Mesenchymal Stem Cells Prevent Hydrocephalus After Severe Intraventricular Hemorrhage

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Background and Purpose—Severe intraventricular hemorrhage (IVH) in premature infants and the ensuing posthemorrhagic hydrocephalus cause significant mortality and neurological disabilities, and there are currently no effective therapies. This study determined whether intraventricular transplantation of human umbilical cord blood-derived mesenchymal stem cells prevents posthemorrhagic hydrocephalus development and attenuates brain damage after severe IVH in newborn rats.

Methods—To induce severe IVH, 100 μL of blood was injected into each lateral ventricle of postnatal day 4 (P4) Sprague-Dawley rats. Human umbilical cord blood-derived mesenchymal stem cells or fibroblasts (1×10⁵) were transplanted intraventricularly under stereotaxic guidance at P6. Serial brain MRI and behavioral function tests, such as the negative geotaxis test and rotarod test, were performed. At P32, brain tissue and cerebrospinal fluid were obtained for histological and biochemical analyses.

Results—Intraventricular transplantation of umbilical cord blood-derived mesenchymal stem cells, but not fibroblasts, prevented posthemorrhagic hydrocephalus development and significantly attenuated impairment on behavioral tests; the increased terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling-positive cells; increased expression of inflammatory cytokines, such as interleukin-1β, interleukin-1α, interleukin-6, and tumor necrosis factor-α; increased astrogliosis; and reduced corpus callosal thickness and myelin basic protein expression after inducing severe IVH.

Conclusions—Intraventricular transplantation of umbilical cord blood-derived mesenchymal stem cells significantly attenuated the posthemorrhagic hydrocephalus and brain injury after IVH. This neuroprotective mechanism appears to be mediated by the anti-inflammatory effects of these cells. (Stroke. 2013;44:XXX-XXX.)

Key Words: animal ■ cell transplantation ■ hydrocephalus ■ inflammation ■ intraventricular hemorrhage ■ newborn

Intraventricular hemorrhage (IVH) is a serious complication of preterm birth, and the number of infants at high risk for developing IVH is increasing as a result of the rise in the absolute number and improved survival of very premature infants with recent advances in neonatal intensive care medicine. Over half of infants with severe IVH (grade ≥3) die or develop posthemorrhagic hydrocephalus (PHH), which requires shunt surgery in up to 70% of cases. IVH is associated with brain damage, especially to the periventricular white matter, which is exacerbated by PHH, and finally results in increased mortality and long-term neurological morbidity, such as seizure, cerebral palsy, and developmental retardation in survivors. Until now, however, there has not been any effective treatment to prevent PHH or ameliorate brain damage after severe IVH in preterm infants, so it remains a major problem of neonatal intensive care.

Although the precise mechanism has not been completely delineated, the pathogenesis of communicating progressive posthemorrhagic ventricular dilatation has been explained by inflammation within subarachnoid spaces attributable to blood contact and deposition of blood products. This obliterative arachnoiditis leads to dysfunction of arachnoid granulations, which reduces cerebrospinal fluid (CSF) resorption and increases intracranial pressure, resulting in venous infarction with decreased cerebral perfusion. Moreover, inflammatory cytokines originating from blood products in the cerebral ventricles may injure the periventricular white matter. Therefore, new therapeutic modalities with anti-inflammatory capabilities to treat PHH and brain damage after severe IVH would be of great value.

Recent preclinical research reported that an anti-inflammatory agent, a cyclooxygenase-2 inhibitor, noticeably reduced reactive gliosis and improved neurological impairment after IVH in a newborn rabbit model. This finding indicates that modulating inflammation could be a key factor in IVH therapy for preterm...
infants. In this context, mesenchymal stem cells (MSCs) may be a promising candidate because accumulating data have demonstrated potent immunomodulating abilities in the brain after stroke or neonatal hypoxic ischemic encephalopathy.\(^\text{11–13}\) Recently, we also reported that transplanting of human umbilical cord blood (UCB)-derived MSCs significantly attenuated several disorders, such as bronchopulmonary dysplasia,\(^\text{4,15}\) acute respiratory distress syndrome,\(^\text{16}\) and middle cerebral arterial occlusion,\(^\text{17}\) through paracrine effects. These findings suggest that transplanting MSCs could be a novel therapy to prevent PHH after severe IVH. In the present study, we investigated whether transplanting human UCB-derived MSCs attenuates PHH and improves neuronal recovery after severe IVH in newborn rats and, if so, whether the protective effects of human UCB-derived MSCs are associated with their anti-inflammatory activity.

