein (GFAP, 1: 500, MAB360, Chemicon). After being washed with PBS, the cells were incubated with Cy3-conjugated anti-mouse IgG (1:1000, AP186C, Chemicon) or anti-rabbit IgG (1:1000, AP182C, Chemicon) for 1 h. Finally, the cell nuclei were stained with diamidinophenylindole (DAPI, 1:1000, Sigma) for 10 min. The cells were examined by confocal microscopy (Carl Zeiss, Germany). To quantify the neuronal differentiation, positively immunostained cells out of ~150 DAPI stained total cells were counted from each six randomly selected fields in triplicate. The experiments were repeated three times independently.

ANTIBODY-BASED ANGIOGENESIS ARRAY ANALYSIS

MSCs were seeded at a density of 40 cells/mm^2 and cultured in EGM2 medium for 48 h. After being washed with PBS, the cells were incubated in endothelial cell basal medium 2 (EBM2) containing 30 μM of fasudil for 48 h. Then, the conditioned medium (CM) was collected from the culture and assayed using a Human Angiogenesis Array Kit (ARY007, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Array images were analyzed using image analysis software (Multi Gauge V3.0, Fuji Photo Film, Tokyo, Japan).

QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (QRT-PCR) ANALYSIS

WJ-MSCs were seeded (60 cells/mm^2) and cultured in EGM2 for 48 h. After being washed with PBS, the cells were starved for 4 h in serum free EBM2 and treated with 10 or 30 μM fasudil for 6 h. Cells were harvested and total RNA was isolated. mRNA expression levels were quantified by real-time PCR on a CFX96 real-time PCR instrument (BioRad, Hercules, CA). 1 μg of each RNA was used as a template for reverse transcription. qRT-PCR was performed using a SYBR green master mix reagents (TOYOBO, JAPAN), the synthesized cDNA and primers for human glial cell line-derived growth factor (GDNF) (sense: CCAACCCAGAGAATTCCAGA, antisense: AGCCGCTGCAGTACC-TAAAA) or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense: CATGACCACAGTCCATGCCATCACT, antisense: TGAGGTCCACCACCCTGTTGCTGTA) according to the manufacturer's instructions. qRT-PCR was carried out under the following conditions: 95°C for 3 min, followed by 45 cycles of 10 s at 95 °C, 10 s at 57 °C, and 10 s at 72 °C. PCR amplification of a housekeeping gene, GAPDH, was performed as a control to allow normalization between samples. The relative expression in each sample was calculated with respect to the standard calibration curve.

WESTERN BLOT (WB) ANALYSIS

Western blotting was performed as described previously [Choi et al., 2013]. In brief, fasudil-treated WJ-MSCs were lysed in lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF, 1 μg/ml pepstatin, and protein inhibitor mix [Roche]). The cell lysate was centrifuged at 14,000 rpm for 30 min, and then the supernatant was subjected to 12% SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane, and the membrane was blocked with 5% skim milk. Blots were incubated with anti-GDNF antibody (1:200, AF-212-NA, R&D Systems) or β-actin and then with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using a chemiluminescent substrate (AbFrontier, Korea).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The CM obtained from culturing MSCs with fasudil (30 μM) in EBM2 for 48 h was concentrated 10-fold using Amicon ultra centrifugal filters (3K, UFC800324, Millipore) and assayed using ELISA (DY212, R&D Systems) to measure secreted GDNF. The ELISA kit for human GDNF that was used exhibits 35% cross reactivity with rat GDNF.

The brains isolated from the experimental rats in each group were cut in 1-mm coronal sections, and the hemisphere sections containing hemorrhage lesions were removed. The isolated tissues were homogenized with lysis buffer, and the tissue lysates were assessed by ELISA according to the manufacturer's protocol.

RAT INTRACEREBRAL HEMORRHAGE (ICH) MODEL

Male Sprague Dawley rats, weighing 240-280 g (7 weeks), were used for the ICH model as described previously [Jeong et al., 2003]. Subject rats were anesthetized by intraperitoneal injection of Zoletil 50 (30 mg/kg, Virbac Lab. Carros, France) and Rumpun (10 mg/kg, Bayer, Leverkusen, Germany) and then placed in a stereotactic apparatus. A surgical incision was made in the midline of the skull. A burr hole was made, and a 26-gauge needle was inserted through the burr hole into the striatum (coordinates: 0.2 mm posterior, 6.0 mm ventral, and 3.0 mm lateral to bregma). ICH was induced by the administration of collagenase type IV (0.23 U in 1 μL volume; Sigma) over a period of 5 min with an infusion pump. Before gentle withdrawal, the needle was allowed to remain in place for an additional 5 min to avoid reflux. The burr hole was sealed with bone wax, and the wound was sutured. Animals were maintained in separate cages at room temperature with free access to food and water under a 12/12-h light/dark cycle. All procedures for the care and use of animals were approved by the Ethics Committee of the Catholic University of Korea (approval No. 2010–0084-01).

CELL TRANSPLANTATION

WJ-MSCs were incubated in EGM2 containing fasudil (10 μM) for 6 h. Then, the cells were dissociated into single cells by brief trypsin-EDTA treatment and suspended in PBS at 2×10^5 cells/3 μL. Randomly selected ICH rats ($n = 9$ each group) at 1 week after ICH surgery received 3 μL of primed- or non-primed MSC suspension. The cells were injected slowly for 5 min into ipsilateral striatum (2 mm cranial to the hemorrhagic lesion). The same volume of PBS was injected into the control group.

