

ORIGINAL ARTICLE

Transplantation of neural stem cells that overexpress SOD1 enhances amelioration of intracerebral hemorrhage in mice

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Previous studies have shown that intraparenchymal transplantation of neural stem cells (NSCs) ameliorates neurologic deficits in animals with intracerebral hemorrhage (ICH). However, massive grafted cell death after transplantation, possibly caused by a hostile host brain environment, lessens the effectiveness of this approach. We focused on the effect of oxidative stress against grafted NSCs and hypothesized that conferring antioxidant properties to transplanted NSCs may overcome their death and enhance neuroprotection after ICH. Copper/zinc-superoxide dismutase (SOD1) is a specific antioxidant enzyme that counteracts superoxide anions. We investigated whether genetic manipulation to overexpress SOD1 enhances survival of grafted NSCs and accelerates amelioration of ICH. Neural stem cells that overexpress SOD1 were administered intracerebrally 3 days after ICH in a mouse model. Histologic and behavioral tests were examined after ICH. Copper/zinc-superoxide dismutase overexpression protected the grafted NSCs via a decrease in production of reactive oxygen species. This resulted in an increase in paracrine factors released by the NSCs, and an increase in surviving neurons in the striatum and a reduction in striatal atrophy. In addition, SOD1 overexpression showed progressive improvement in behavioral recovery. Our results suggest that enhanced antioxidative activity in NSCs improves efficacy of stem cell therapy for ICH.

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Keywords: cell transplantation therapy; copper/zinc-superoxide dismutase; intracerebral hemorrhage; neural stem cell; reactive oxygen species

INTRODUCTION

Intracerebral hemorrhage (ICH) accounts for ~10% to 15% of all strokes that occur in the world each year, and hypertension is the main cause of spontaneous ICH. The prognosis of ICH is poor, with an overall 30-day mortality rate of ~40%.¹ Most survivors experience persistent, severe neurologic deficits.² Medical therapy for ICH, such as mechanical removal of the hematoma, pharmacological prevention of edema formation, and reduction in intracranial pressure, shows only limited effectiveness.³ It is important to explore alternative approaches.

Intracerebral hemorrhage results in both primary and secondary injury. The primary injury results from disruption of adjacent tissue and mass effect.⁴ Secondary injury occurs with the development of edema, free radical formation, inflammation, and direct cellular toxicity caused by the hematoma and subsequent degradation byproducts.⁵ Neuroprotective strategies might prevent additional cell loss in the perihematomal area caused by secondary injury, thereby improving outcome after ICH.

Previous studies have shown that intraparenchymal transplantation of stem cells ameliorates neurologic deficits in animals with ICH.^{6–8} Stem cell transplantation might not only result in integration of the transplanted cells into the host brain, but might also contribute to neuroprotection. The optimal time window for transplantation after ICH is yet to be determined. Neuroprotective mechanisms might be most effective if the stem cells are

transplanted early after the insult. However, the host's hostile environment during the acute stage after ICH impairs survival of the grafted cells.^{9–12} Furthermore, there is little information regarding the mechanism involved in the death of grafted cells. Increased free radicals and reactive oxygen species (ROS) production have important roles in ICH damage. Hemoglobin (Hb) and its breakdown products, such as iron, are the major sources of ROS and mediate the pathologic process after ICH.^{13,14} Therefore, ROS production after ICH might be involved in the accelerated death of the grafted stem cells, resulting in diminished effectiveness of stem cell therapy.

Copper/zinc-superoxide dismutase (SOD1) is a dimeric cytosolic enzyme that detoxifies superoxide anions to H₂O₂. We have shown that SOD1 overexpression reduces oxidative stress induced by Hb, resulting in the reduction of blood–brain barrier disruption and the decrease in apoptotic cell death in the rat brain.¹⁴ We have also reported that transplantation of neural stem cells (NSCs) that overexpress SOD1 provides extended survival of these grafted cells and facilitates functional recovery in ischemic stroke animals.¹⁵

The purpose of this study was to determine whether ROS cause grafted cell death and whether SOD1 overexpression in NSCs can increase their survival after early transplantation in mice with ICH and can enhance functional recovery. We also sought to investigate whether NSCs that overexpress SOD1 enhance neuroprotection *in vitro* after they are treated with Hb.