**Methods**

**Cell Preparation**

This study was approved by the Institutional Review Board of Samsung Biomedical Research Institute, Seoul, Korea. As previously reported, UCB was collected from umbilical veins after neonatal delivery with informed consent from pregnant mothers, and MSCs were isolated and cultivated.\(^\text{16,19}\) We confirmed the differentiation potential and karyotypic stability of the human UCB-derived MSCs up to 11 passages.\(^\text{16–20}\) Human fetal lung fibroblasts (MRC-5; Korean Cell Line Bank No.10171) were obtained from the Korean Cell Line Bank (Seoul, Korea). Human UCB-derived MSCs from a single donor were cultured with super paramagnetic iron oxide particles (Feridex I. V., Taejon Pharmaceutical Co Ltd, Seoul, Korea). Human UCB-derived MSCs significantly attenuated several disorders, such as bronchopulmonary dysplasia, acute respiratory distress syndrome, and middle cerebral arterial occlusion, through paracrine effects. These findings suggest that transplanting MSCs could be a novel therapy to prevent PHH after severe IVH.

**Animal Model**

The experimental protocols described herein were reviewed and approved by the Animal Care and Use Committee of Samsung Biomedical Research Institute, Seoul, Korea. This study was also performed in accordance with Institutional and National Institutes of Health Guidelines for Laboratory Animal Care. Newborn Sprague-Dawley rats (Orient Co, Seoul, Korea) in culture medium to cultured with super paramagnetic iron oxide particles (Feridex I. V., Taejon Pharmaceutical Co Ltd, Seoul, Korea) in culture medium to induce endocytosis and were dual-labeled with PKH26GL Red Fluorescent Cell Membrane Labeling Kit (Sigma-Aldrich, St. Louis, MO) for transplantation according to the manufacturer protocol, as previously reported.\(^\text{14,15,17}\) The localization of transplanted donor cells was confirmed as described in the online-only Data Supplement.

**In Vivo MRI and Assessment of the Ventricle to Whole Brain Volume Ratio**

Brain MRI was performed on neonatal rats to confirm severe IVH at P5 and to monitor subsequent PHH at P11 and P32 by an investigator blinded to the treatment groups. MRI was performed using a 1.0-Tesla MRI system (Bruker-Biospin, Fallanden, Switzerland) as described previously and in the online-only Data Supplement.\(^\text{17}\) The ventricle to whole brain volume ratio was calculated for each pup. Two blinded independent examiners calculated the volume ratio by manually outlining the ventricle and the whole brain in 12 MRI slices using ParaVision software (version 2.0.2, Bruker, BioSpin, Karlsruhe, Germany) as previously described.\(^\text{17}\) Volume estimates were then made according to Cavalieri’s principal,\(^\text{17}\) and the ventricle to whole brain volume ratio was calculated to determine the extent of PHH after severe IVH.
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Functional Behavioral Tests
Negative geotaxis tests were performed at P11, P18, P25, and P32, as described in the online-only Data Supplement. The time required for pups to rotate 180° to face uphill after release was recorded. The values were analyzed by date, and the average time from 3 trials was used as the final result. Rotarod tests were performed consecutively at P30 to P32 to assess long-term effects on motor function by analyzing the latency to fall on a treadmill test.22,23 All animals were tested 3 times on 3 consecutive days with a 15-minute intertrial interval. Because rats have the ability to learn rotarod tests, the values were analyzed by date, and the average latency to fall from 3 trials was used as the final result. All behavioral tests were conducted by 2 independent evaluators who were blind to the groups.

Measurement of Corpus Callosum Thickness
Sections (4-μm thick) were cut from paraffin blocks and stained with hematoxylin and eosin. The thickness of the anterior corpus callosum was measured in the midline of the coronal sections at the level of the medial septum area (+0.95 mm to −0.11 mm/Bregma) on hematoxylin and eosin stained slides.