BEHAVIORAL TESTS

Motor function was determined using the rotarod and limb placing tests. In the rotarod test, the rats were placed on the rotarod cylinder, and the time that the animals remain on the rotarod was measured. The speed was slowly increased, from 4 to 40 rpm, within a period of 5 min. The animals were trained for 7 days, and the maximum time score was recorded with three rotarod measurements 1 day before ICH induction. The limb placing test was perform using a method modified from a previously described test [Kim et al., 2011]. This test consists of three limb placing tasks that assess the sensorimotor integration of the forelimb and the hindlimb by confirming responses to tactile and proprioceptive stimulation. In the first task, the rat was suspended 10 cm over a table and observed to stretch toward the table. Second, the rat was placed along the edge of the table, with its limbs suspended and allowed to move freely. The rat was checked for lateral placement of the limbs. Finally, each limb was gently pulled down, and retrieval and placement were checked. The three tasks were scored in the following manner: normal performance, 0 points; performance with a delay and/or incomplete, 1 point; no performance, 2 points. A total of 9 points indicates maximal neurological deficit, and 0 points indicates no neurological deficit.

IF AND IMMUNOHISTOCHEMISTRY (IHC) OF THE BRAIN TISSUE

Each rat ($n = 3$/group) was anesthetized and perfused through the aorta with 4% paraformaldehyde in PBS. The brain was removed from the skull and post-fixed in 4% paraformaldehyde by immersion for 24 h and then transferred into 30% sucrose for 1–2 days until the brain sank to the bottom of their container. The brain was cut into 10 μm coronal sections in a cryostat (Leica) throughout the striatum. The brain sections were immunostained on free-floating sections. Brain sections were pre-incubated in PBS containing 5% normal goat serum (NGS, Vector Laboratories, Burlingame, CA) and 0.2% Triton X-100 for 30 min at RT. The sections were then incubated for 1 h with primary antibody to NF-H (1:200, Chemicon), human mitochondria (1:200, MAB1273, Chemicon), MAP2 (1:200, AB5622, Chemicon), GFAP (1:400, Dako), von Willebrand factor (vWF, 1:100, AB7356, Chemicon), or GDNF (1:200, AF-212-NA, R&D Systems) in PBS containing 5% NGS. After being rinsed in PBS, the sections were incubated with matching fluorescent secondary antibodies and examined by fluorescence microscopy (Carl Zeiss). The brain sections were also processed for horseradish peroxidase (HRP) IHC for vWF, GDNF, and HIF-1α. The brain sections were incubated with primary antibody against vWF (1:100, AB7356), GDNF (1:200, AF-212-NA) and HIF-1α (1:200, NB 100–105, Novus Biologicals), followed by secondary antibodies conjugated with HRP. Visualization was performed using the EDLA System (K4065A, K4065B, Dako) according to the manufacturer's instructions. To quantify the level of differentiation into neuron-like cells in the transplanted WJ-MSCs, double-positive cells out of 500∼1500 total transplanted (HuMit +) cells were counted from four sections and four fields per group (n = 3).

STATISTICAL ANALYSIS

Data are presented as the mean ± standard error (SE). Statistical analyses were performed by a one-way ANOVA with Tukey's post hoc test to compare differences between individual groups using GraphPad Prism (Graphpad, San Diego, CA). *P*-values smaller than 0.05 were considered to indicate statistical significance.

RESULTS

WJ-MSCS DIFFERENTIATE INTO NEURON-LIKE CELLS WITH FASUDIL PLUS COCL$_2$

Similar to human BM-MSCs treated with ROCK inhibitors and $CoCl_2$ [Lee et al., 2010a], WJ-MSCs were also able to differentiate into neuron-like cells upon treatment with Y27632 and $CoCl_2$ (Supplementary Fig. S1). EGM2 medium showed a better option for neuronal induction compared to M199 or DMEM based culture media. Since treatment with 30 μM of fasudil for 72 h is effective for differentiation of BM-MSCs into neuron-like cells, we tested whether 30 μM of fasudil could induce differentiation of WJ-MSCs into neuron-like cells. WJ-MSCs cultured in EGM2 in the presence of fasudil and/or $CoCl_2$ exhibited neuron-like morphology with multiple branches and long and thin processes (Fig. 1A). When total branch length/cell was analyzed in a semi-quantitative manner, treatment with fasudil or fasudil plus $CoCl_2$ was found to markedly induce the formation of long processes in WJ-MSCs (fasudil plus $CoCl_2$ = 18.3 ± 1.4 vs. vehicle control = 1.0 ± 0.3 of relative total branch length, $P < 0.001$). Culturing WJ-MSCs in hypoxic chamber (∼1% oxygen concentration) with fasudil also showed a synergistic effect on neuronal differentiation (data not shown).