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MATERIALS AND METHODS

Animals

All animals were treated in accordance with Stanford University guidelines and the animal protocols were approved by Stanford University's Administrative Panel on Laboratory Animal Care. Homozygous green fluorescent protein (GFP) transgenic (Tg) mice (C57BL/6-Tg [UBC-GFP] 30Scha/J; The Jackson Laboratory, Bar Harbor, ME, USA) were bred with heterozygous SOD1 Tg mice (C57BL/6 background, backcrossed with C57BL/6 for more than 10 generations) to generate heterozygous GFP Tg mice (wild-type (WT) mice) and heterozygous SOD1/GFP double Tg mice (Tg mice).¹⁶ These animals were used for isolation of NSCs. We also used C57BL/6 mice (The Jackson Laboratory) for ICH models and for preparation of Hb.

Isolation and Culture of Neural Stem Cells

Neural stem cells were isolated from the subventricular zones of postnatal day 1 WT mice and Tg mice as described.¹⁵ In brief, bilateral subventricular zones were dissected and mechanically dissociated. The cells were collected and suspended in Neurobasal-A medium (Invitrogen, Carlsbad, CA, USA) containing B-27 supplement (Invitrogen), L-glutamine (Invitrogen), 20 ng/mL mouse fibroblast growth factor basic (PeproTech, Rocky Hill, NJ, USA), and 10 ng/mL mouse epidermal growth factor (PeproTech). Cells were grown as adherent monolayers. The medium was changed every 2 days and cells were passaged weekly. Cells that had been passaged 5 to 10 times were used for the experiments.

Preparation of Hemoglobin for Treatment in Neural Stem Cells

In prior cell culture studies, Hb was shown to be an oxidative cytotoxin.^{17,18} Hemoglobin was prepared as described.¹³ The mice were killed with isoflurane and blood was drawn by cardiac puncture. After centrifugation of the blood at 1,250 g for 5 minutes at 4°C, the supernatant was removed and the pellet was washed, resuspended in sterile saline, and lysed by two freeze-thaw cycles. The sample was then centrifuged again and most of the supernatant was removed. The Hb concentration of this sample was determined with an Hb assay kit (BioChain, Newark, CA, USA). Hemoglobin was then diluted with sterile saline, aliquoted, and stored at -80°C until used.

Cell Viability Assay

Cell viability was assessed with a cell proliferation reagent using a WST-1 assay kit (Roche Diagnostics, Indianapolis, IN, USA).¹⁹ The NSCs were cultured on 96-well plates and were exposed to different doses of Hb for 24 hours. The NSCs were then washed with phosphate-buffered saline (PBS) and incubated with 90 μ L of new medium with 10 μ L WST-1 assay reagent for 1.5 to 2 hours. The absorbance of the samples was measured at 450 nm using a microplate reader. We used four samples for each condition and repeated this experiment six times for each condition ($n = 6$).

Assessment of Cell Death *In Vitro*

Cell death was assessed by LIVE/DEAD Viability/Cytotoxicity assay kit (Life Technologies, Grand Island, NY, USA).²⁰ The NSCs were cultured on 8-well chamber slides (ThermoFisher Scientific, Waltham, MA, USA) and were exposed to 20 μ mol/L Hb for 12 hours. The NSCs were then washed with PBS and incubated with 4 μ mol/L ethidium homodimer-1 and 2 μ mol/L calcein AM for 15 minutes. Calcein is retained very well within live cells, producing an intense uniform green fluorescence. Ethidium homodimer-1 enters cells with damaged membranes and produces a bright red fluorescence in dead cells.

Western Blot Analysis *In Vitro*

To investigate whether Hb induces changes in SOD1 expression in NSCs, Western blotting was performed. We also investigated whether overexpression of SOD1 involves the phosphorylated serine threonine kinase, pAkt, in NSCs under normal conditions and after exposure to Hb. After exposure to 20 μ mol/L Hb for 4, 8, and 24 hours, 50 μ mol/L Hb for 4 hours, and 100 μ mol/L H₂O₂ for 4 hours, the NSCs were washed and treated with cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) and used as whole-cell lysate samples. Protein concentrations were examined by comparison with a known concentration of bovine serum albumin using a kit (ThermoFisher Scientific). Equal amounts of the samples (20 μ g) were