TUNEL Assay
Cell death in the periventricular white matter was assessed with the immunofluorescent terminal deoxynucleotidyltransferase-mediated deoxuryridine triphosphate nick-end labeling (TUNEL) technique (kit S7110 ApopTag, Chemicon, Temecula, CA). A blinded evaluator counted the TUNEL-positive nuclei in the periventricular area, including the corpus callosum and caudate nucleus, on coronal brain sections. Three coronal sections (+0.95 mm to −0.11 mm/Bregma) were counted from each brain and 3 random nonoverlapping fields from each section.

Immunohistochemistry
Immunohistochemical analysis of gliosis (neuronal specific glial fibrillary acidic protein [GFAP]), myelination (myelin basic protein [MBP]), reactive microglia (ED-1), and neuron-specific marker (neuronal nuclear antigen) was performed on deparaffinized 4-μm thick brain sections as previously reported.17 Three coronal sections (+0.95 mm to −0.11 mm/Bregma) were stained from each brain, and 3 random nonoverlapping fields were assessed from each section. The immunofluorescent GFAP or MBP staining intensity was measured in 3 random fields of the corpus callosum area on each of 3 coronal sections by a blinded observer using Image J software (National Institutes of Health).

ELISA
Interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α concentrations in tissue homogenates and CSF (online-only Data Supplement) were measured using the Milliplex MAP ELISA Kit according to the manufacturer protocol (Millipore, Billerica, MA).

Statistical Analyses
Estimation of sample size was based on the preliminary results of the pilot study. Data are expressed as the mean±SD. For continuous variables with a normal distribution, statistical comparison between groups was performed by 1-way ANOVA test with Bonferroni correction. A P<0.05 was considered significant. Stata software (version 11.0, Stata Corp LP, College Station, TX) was used for analyses.

Results
Survival and Body Weight
IC rats had the least weight gain than other groups, but transplanting MSCs or fibroblasts did not significantly improve weight gain (final weight at P32: 151±14 g, 139±17 g, 143±7 g, and 145±16 g in NC, IC, IF, and IM, respectively). There was no significant difference in survival among groups: 10 of 10 (100%), 15 of 18 (83%), 12 of 14 (86%), and 14 of 16 (88%) in NC, IC, IF, and IM group, respectively.

Serial Brain MRI
Ventricles dilated and filled with blood were confirmed by brain MRI 1, 7, and 28 days after inducing IVH (P5, P11, and P32, respectively). Figure 2 displays serial brain MRIs of each group at each time point (P5, P11, and P32). At P5, 1 day before MSC transplantation, the ventricular dilatation severity, presented as a ratio ventricle to whole brain volume, did not differ among the IC, IF, and IM groups (13.9±5.4%, 12.3±3.1%, and 16.3±5.9%, respectively). At P11 and P32, after cells or vehicle transplantation, the IC group presented
active progression of ventriculomegaly, which was significantly attenuated in the IM (*P<0.05), but not in the IF (ventricle to whole brain ratio at P11: 1.5±0.1%, 15.1±10.1%, 13.9±6.6%, and 9.0±4.7%; at P32: 1.6±0.0%, 19.3±14.1%, 19.1±9.3%, and 9.6±7.5% in NC, IC, IF, and IM, respectively). These results suggest that transplanting MSCs significantly attenuates PHH after severe IVH.

**Functional Behavior Tests**

To assess the sensorimotor function, the negative geotaxis test and rotarod test were performed.

In the negative geotaxis test, IC animals showed a marked functional impairment over time, and MSC treatment (IM) significantly improved this performance at P18 and P32 (duration in seconds at P32: 3.1±1.8, 5.4±3.1, 6.3±2.8, and 3.4±1.5 in the NC, IC, IF, and IM groups, respectively; IC vs IM, *P<0.05*). Fibroblast treatment (IF), however, was not different from IC in the negative geotaxis test at any time point (Figure 3A). The rotarod test was done at P30, P31, and P32. Although initial rotarod test results at P30 were not significantly different between groups, the IC group had a significantly shorter latency to falling at P31 and 32 compared with the NC group. This impaired function significantly improved over time after MSC (but not fibroblast) transplantation (duration in seconds at P32: 176±14.1, 74±9.3, 91±7.5, and 131±9.3 in the NC, IC, IF, and IM groups, respectively; IC vs IM, *P<0.05*; Figure 3B).