To evaluate the induction levels of neuronal markers by treatment with fasudil plus $CoCl_2$, the induced cells were stained by IF. Expression of nestin, a neural stem cell maker, was decreased by $CoCl_2$ treatment alone (16.6 ± 5.4%) and by co-treatment (fasudil plus $CoCl_2$ (13.4 ± 3.4%) compared to the control cells (28.5 ± 3.8%) (Fig. 1B). Most of the cells were β-tubulin III positive (immature neuronal marker) after treatment with $CoCl_2$ alone (93.4 ± 2.2%) or fasudil plus $CoCl_2$ (86.0 ± 6.2%) compared to the control cells (77.7 ± 11.3%). Additionally, expression of NSE and NF-H (mature neuronal markers) was also increased upon $CoCl_2$ treatment alone (95.6 ± 4.0 and 88.6 ± 2.9%, respectively) and co-treatment (97.2 ± 2.9 and 91.1 ± 8.0%, respectively) compared to the each control (18.5 ± 1.5% and 16.0 ± 2.8%, respectively). Both $CoCl_2$ and fasudil promoted the expression of MAP2 (91.0 ± 4.6 and 34.8 ± 7.5%, respectively), resulting in increase of its expression by co-treatment (93.0 ± 5.2%, respectively) compared to untreated control cells (6.9 ± 1.8%). Only a small number of cells expressed GFAP (astrocyte marker), and there was no noticeable change following $CoCl_2$ treatment alone or co-treatment. Thus, co-treatment with fasudil and $CoCl_2$ effectively induced the expression of neuronal markers in WJ-MSCs. This result is consistent with the previous report that rat BM-MSCs differentiate into neuron-like cells after co-treatment with Y27632 and $CoCl_2$ [Pacary et al., 2006].

EXPRESSION OF GDNF IN WJ-MSCS ARE INCREASED BY FASUDIL TREATMENT

Because an indirect paracrine effect is one of the major mechanisms in the therapeutic effects of MSC transplantation [Seo and Cho, 2012], we investigated whether fasudil could affect the paracrine effects of MSCs. CM collected from MSCs treated with fasudil or PBS was subjected to analysis using an antibody-based angiogenesis array kit (Fig. 2A). Surprisingly, secretion of GDNF, a neurotrophic factor, was specifically increased, about 2.6-fold from fasudil treatment, whereas other angiogenic factors, including IGFBP3, IL8, angiopoietin-1, and FGF-7, showed less than 1.5-fold increases. Quantitative RT-PCR and WB analysis (Figs. 2B and C) confirmed the increase of GDNF expression at the levels of transcription (about 10.7-fold) and protein synthesis (about 2.4-fold) from fasudil (30 μM) treatment. $CoCl_2$ treatment showed no effect on GDNF expression in WJ-MSCs. ELISA data also showed an increase in GDNF secretion following treatment with fasudil (Fig. 2D). In the secretion profiles, VEGF was not the major angiogenic factor in WJ-MSCs (Fig. 2A), and its level was not changed upon fasudil treatment. Other angiogenic factors, IL-8 and IGFBP3, were present in the CM in substantial amounts without change upon fasudil treatment. This observation is also consistent with the secretion profile of WJ-MSCs of other donor type [Choi et al., 2013].

TRANSPLANTATION OF PRIMED WJ-MSCS IMPROVES FUNCTIONAL RECOVERY IN ICH RATS

We first examined in what time frame HIF-1α upregulation occurred in the ICH model. When HIF-1α was stained in brain tissues derived from the ICH rats, we found that high levels of HIF-1α were detected at the border of the injury region 1 week after injury (Supplementary