loaded per lane and analyzed by SDS-PAGE on a 10% NuPAGE Bis-Tris gel (Invitrogen) and then immunoblotted. The primary antibodies were a 1:3,000 dilution of rabbit polyclonal anti-SOD1 (molecular weight: ~19 kDa) (Enzo Life Sciences, Farmingdale, NY, USA), a 1:500 dilution of rabbit polyclonal anti-pAkt (Ser473) (molecular weight: ~60 kDa) (Cell Signaling Technology), a 1:2,000 dilution of rabbit polyclonal anti-Akt (molecular weight: ~60 kDa) (Cell Signaling Technology), and a 1:100,000 dilution of mouse monoclonal anti- β -actin (molecular weight: ~42 kDa) (Sigma-Aldrich, St Louis, MO, USA). After incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (Cell Signaling Technology) or anti-rabbit IgG (Cell Signaling Technology), the antigen was detected by SuperSignal West Pico substrates (ThermoFisher Scientific). Images were captured with a GS-700 imaging densitometer (Bio-Rad, Hercules, CA, USA) and the results were quantified using MultiAnalyst software (Bio-Rad).

Immunofluorescent Staining

For immunocytochemistry, the NSCs were cultured on 8-well chamber slides, then washed with PBS, and fixed with 4% paraformaldehyde in PBS. For immunohistochemistry, the animals were anesthetized and perfused with PBS followed by 4% paraformaldehyde in PBS. The brains were postfixed overnight in the same fixative at 4°C. For cryosectioning, fixed tissue was cryoprotected in 10% sucrose in PBS overnight at 4°C, then in 20% sucrose in PBS overnight at 4°C, and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA). Cryostat sections (20 μ m) were cut and affixed to glass slides. Cells or tissue sections were subsequently incubated overnight at 4°C in an appropriate mixture of primary antibodies. The following antibodies were used: rabbit anti-GFP (1:200, Invitrogen), goat anti-GFP (1:200, LifeSpan BioSciences, Seattle, WA, USA), mouse anti-Nestin (1:100, BD Biosciences Pharmingen, San Jose, CA, USA), mouse anti-mitogen-activated protein 2 (MAP2) (1:200, Sigma-Aldrich), rabbit anti-glial fibrillary acidic protein (GFAP) (1:200, Millipore, Billerica, MA, USA), mouse anti-NG2 (1:200, Abcam, Cambridge, MA, USA), and rabbit anti-Ki-67 (1:200, Abcam). After three washes, cells or tissue sections were incubated for 1 hour with a 1:500 dilution of the following secondary antibodies: Alexa Fluor 488-conjugated donkey anti-rabbit IgG, Alexa Fluor 594-conjugated donkey anti-rabbit IgG, Alexa Fluor 488-conjugated donkey anti-goat, and Alexa Fluor 594-conjugated donkey anti-mouse IgG (all from Invitrogen). Slides were covered with VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole. The samples were examined by confocal or fluorescence microscopy (AxioPlan 2; Carl Zeiss, Thornwood, NY, USA).

Oxidative Damage

Production of superoxide anions *in vitro* was shown by oxidized hydroethidine as described.²¹ The cell culture was incubated with 5 μ mol/L hydroethidine solution (Invitrogen) for 15 minutes, followed by fixation with 4% paraformaldehyde in PBS. Slides were covered with VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole and observed with a fluorescence microscope. The oxidized hydroethidine fluorescence was examined at an excitation of 510 nm and emission of 580 nm and quantified with ImageJ software (version 1.42q; NIH, Bethesda, MD, USA). For an *in vivo* study, we observed the carbonyl proteins as indicators of oxidative protein damage, with the use of a commercial kit (Millipore).¹⁴ The mice were killed 2 days after NSC transplantation and tissue sections were prepared. Wild-type NSCs were also transplanted into the striatum without ICH as a control. The sections were incubated with 2,4-dinitrophenylhydrazine, and 2,4-dinitrophenyl-derivatized carbonyl proteins were detected by immunostaining with an anti-2,4-dinitrophenyl biotinylated antibody (1:200) and streptavidin-Cy3 (1:500). Fluorescence intensity was examined at an excitation of 510 nm and emission of 580 nm and quantified with ImageJ software.