**Cell Death, Reactive Gliosis, and Myelination**

To determine whether MSC treatment attenuated periventricular cell death and reactive gliosis after severe IVH, the number of TUNEL-positive cells and the density of GFAP-positive cells in the periventricular area were assessed in brain tissue at P32 by immunohistochemistry. The number of TUNEL-positive cells and density of GFAP-positive cells increased in the CSF and periventricular tissue compared with the NC group (Figure 4). Myelination in the periventricular area was also evaluated at P32. The optical density of MBP, by immunostaining, indicated that myelination was significantly reduced in IC animals compared with NC animals, and this impaired myelination was significantly improved in IM but not in IF animals (Figure 5A and 5B).

**Corpus Callosum Thickness**

As ventricular dilatation progressed, the IVH groups presented a significantly thinner anterior corpus callosum (measured in the midline of coronal sections at the level of the medial septum) at P32 than the NC group (*P<0.01*, 1-way ANOVA; Figure 5C and 5D). Transplanting MSCs, but not fibroblasts, significantly attenuated compression of the periventricular corpus callosum induced by PHH after severe IVH (IC, 0.25±0.06 mm; IF, 0.22±0.07 mm; IM, 0.44±0.07 mm; *P=0.49*, *P<0.05*; respectively; Figure 5D).

**Inflammatory Cytokines in CSF and Brain**

To determine whether transplanted MSCs attenuated brain inflammation induced by severe IVH, we analyzed inflammatory cytokine concentrations, including *IL-1α*, *IL-1β*, *IL-6*, and *TNF-α* in the CSF at P18 and P32 and in periventricular brain tissue homogenates at P32.

Levels of inflammatory cytokines in the CSF and periventricular tissue revealed a statistically significant difference between groups (*P<0.01*; 1-way ANOVA). In the IC and IF groups, *IL-1α* (*P<0.001* vs NC), *IL-1β* (*P<0.001* vs NC), *IL-6* (*P<0.001* vs NC), and *TNF-α* (*P<0.001* vs NC) were increased in the CSF and periventricular tissue compared with the NC group (Figure 6A–6C). This increase in inflammatory cytokines was significantly attenuated by intraventricular MSC transplantation (CSF at P18: *IL-1α*, *P<0.001* vs IC; *IL-1β*, *P<0.001* vs IC; *IL-6*, *P<0.01* vs IC; *TNF-α*, *P<0.001* vs IC; CSF at P32: *IL-1α*, *P<0.05* vs IC; *IL-1β*, *P<0.01* vs IC; *IL-6*, *P<0.001* vs IC; *TNF-α*, *P<0.01* vs IC; periventricular tissue at P32: *IL-1α*, *P<0.01* vs IC; *IL-1β*, *P<0.01* vs IC; *IL-6*, *P<0.01* vs IC; *TNF-α*, *P<0.01* vs IC; *IL-6*, *P<0.01* vs IC; *TNF-α*, *P<0.01* vs IC).

Figure 3. Transplantation of human umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs) improved sensorimotor function. Sensorimotor functional outcomes on the negative geotaxis test (A) and rotarod test (B). Data are expressed as mean±SEM. IC indicates intraventricular hemorrhage (IVH) control rats; IF, IVH with human fibroblast transplantation; IM, IVH with UCB-derived MSC transplantation; and NC, normal control rats. *P<0.05* vs NC; †*P<0.05* vs IVH-M; #*P<0.05* vs IC.
IL-6, $P<0.001$ vs IC; TNF-α, $P<0.001$ vs IC). However, none of significant improvement was observed in the IF (IC vs IF, $P>0.05$). The number of macrophages (ED-1–positive cells) in the periventricular area was assessed in the brain tissue at P32. IC and IF presented a significantly higher number of macrophages compared with NC, and MSC transplantation significantly attenuated the number of ED-1–positive cells ($P<0.05$; Supplemental Figure III). However, no significant difference in number of ED-1–positive cells was observed between IF and IC.