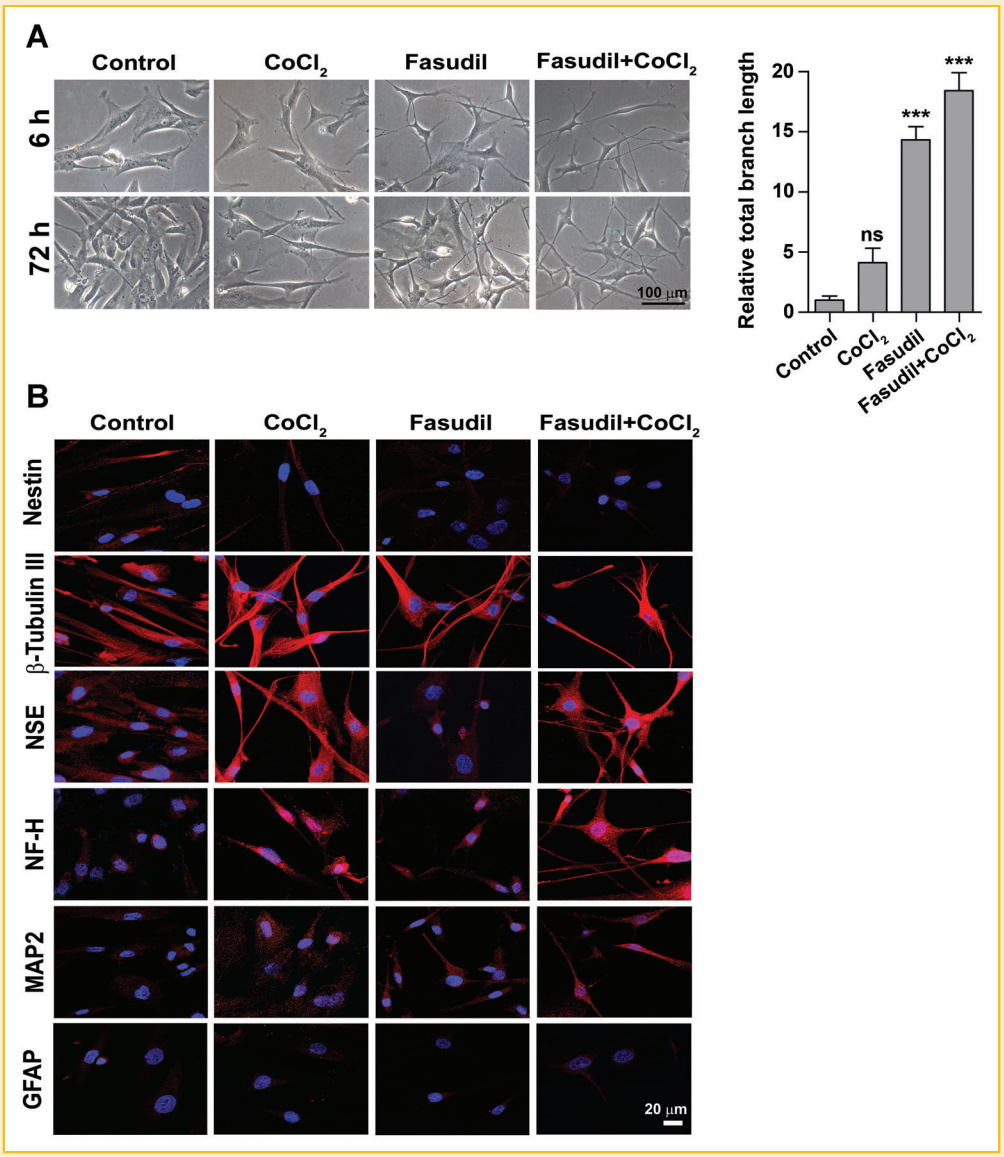

Fig. 1. Treatment of WJ-MSCs with fasudil/$CoCl_2$ induces differentiation into neuron-like cells. (A) WJ-MSCs were incubated with 100 μM of $CoCl_2$ and/or 30 μM of fasudil in EGM2 for 72 h, and the morphological changes in induced cells were examined. Relative total branch length per cell was assessed after 6 h of treatment of fasudil and/or $CoCl_2$. *** $P < 0.001$ vs. untreated control. Representative data from four independent experiments performed in triplicate are shown. (B) Induced cells were stained for nestin, β-tubulin III, NSE, NF-H, and MAP2 for neurons, GFAP for glia (in red), and nuclei (DAPI, blue).

Fig. 2). A detectable level of HIF-1α was also observed in a narrower region even at 5 weeks. We also observed that transplanted MSCs (HuMit-positive cells) migrated to the border region 2 weeks after transplantation (Supplementary Fig. S3). Thus, WJ-MSCs were primed only with fasudil (6 h) for cell transplantation because the HIF-1α upregulated area is the place where the WJ-MSCs home to and differentiate into neuron-like cells. A low concentration (10 μM) of fasudil was used to avoid cellular toxicity, since 10 μM fasudil showed effective differentiation of WJ-MSCs into neuron-like cells (Supplementary Fig. S4). MSCs primed with fasudil (2×10^5 cells) or non-primed MSCs were transplanted in ICH rats 7 days after injury, and behavioral tests were performed for up to 7 weeks. Each group showed severe neurological deficits just after surgery and partial recovery during the first week after injury (Figs. 3A and B). There was no significant difference in the behavioral test between the groups 1 week after injury. One week after transplantation, the ICH rats receiving primed or non-primed MSCs showed significantly improved behavioral performance compared with the control PBS group, and the promoting effects persisted for at least up to 7 weeks on the rotarod test (Fig. 3A). Additionally, the primed MSC group showed significantly increased functional recovery compared with the non-primed MSC group at 4 weeks post-transplantation transcription. In the limb placing test, the primed and non-primed MSC groups showed statistically significant improvements beginning at 3 and 5 weeks post-transplantation, respectively, compared with the