Intracerebral Hemorrhage Model with Autologous Blood Infusion

We used an experimental ICH procedure described previously,^{22,23} with some modifications. Male C57BL/6 mice (14 to 15 weeks old, 25 to 30 g) were anesthetized with 2.0% isoflurane in 30% oxygen and 70% nitrous oxide and placed in a stereotaxic frame. The rectal temperature was controlled at 37.0 \pm 0.5°C with a homeothermic blanket. A midline scalp incision was made and a hole was drilled in the right side of the skull (0.0 mm anterior and 2.5 mm lateral of the bregma) in preparation for the infusion. The mouse tail tip was cut off and some drops of blood were allowed to fall on parafilm. The blood (20 μ L) was collected without the use

of an anticoagulant and used for injection. A 30-gauge needle attached to a 50- μ L Hamilton microsyringe was inserted 3.5 mm ventral from the surface of the skull, with adjustment of the stereotaxic arm to a point 5° medially relative to the vertical axis. After waiting 2 minutes, the needle was withdrawn 0.5 mm, and left for another 5 minutes. Ten microliters of blood was injected over 10 minutes using a microsyringe pump. The remaining 10 μ L of blood was injected using the same procedure. After infusion, the needle was left in place for 25 minutes and then slowly removed (Supplementary Figure 1A). Animal mortality was rare during surgery (<5%) and was related mostly to respiratory disorder. No animals died after surgery. All underwent behavioral testing 1 day after ICH and those with low behavioral scores were excluded from the experiment (cylinder test <0.6, corner turn test <0.8; 40% were excluded).

Intracerebral Transplantation

The mice were anesthetized 72 hours after ICH and the NSCs were transplanted using a 10- μ L Hamilton syringe with a 31-gauge needle. The mice were given two 1.0- μ L deposits of single-cell suspension in PBS (1×10^5 cells/ μ L) along the anterior–posterior axis into the striatum at these coordinates: (1) anterior–posterior (A–P), 0.5; medial–lateral (M–L), 2.5; dorsal–ventral (D–V), 3.0; (2) A–P, -0.75; M–L, 2.5; D–V, 3.0. These targets approximated the edge of the ICH. Deposits were delivered at 0.5 μ L/minute, and the needle was left in place for 5 minutes post injection. The control group had PBS injected without NSCs. In the WT group, the mice had grafted WT NSCs, and in the Tg group, the mice had grafted Tg NSCs.

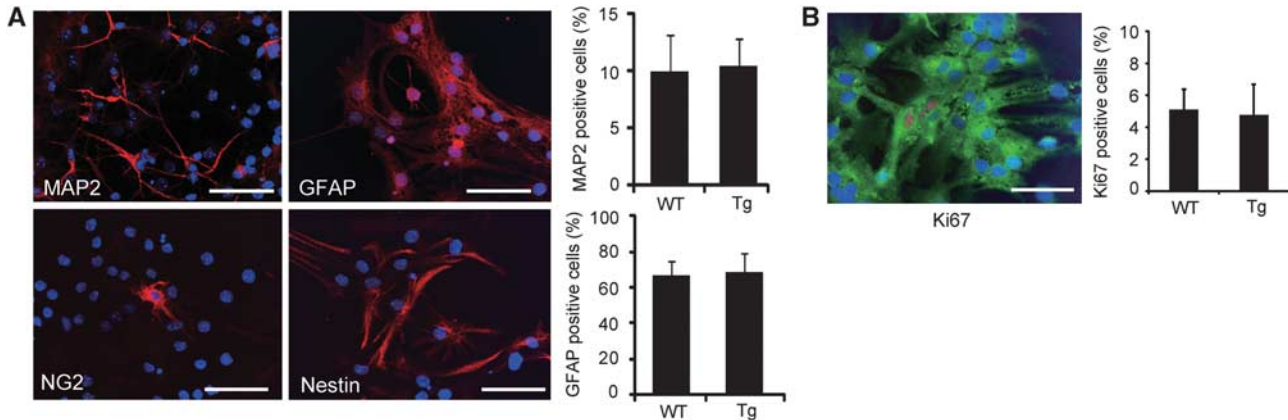


Figure 1. Cell differentiation and proliferation *in vitro*. (A) Neural stem cells (NSCs) were stained with the neuronal marker mitogen-activated protein 2 (MAP2) (red), glial fibrillary acidic protein (GFAP) (red), the oligodendrocytic marker NG2 (red), and the immature cell marker Nestin (red), and 4',6 diamidino-2-phenylindole (blue). The NSCs differentiated into neurons, astrocytes, and oligodendrocytes. Some cells were still stained with Nestin. Scale bars, 50 μ m. (B) Fluorescent staining with the proliferation marker Ki-67 (red), green fluorescent protein (green), and 4',6 diamidino-2-phenylindole (blue) after culturing in differentiation medium. Scale bar, 50 μ m. WT, wild-type; Tg, transgenic.