Confirmation of Donor Cells
T2-weighted image revealed a low-signal intensity area reflecting the presence of transplanted super paramagnetic iron oxide-tagged MSCs. The persistence of this signal in the bilateral periventricular areas for 28 days after inducing IVH (P32) suggests that MSCs migrated to the injured area (Supplemental Figure IIA). This conclusion is further supported by deposition of PKH26-positive red fluorescent donor cells, especially in the periventricular area, in IM animals but not in the IC group (Supplemental Figure IIB).

Discussion
Severe IVH and the ensuing PHH are common and serious complications of premature birth that increased mortality and neurological morbidities. Currently, there are no clinically effective therapies available; therefore, developing an appropriate animal model to simulate clinical IVH in premature infants is essential for elucidating its pathophysiologic mechanism and testing the efficacy of potential new treatments. Cherian et al.\(^{24}\) reported ventriculomegaly in 65% of Wistar rat pups after biventricular injection of 80 uL fresh maternal whole blood at P7, with a 20% mortality rate. In the present study, we consistently induced PHH after severe IVH with a comparable 17% mortality rate using P4 instead of P7 rats, because severe IVH is common in less mature infants.\(^{25}\) We also increased the volume of blood infused into each ventricle to 100 uL because the IVH severity correlates with the likelihood of PHH developing.\(^{3}\) Our data showing persistent PHH, impaired behavioral function, and histological abnormalities after severe IVH indicate that our newborn rat pup model is suitable and appropriate to research preterm IVH and the ensuing PHH.

In the present study, intraventricular transplantation of human UCB-derived MSCs, but not fibroblasts, significantly attenuated PHH after severe IVH, impaired behavioral function on the negative geotaxis and rotarod tests, histological abnormalities such as increased astrocytic gliosis and TUNEL-positive cells, and reduced corpus callosum thickness and MBP. We previously showed the protective effects of transplanting human UCB-derived MSCs against various disorders, such as bronchopulmonary dysplasia,\(^{14}\) acute respiratory distress syndrome,\(^{15}\) and neonatal stroke.\(^{16}\) Moreover, the safety and feasibility of MSCs have been demonstrated in ongoing clinical trials, including a phase I clinical trial of MSC transplantation for bronchopulmonary dysplasia (BPD).
Taken together, these findings strongly support the potential use of human UCB-derived MSCs as a novel therapeutic modality for severe IVH, for which effective treatments have not been established. Recently, MSCs have generated great interest as a potent therapeutic modality for brain injury as a result of increasing evidence of their multipotency, including anti-inflammatory, antioxidative, and angiogenic activities.26–28 In addition, among the various MSCs sources, including bone marrow, adipose tissue, and UCB,29 UCB is considered promising because of its availability, high proliferation capacity,19,29 and low immunogenicity.30 Furthermore, the low expression of human leukocyte antigen major histocompatibility complex class I and the deficit of major histocompatibility complex class II molecules in UCB-MSCs suggest they will be well tolerated, even from allogeneic sources.31 Our previous studies showed that xenotransplantation of human UCB-MSCs into immunocompetent wild-type rats did not result in any of the apparent gross or microscopic findings consistent with abnormal immunologic reactions.14,15

Besides attenuating PHH and abnormal histology, improving functional outcome is important for the clinical application of MSCs for severe IVH. In the present study, the initial IVH was severe and sensorimotor function improved only after transplanting MSCs, not fibroblasts. In rats, the postural reflex to face uphill on a slanted slope develops in the second week of life.32 We found that, although the initial negative geotaxis test performed at P11 did not differ significantly between the groups, MSC transplantation significantly improved the outcome on follow-up tests at P25 and P32. These findings suggest that increased pressure attributable to progressive hydrocephalus after severe IVH might contribute to periventricular white matter injury and demyelination, as evidenced by corpus callosum thinning and reduced MBP expression.33,34 In addition, the protective effects of MSC transplantation might be attributable, at least in part, to preventing PHH. The improved

![Figure 5](http://stroke.ahajournals.org/)