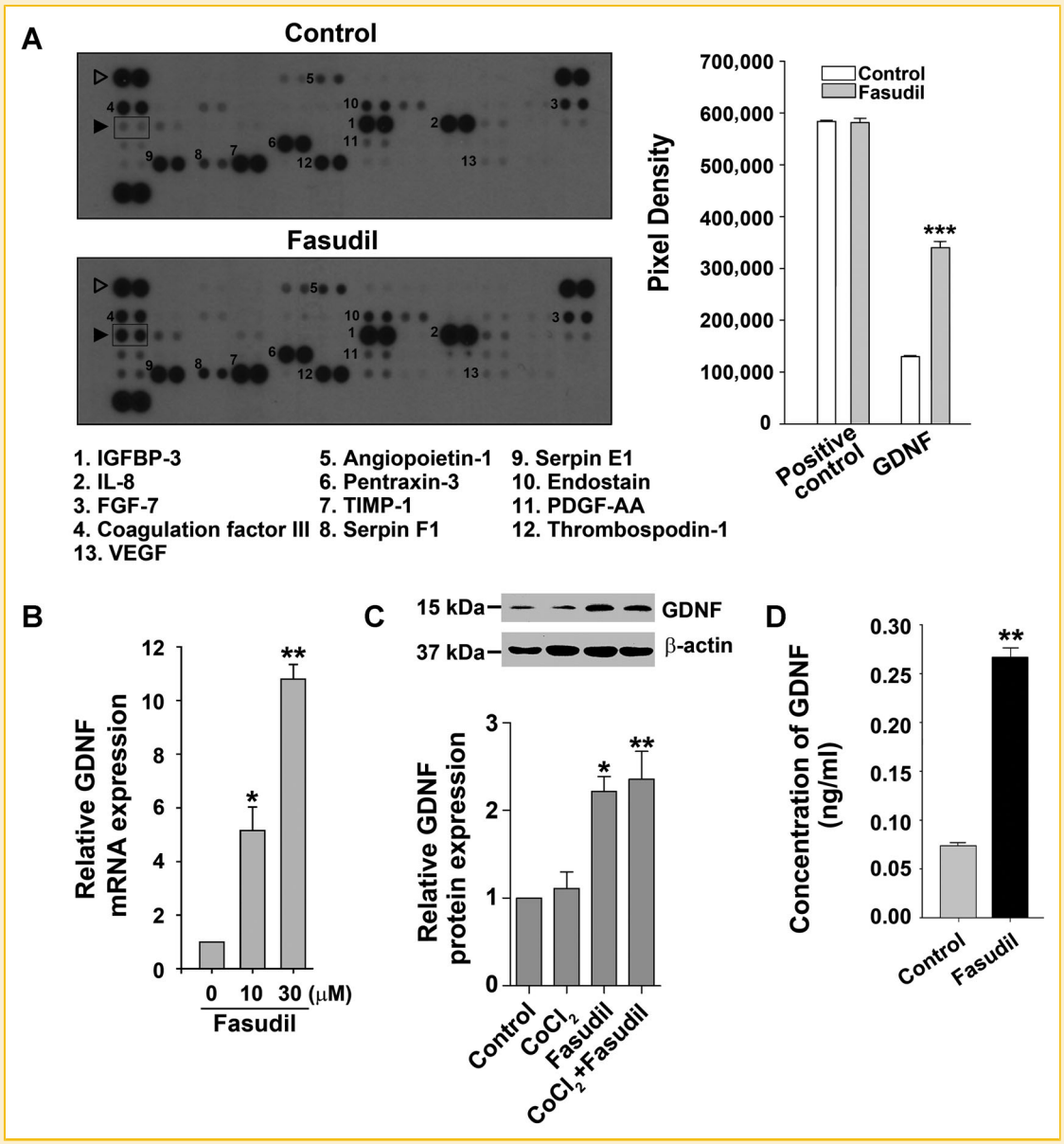

Fig. 2. Fasudil increases the expression of GDNF at the mRNA and protein levels in vitro. (A) Each CM prepared from WJ-MSCs cultured in the presence or absence (control) of fasudil was analyzed using an antibody-based angiogenesis array kit. Closed arrow heads indicate GDNF spots; open arrow heads indicate positive controls. The graph shows the relative spot pixel densities of the array images of GDNF. *** $P < 0.001$ vs. untreated control. (B) WJ-MSCs were treated with fasudil for 6 h and then processed for total RNA isolation. After cDNA synthesis, qRT-PCR was performed using human GDNF- or GAPDH-specific primers. * $P < 0.05$, ** $P < 0.01$ versus untreated control. (C) WJ-MSCs were treated with fasudil and/or $CoCl_2$ for 48 h and then processed for WB analysis. Densitometric quantitation of the Western blots was performed. The density of each protein band was normalized to that of β-actin. Values represent the mean of three independent WB experiments. * $P < 0.05$, ** $P < 0.01$ vs. untreated control. (D) The CM obtained after induction with fasudil for 48 h was concentrated 10-fold and was subjected to ELISA. Three independent experiments were performed in triplicate using WJ-MSCs of different origins, and the data shown are representative. ** $P < 0.01$ vs. untreated control.

control PBS group, and these improvements persisted for at least up to 7 weeks (Fig. 3B). The primed MSC group also showed increased functional recovery, compared with the non-primed MSC group, at 3 weeks post-transplantation; however, this difference was not statistically significant. Overall, the primed MSC group showed improved behavioral recovery compared with the non-primed MSC group.

PRIMING WITH FASUDIL INCREASES GDNF LEVELS AND MAP2 AND NF-H POSITIVE-GRAFTED CELLS IN THE ICH LESION

First, we examined the vessel density for the brain tissues derived from the primed- and non-primed MSC groups 3 weeks after transplantation. Both groups showed increased vessel density, compared with the control group, while no difference was observed between the two MSC groups (Fig. 4A). This was consistent with the

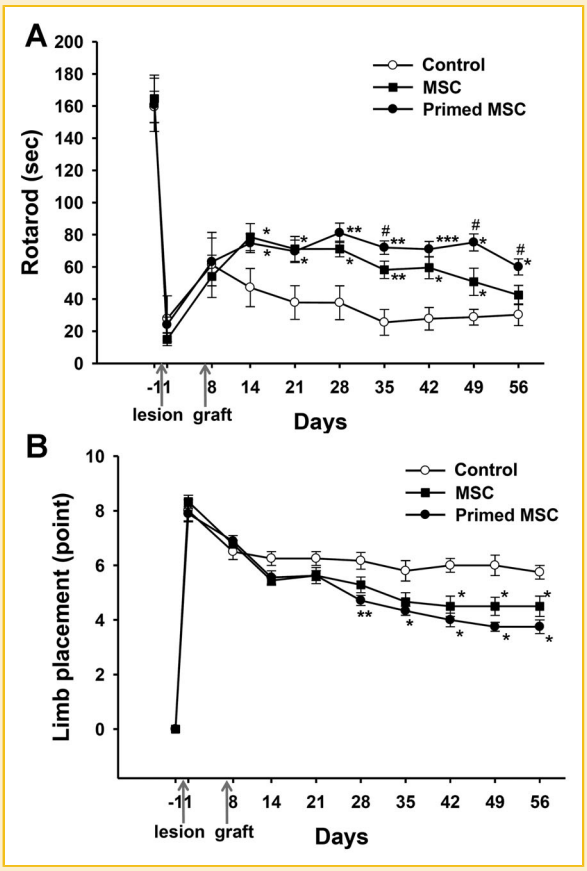

Fig. 3. Transplantation of primed MSCs improves the behavioral recovery of ICH rats. Functional recovery was demonstrated in the ICH rats ($n = 9$) following transplantation of primed with fasudil or non-primed MSCs by rotarod (A) and limb placing tests (B). *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control (PBS), # $P < 0.05$ vs. non-primed MSC group.