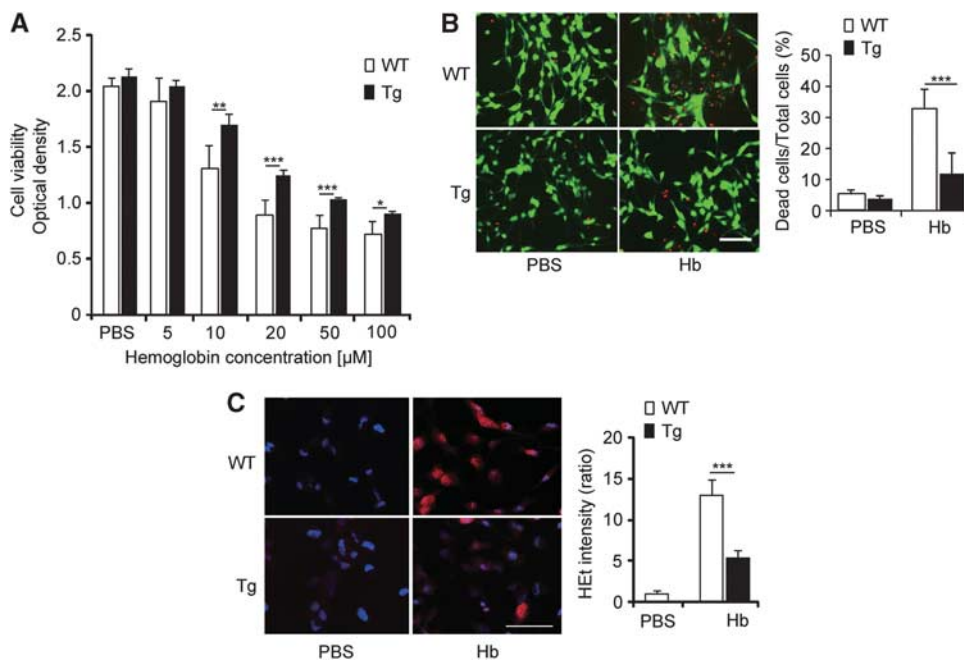


Figure 2. Reduced death in copper/zinc-superoxide dismutase transgenic (Tg) neural stem cells after exposure to hemoglobin (Hb). (A) WST-1 assay after exposure to Hb. (B) Neural stem cell death analyzed by LIVE/DEAD assay. Live cells (green) and dead cells (red) after exposure to Hb. Scale bar, 100 μ m. (C) Superoxide anion production analyzed by hydroethidine (HET) (red) and 4',6 diamidino-2-phenylindole (blue) after exposure to Hb. Scale bar, 50 μ m. * P <0.05, ** P <0.01, *** P <0.001. WT, wild-type; PBS, phosphate-buffered saline.