Figure 5. Stem cell transplantation improved delayed myelination and attenuated corpus callosum thinning induced by posthemorrhagic hydrocephalus. **A**, Representative immunofluorescence photomicrographs of the periventricular area in each group with staining for myelin basic protein (MBP; green) and 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue; original magnification ×400; scale bars=25 μm). **B**, Average MBP density in each group. **C**, Representative optical microscopic photographs of the corpus callosum stained with hematoxylin and eosin (original magnification ×100; scale bars=100 μm). **D**, Corpus callosum thickness in each group. Data are expressed as mean±SEM. IC indicates IVH control rats; IF, IVH with human fibroblast transplantation; IM, IVH with human UCB-derived MSC transplantation; and NC, normal control rats. *P<0.05 vs NC; †P<0.05 vs IVH-M; ‡P<0.05 vs IC.
rotarod tests results at P31 and P32 after MSC transplantation imply that the protective effects might persist into human adolescence. None of these advantages were observed after fibroblast transplantation group, even though fibroblasts are morphologically similar to MSCs.

The precise mechanism by which human UCB-derived MSCs protect against severe IVH-induced brain injury has not yet been elucidated. Inflammatory responses of the choroid plexus and the ependymal lining of the ventricles caused by blood products and inflammatory cytokines play a pivotal role in the development of PHH and brain injury after severe IVH. The present study, transplanting human UCB-derived MSCs not only prevented PHH and significantly attenuated the impaired behavioral function and histological abnormalities, but also significantly downregulated the increased inflammatory cytokines, such as IL-1α, IL-1β, IL-6, and TNF-α, observed after severe IVH. Furthermore, PKH26-positive donor cells showed little colocalization with GFAP (astrocytic protein) or neuronal nuclear antigen, indicators of astrogliosis or neuronal differentiation, respectively (Supplemental Figure IV). Overall, these findings suggest that human UCB-derived MSCs protection against PHH and brain damage after severe IVH might be primarily mediated by or associated with their anti-inflammatory effects rather than their regenerative capabilities.

Although the anti-inflammatory action of MSCs has been reported in various disorders, including BPD, acute respiratory distress syndrome, and neonatal stroke, the mechanism remains to be delineated. Anti-inflammatory effects of MSC transplantation might include immune modulation and delivery of various growth factors and cytokines, such as vascular endothelial growth factor, hepatocyte growth factor, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, and IL-10. By secreting these trophic factors, MSCs might promote a shift from a proinflammatory environment toward an anti-inflammatory or tolerant environment. Further studies will be necessary to identify the trophic factors secreted from transplanted stem cells that might mediate this anti-inflammatory effect.

Determining the therapeutic time window is essential to translate these experimental results to clinical trials. In the present study, we demonstrated that MSC transplantation was effective 2 days after inducing severe IVH. Having proven the efficacy of delayed MSC transplantation, our next step will be to test the efficacy of MSC transplantation after longer delays, for example, 1 week after inducing IVH.

Determining the optimal route to transplant MSCs is another issue that must be addressed. In the present study, MSCs were administered intraventricularly because local intraventricular stem cell administration is more effective than systemic intravenous delivery. CSF circulation could allow for less invasive delivery, such as intraspinal transplantation, although further studies are necessary to confirm this. Further study for appropriate therapeutic dose of cells is also required to optimize therapeutic effects of MSCs for the attenuation of brain injury after severe IVH.

In summary, intraventricular transplantation of human UCB-derived MSCs, but not fibroblasts, significantly attenuated PHH, impaired behavioral function, increased TUNEL- and GFAP-positive cells, and reduced corpus callosum thickness and MBP expression, which are observed after inducing severe IVH. Our findings suggest that the protective effects of MSCs might be mediated by their anti-inflammatory effects rather than their regenerative capabilities.

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Disclosures

Dr Oh would like to declare the following potential conflict of interest situation arising from the position in a company as board membership and ownership of stocks. The other authors have no conflicts to report.

References

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Title: Mesenchymal stem cells prevent hydrocephalus after severe intraventricular hemorrhage
**Supplemental methods**

*Human umbilical cord blood (UCB) derived mesenchymal cell (MSC)*

These cells expressed CD105 (99.6%) and CD73 (96.3%), but not CD34 (0.1%), CD45 (0.2%), and CD14 (0.1%)\(^1\). They were positive for human leukocyte antigen (HLA)-AB (96.8%), but generally not for HLA-DR (0.1%). The cells also expressed pluripotency markers such as octamer-binding transcription factor 4 (Oct 4; 30.5%)\(^2\) and stage-specific embryonic antigen 4 (SSEA-4; 67.7%)\(^3\). Human UCB-derived MSCs differentiated into various cell types such as respiratory epithelium, osteoblasts, chondrocytes, and adipocytes with specific *in vitro* induction stimuli\(^1,4-6\). We confirmed the differentiation potential and karyotypic stability of the hUCB-MSCs up to the 11th passage.