similar secretion profiles of angiogenesis-related proteins observed between fasudil-treated and untreated MSCs. Based on previous results, we also examined whether priming with fasudil could affect GDNF levels in the ICH lesion. In the lesion area, the level of GDNF was higher in the primed MSC group than in the non-primed MSC group (Fig. 4B). To confirm this, we also assessed the brain tissues from the ICH rats by ELISA. A higher level of GDNF (about 1.8-fold) was detected in the brain extracts of the primed MSC group compared with those of the non-primed group by ELISA (0.94 vs. 0.52 ng/g tissue; Fig. 4C). Thus, we conclude that fasudil induces expression of GDNF in transplanted WJ-MSCs in vivo.

Next, we examined whether priming with fasudil could increase differentiation into neuron-like cells in the transplanted MSCs. The phenotype of transplanted MSCs in the ICH lesion 3 weeks after transplantation was evaluated by double-fluorescence immunolabeling with HuMit and MAP2, NF-H, or GFAP (Fig. 5). Notably, the percentage of cells doubly positive for HuMit/MAP2 in the non-primed and the primed MSC groups was $3.71 \pm 1.94\%$ and $52.29 \pm 10.15\%$, respectively. In addition, the percentage of cells doubly positive for HuMit/NF-H in the non-primed and the primed MSC groups was $17.98 \pm 7.27\%$ and $26.97 \pm 5.14\%$, respectively (no statistical significance). The percentage of differentiated GFAP-positive cells was similar between the groups. Thus, these results indicate that priming MSCs with fasudil before transplantation increased MSC differentiation into neuron-like cells.

DISCUSSION

Improvement in cell therapy using MSCs is required to increase the effectiveness of therapeutic applications. The present study demonstrated that treatment with the ROCK inhibitor fasudil under hypoxic conditions (as mimicked by $CoCl_2$ treatment) efficiently induced the differentiation of WJ-MSCs into neuron-like cells. More importantly, prior priming of WJ-MSCs with fasudil improved the outcome of WJ-MSC transplantation in a rat ICH stroke model by up-regulating GDNF and increasing differentiation into neuron-like cells. Thus, priming with fasudil may be useful as a strategy for MSC transplantation, promoting regeneration and functional recovery in stroke.

In this study, we also found the optimal condition for WJ-MSC′s differentiation into neuron like cells. Pacary et al. showed neuronal differentiation of rodent BM-MSCs by treatment of ROCK inhibitor and $CoCl_2$ in 20% FBS-containing medium [Pacary et al., 2006]. We found that differentiation of WJ-MSCs by ROCK inhibitor and $CoCl_2$was more efficient in EGM2 compared to M199- or DMEM based media. EGM2 may be a good choice for priming MSCs with ROCK inhibitor. Since Y27632 blocks the inhibitory effect of neurite outgrowth inhibitor CSPG on morphological changes of MSCs into neuron-like cells [Lim and Joe, 2013], priming MSCs with fasudil may also provide a another benefit in cell transplantation.

Non-primed MSCs also significantly improved functional recovery, compared with the PBS control, albeit at a lower level than primed MSCs. Additionally, both primed and non-primed MSCs increased vessel numbers to similar levels, which could be mediated by the similar secretion profiles of angiogenic factors. Notably, protease inhibitors, such as TIMP-1, were also highly secreted from WJ-MSCs. Because MMPs have been implicated in extensive vascular damage in stroke [Kim and Joh, 2012], such inhibitors from WJ-MSCs might play a role in ameliorating the ischemic damage in stroke. The efficacy of WJ-MSCs in cell therapy is also supported by a recent report that WJ-MSCs promote functional recovery by producing growth-promoting factors after ischemia generated by middle cerebral artery occlusion and reperfusion [Lin et al., 2011]. WJ-MSCs are readily attainable, young cells with multipotent differentiation ability and have a low frequency to confront pathological situations compared to adult BM-MSCs [Troyer and Weiss, 2008]. WJ-MSCs can also be used for allogeneic transplantations [Weiss et al., 2008]. Thus, our study also suggests that WJ-MSCs may be an attractive alternative source for cell therapy in stroke in place of BM-MSCs.

Previously, WJ-MSCs have been shown to differentiate into dopaminergic neurons in vitro [Mitchell et al., 2003; Fu et al., 2006]. The therapeutic application of such induced cells has been demonstrated in the treatment of Parkinson′s disease, and the transplanted cells were identified by positive tyrosine hydroxylase staining at least 4 months after transplantation. In our study, we found that priming WJ-MSCs with fasudil results in significantly increased double-positive cells ($MAP2^+/HuMit^+$) compared with