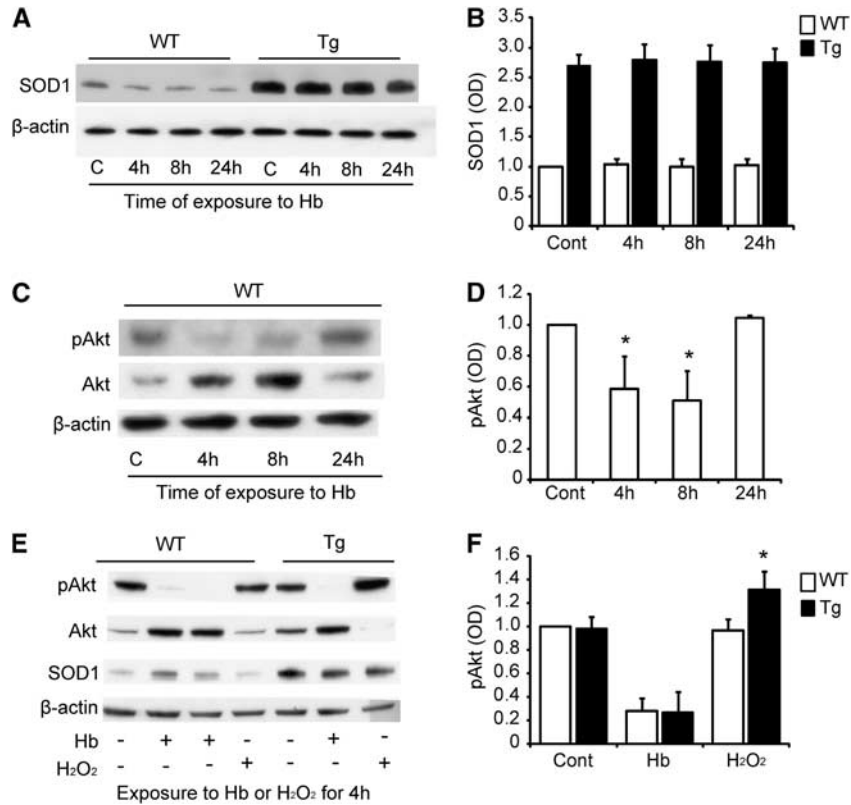


Figure 3. Copper/zinc-superoxide dismutase (SOD1) and phosphorylated serine threonine kinase (pAkt) expression in neural stem cells (NSCs) *in vitro*. **(A, B)** Western blot analysis of SOD1 expression after exposure to 20 $\mu\text{mol/L}$ hemoglobin (Hb). **(C, D)** Western blot analysis of pAkt expression after exposure to 20 $\mu\text{mol/L}$ Hb in wild-type (WT) NSCs. **(E, F)** Western blot analysis of pAkt expression after exposure to 50 $\mu\text{mol/L}$ Hb and 100 $\mu\text{mol/L}$ H₂O₂ for 4 hours. * $P < 0.05$. Tg, SOD1 transgenic; C and Cont, control; OD, optical density.

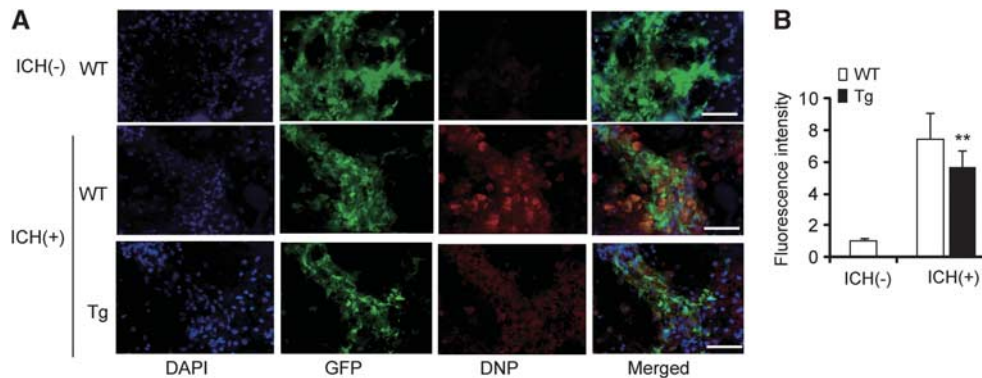


Figure 4. Reduced oxidative stress in copper/zinc-superoxide dismutase transgenic (Tg) neural stem cells (NSCs) *in vivo*. **(A)** Fluorescent staining with 4',6-diamidino-2-phenylindole (DAPI) (blue), green fluorescent protein (GFP) (green), and 2,4-dinitrophenylhydrazine and 2,4-dinitrophenyl (DNP) (red) in brain sections 2 days after transplantation. **(B)** DNP signals increased in the wild-type (WT) NSCs, but this increase was significantly reduced in the Tg NSCs ($n = 5$). Scale bars, 50 μm . ** $P < 0.01$. ICH, intracerebral hemorrhage.

Detection of Paracrine Factors

Fresh brain was removed 5 and 14 days after ICH in each group (WT, Tg, PBS control) (Supplementary Figure 1B). Blocks of tissue were cut from 1 mm rostral to 2 mm caudal to the bregma. From these samples, the NSC-transplanted side of the striatum was dissected with the use of a microscope and used as samples that were treated with cell lysis buffer (Cell Signaling Technology) and used as whole-cell lysate samples. Commercial glial cell-derived neurotrophic factor (GDNF) (Promega, Madison, WI, USA) and vascular endothelial growth factor (VEGF) (R&D

Systems, Minneapolis, MN, USA) ELISA kits were used to quantify the concentration of GDNF and VEGF in each sample.