**Sample preparation**

Whole brain tissue was harvested after transcardiac perfusion with ice-cold PBS. For histologic analyses, the brain was fixed with 4% paraformaldehyde overnight at room temperature and then embedded in paraffin wax (NC, n=5; IC, n=7; IM, n=5; IF, n=7) or incubated in a 30% sucrose solution and frozen for cell confirmation (IC, n=1; IM, n=3). For biochemical analyses, tissue of the periventricular zone of the brain including the corpus callosum was selectively obtained, snap-frozen in liquid nitrogen, and stored at -80°C (NC, n=5; IC, n=7; IM, n=6; IF, n=5). At P18 and P32, CSF was obtained by cisternal tap and stored at -80°C for later biochemical analyses (NC, n=10; IC, n=15; IM, n=14; IF, n=12).

*In vivo magnetic resonance imaging (MRI)*

MRI examination was performed under anesthesia with 1.5-2% isoflurane in oxygen-enriched air using a 7.0-teslar MRI system (Bruker-Biospin, Fällanden, Switzerland). MRI was performed with a 20 cm gradient set capable of providing a rising time of 400 mTm\(^1\). Images were acquired for a total of 12 coronal slices with 1.0 mm slice thickness and no interslice gap. For T2-weighted images (T2WI), fast spin echo sequence was used to acquire the image using repetition time (TR)=3,000 ms, time to echo (TE)=60 ms, field of view (FOV)=25.6 mm x 25.6 mm, matrix size=256 x 256, number of excitations (NEX)=12. For T2*WI, a FLASH (fast low angle shot) sequence was used to acquire the images using TR=208 ms, TE=10 ms, flip angle=15°, FOV=25.6 mm x 25.6 mm, matrix size=256 x 256, NEX=12. One MRI session took 30 minutes per pup on average and all sessions included T2WI and T2*WI.

**Functional behavioral tests**

A negative geotaxis test, based on the innate reflex rotation to face uphill when placed head down on an inclined wooden platform\(^7\), was performed on the 7\(^{th}\), 14\(^{th}\), 21\(^{st}\), and 28\(^{th}\) day after IVH induction (P11, 18, 25, and 32). Pups were gently held for 3-5 seconds in a head downward position on a slanted slope and the time required for the pups to rotate 180° to face uphill after release was recorded. The pups were observed for up to 60 seconds and if the pup could not complete this test in 60 seconds or fell down from the slope more than three times, the score was recorded as 60 seconds.

Rotarod tests were performed consecutively on the 26\(^{th}\) to 28\(^{th}\) day after IVH induction (P30-32). The rotation speed of the treadmill was accelerated from 4 to 40 rpm over 100 seconds for a maximum of 3 minutes. Because rats have the ability to learn rotarod tests, the values were analyzed by date and the average latency to fall from three trials was used as the final result.
**TUNEL assay**

Paraffin section slides were deparaffinized, rehydrated, digested with Proteinase K (20 µg/ml in PBS; Sigma) at room temperature for 15 minutes, and washed in PBS for 10 minutes. Sections were then incubated with equilibration buffer for 1 minute and immediately incubated with working strength TdT enzyme in a humidified chamber at 37°C for 1 hour. Each section was immersed in a stop/wash buffer and gently rinsed with PBS. Fluorescein isothiocyanate (FITC)-labeled anti-digoxigenin conjugate was applied to the sections, which were then incubated at room temperature for 30 minutes in the dark. The immunofluorescent terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) technique (kit S7110 ApopTag, Chemicon, Temecula, CA, USA) was applied according to the manufacturer’s protocol to detect cell death in the periventricular white matter. The brain slides were counterstained with DAPI (H-1200, Vector Laboratories, Inc., Burlingame, CA, USA) to determine the degree of DNA fragmentation in cell nuclei and visualized by confocal microscopy (Biorad Radiance 2100, Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a krypton/argon laser at 400x magnification. Images were obtained using Laser shop 2000 software (Bio-Rad Laboratories, Inc.).