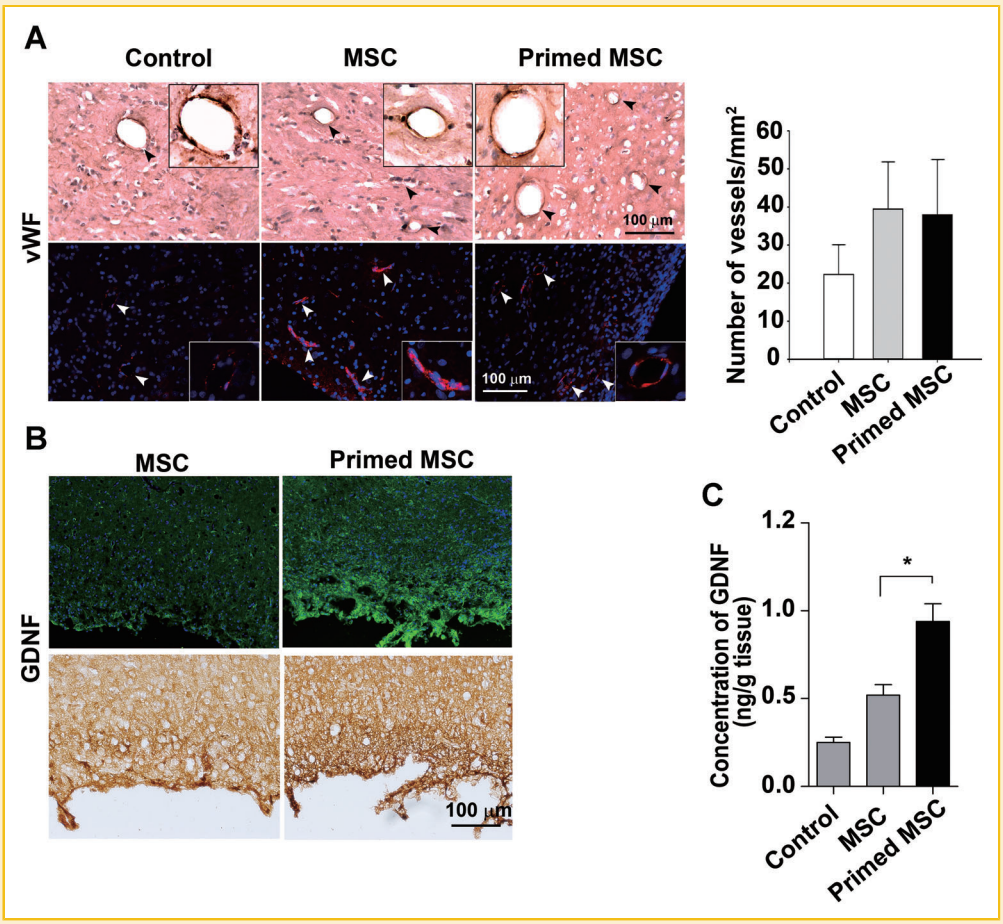

Fig. 4. Priming MSCs with fasudil increases GDNF production in the ICH lesion. (A) To evaluate any pro-angiogenic effect, four brain sections ($n = 3$/group) were stained with anti-vWF. The positively stained vessels (arrow head) within the border of the hemorrhage lesion were counted in four fields of each section. The total number of vessels/mm^2 area is presented. Boxes indicate the magnified fields. (B) Staining with anti-human GDNF antibody also showed an increase of GDNF expression upon priming with fasudil. (C) The ELISA analysis of the brain tissue extract from the ICH rats also showed an increase in the GDNF level in the primed MSC group. * $P < 0.05$.

non-primed cells. Thus, our results support that priming MSCs with fasudil may provide an advantage in MSC therapy for stroke.

When we examined the paracrine effects of fasudil-primed WJ-MSCs through antibody-based protein array analysis, unexpectedly, we found that GDNF was the only factor among the paracrine factors tested that was markedly increased by fasudil treatment (2.6-fold). GDNF, a potent neurotrophic factor, promotes the survival of central nervous system neurons in vitro [Lin et al., 1993]. GDNF also provides favorable outcomes in animal models of ischemic stroke [Wang et al., 1997; Kobayashi et al., 2006]. However, repeated administration of GDNF through the intracerebral or intraventricular space is required in experimental animals to sustain neuroprotective and neurotrophic activity because the beneficial effects of GDNF are transient and short lived. Thus, gene therapy with GDNF has been attempted using stem cells as vehicles [Horita et al., 2006; Lee et al., 2009]. Considering the uncertainty regarding the safety of gene therapy, brain transplantation of primed WJ-MSCs that produce high levels of GDNF will be an attractive strategy to deliver GDNF steadily in the ICH lesion site. In this study, 1.8-fold higher levels of GDNF were detected in the brain extracts of the primed MSC group compared to those of the non-primed group. It appears that the fasudil-primed MSCs were effective in secreting GDNF in the ICH lesion site, and this might result in more improvement in motor performance by promoting endogenous neurogenesis. Recently, human BM-MSCs alone have been reported to protect catecholaminergic and serotonergic neuronal perikarya and the transport function from oxidative stress by the secretion of GDNF [Whone et al., 2012]. Thus, upregulation of GDNF by fasudil may be an important benefit obtained by fasudil treatment in cell therapy for neurodegenerative disease.

Notably, transcriptomic profiling of astrocytes treated with fasudil (100 μM) revealed an up-regulation of genes associated with motility, cytoskeletal reorganization, extracellular matrix, and, unexpectedly, EAAT2, BDNF, anti-oxidant species, and metabolic and signaling genes [Lau et al., 2012]. Moreover, changes in Rho-ROCK activation and in cytoskeletal tension, which controls cell shape, affect MSC differentiation [McBeath et al., 2004]. Thus, it cannot be ruled out that the morphological change into neuron-like cells caused by ROCK inhibitors might trigger the expression of GDNF or neuronal markers. In future research, the underlying mechanism of GDNF up-regulation and the optimal concentration of fasudil for priming MSCs for in vivo functions should be addressed to