**Immunohistochemistry**

Brain coronal sections were incubated with the following primary antibodies: GFAP (rabbit polyclonal, Dako, Glostrup, Denmark; 1:1000 dilution) and MBP (rabbit polyclonal, Abcam, Cambridge, MA, USA; 1:1000 dilution), ED-1 (mouse monoclonal, Millipore, Concord Road, MA, USA; 1:100 dilution), and NeuN (Millipore, Billerica, MA, USA; 1:200 dilution). Three coronal sections (+0.95 mm to -0.11 mm/Bregma) were stained from each brain as previously reported. The immunofluorescent GFAP or MBP staining intensity was measured in six random fields of the right and left periventricular area including corpus callosum and hippocampus on each of three coronal sections by a blinded observer using Image J software (National Institutes of Health, USA). The number of ED-1 positive cells was determined in three non-overlapping three random fields of periventricular area including corpus callosum and hippocampus on each of three coronal sections by a investigator who was blind to the groups. Cryosections (10 µm thick) were mounted with Vectashield mounting solution containing 4′,6′-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (H-1200; Vector, Burlingame, CA). The MSCs were immunostained using PKH26 red fluorescence; the 40× objective images were stained with DAPI to detect nuclear signals. The sections were analyzed by confocal microscopy (Biorad Radiance 2100, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Enzyme-linked immunosorbent assay (ELISA)**

Frozen samples of the periventricular zone of brain tissue were homogenized and centrifuged at 8,000 g for 20 minutes at 4°C. The protein content in the supernatant was measured using the Bradford method with bovine serum albumin (Sigma-Aldrich) as a standard.

**Tracking of donor cells**

The localization of transplanted donor cells that were double-labeled with SPIO and PKH26 was observed by T2*W brain MRI at P11 and P32. After sacrifice, 10-µm thick cryosections were cut from the brains at the medial septum area (+0.95 mm to -0.11/Bregma) and mounted with Vector shield mounting solution containing DAPI (H-1200, Vector Laboratories, Inc., Burlingame, CA, USA). The localization of PKH26-positive red fluorescent cells in the coronal sections was assessed by confocal microscopy (Biorad Radiance 2100, Bio-Rad Laboratories, Inc., Hercules, CA, USA).
Supplemental Figures and Figure Legends

Figure S1. Preliminary IVH modeling in P4 newborn rats. Four weeks after injecting 40 ul, 100 ul, and 200 ul blood intraventricularly at P4, ventricular dilatation was assessed by MRI and on corresponding mid-coronal tissue sections.
Figure S2. Localization of grafted human UCB-derived MSCs that were tagged with super-paramagnetic iron oxide (SPIO) and PKH26 along the periventricular area in rat brains. Donor cells were confirmed by T2* MRI as low signal-intensity in the periventricular areas reflecting SPIO (A) and by PKH26 positivity (red) merged with DAPI staining (blue) in corresponding periventricular tissue (red arrow) (B) (original magnification x400; scale bars, 25 µm).
Figure S3. Stem cell transplantation attenuates reactivated microglia induced by severe IVH. A, Representative immunofluorescence photomicrographs of the periventricular area with staining for ED-1 positive cells (green), and DAPI (blue) (original magnification; x600, scale bars; 20 µm). B, Average number of ED-1 positive cells and in the periventricular area. Data are expressed as mean ± SEM. NC, normal control rats; IC, IVH control rats; IM, IVH with human UCB-derived MSC transplantation; IF, IVH with human fibroblast transplantation; DAPI, 4’,6’-diamidino-2-phenylindole dihydrochloride hydrate. * P <0.05 vs. NC, # P <0.05 vs. IC, † P <0.05 vs. IVH-M.
Figure S4. Co-localization of donor cells with glial and neuronal marker. Glial fibrillary acidic protein (GFAP) and Neuronal specific nuclear protein (NeuN) stains in the periventricular area with the co-localization of PKH26-tagged MSCs (arrow). Red, PKH26 stain; green, GFAP, or NeuN stain; blue, DAPI stain (original magnification; x400, scale bar = 25 µm). DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride hydrate.
Supplemental References