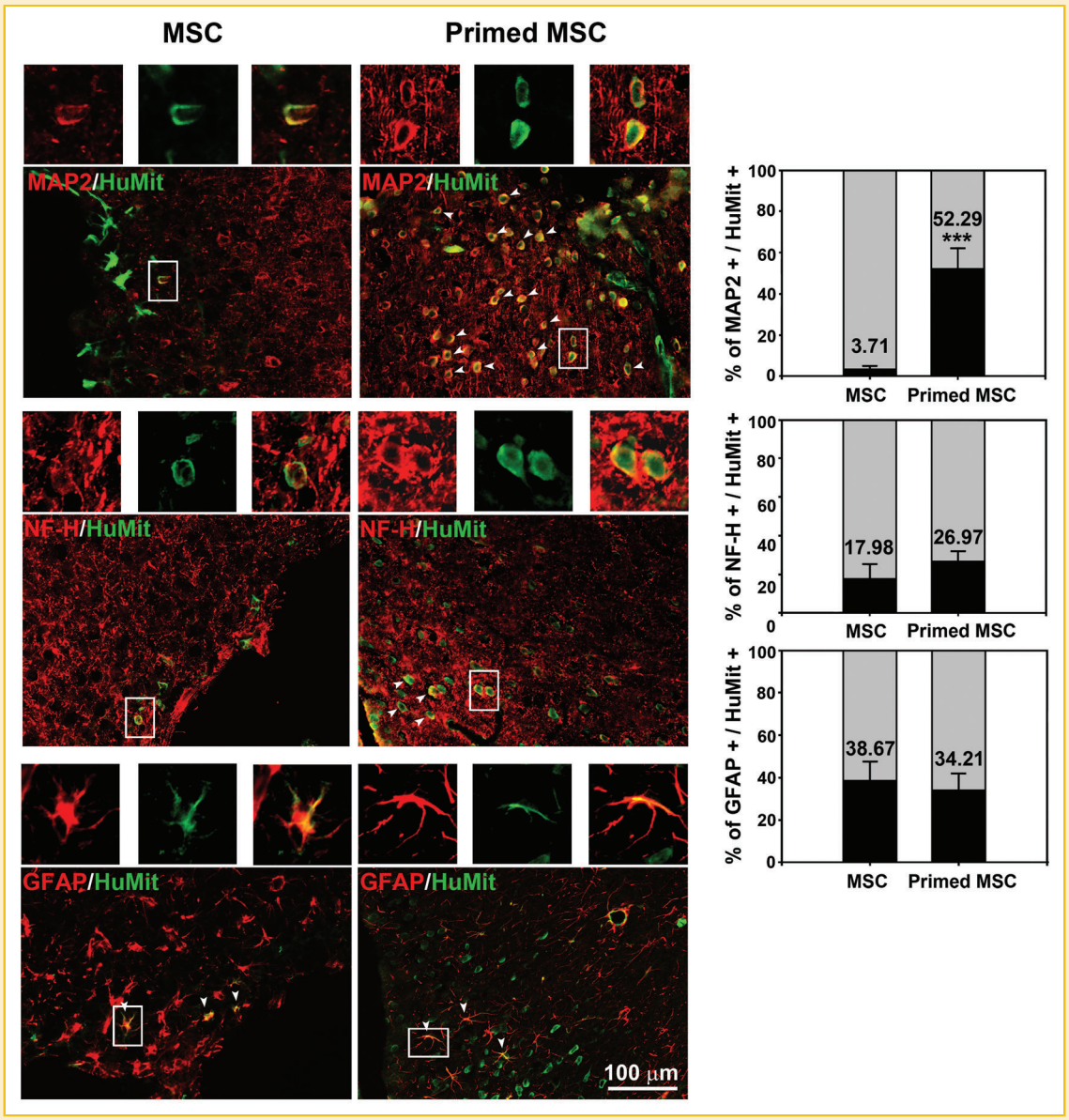

Fig. 5. Priming MSCs with fasudil increases MAP2 and NF-H positive-grafted cells in the ICH lesion. Three weeks after cell transplantation, HuMit-positive cells (engrafted cells, green) were found in the lesion border, and some of them were also positive for MAP2, NF-H, and GFAP (red). The double-positive cells were counted and displayed as a percentage of total HuMit-positive cells. Boxes indicate the magnified fields. Arrowheads indicate HuMit-positive cells colocalized with MAP2, NF-H, or GFAP. *** $P < 0.001$ vs. non-primed MSC group.

identify the optimal conditions for efficient cell therapy. Systemic administration of fasudil can be accompanied with transplantation of MSCs primed with fasudil as another option for improving therapeutic efficacy, since systemic administration of fasudil alone is effective in acute stroke [Rikitake et al., 2005]. Chemotactic factors, such as G-CSF, could also be used to enhance the therapeutic effect of fasudil-primed MSCs by improving the migration of transplanted cells after stroke [Popa-Wagner et al., 2010].

In summary, our study explains the potential therapeutic benefits of readily obtainable WJ-MSCs for the treatment of stroke in ICH rats. More importantly, it provides evidence for an increased therapeutic benefit of WJ-MSCs in ICH rats by priming cells with fasudil, the only ROCK inhibitor currently used clinically. The underlying mechanism is likely to involve the up-regulation of GDNF and the increased differentiation into neuron-like cells. Thus, a strategy of priming MSCs with fasudil before transplantation may be useful for cell therapy in stroke.

ACKNOWLEDGMENTS

We thank Dr. Seung U. Kim (University of British Columbia, Canada) for helpful comments on the manuscript. This work was supported by the National Research Foundation of Korea grants by the Korean Government (NRF-2009-0079656, NRF-2012R1A5A2047939).

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