Quantification of Survival of Transplanted Neural Stem Cells

To quantify survival of the grafted cells, the animals were killed 14 and 35 days after ICH and tissue sections were prepared. After immunostaining with GFP, as described above, the transplanted GFP-positive cells were counted using unbiased computational stereology (fractionator method,

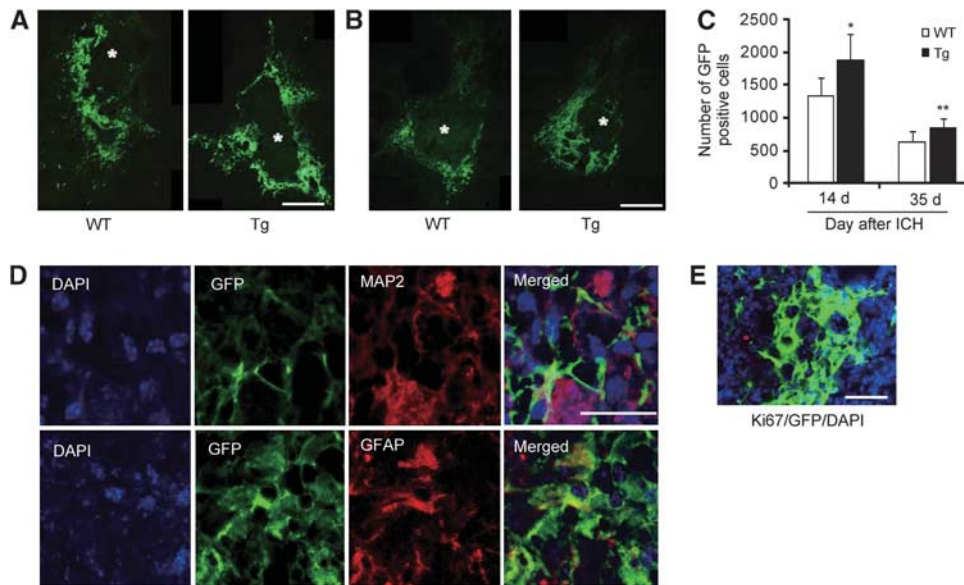


Figure 5. Increased cell survival *in vivo*. Fluorescent staining with green fluorescent protein (GFP) (green) revealed that the grafted cells surrounded the intracerebral hemorrhage (ICH) lesion 14 days (A) and 35 days (B) afterwards. The images were taken at a $\times 40$ objective, and were put together to make an entire image of the grafted cells and ICH using the STEREOINVESTIGATOR system (MicroBrightfield, Williston, VT, USA). * represents ICH. Scale bars, 200 μm . (C) The number of surviving grafted cells significantly increased in the copper/zinc-superoxide dismutase transgenic (Tg) neural stem cell (NSC) group compared with the wild-type (WT) NSC group 14 and 35 days after ICH ($n = 7$). (D) Fluorescent staining with GFP (green), 4',6 diamidino-2-phenylindole (DAPI) (blue) and mitogen-activated protein 2 (MAP2) (red), or glial fibrillary acidic protein (GFAP) (red) revealed that the grafted NSCs differentiated into neurons (MAP2⁺) and astrocytes (GFAP⁺) 32 days after transplantation. (E) Fluorescent staining with GFP (green), DAPI (blue), and the proliferation marker Ki-67 (red) revealed that no Ki-67-positive grafted cells were observed 32 days after transplantation. Scale bar, 20 μm . * $P < 0.05$, ** $P < 0.01$.

using STEREOINVESTIGATOR software (MicroBrightfield, Williston, VT, USA)), as described.¹⁸ All the GFP-positive cells were counted on 12 serial coronal sections per brain (0.25 mm apart).

Assessment of Neural Stem Cell Proliferation and Differentiation

After culturing in differentiation medium containing 1% fetal bovine serum for 5 days, the NSCs were stained with a proliferation marker (Ki-67) or lineage-specific marker (Nestin, MAP2, GFAP, NG2) to assess NSC proliferation and differentiation *in vitro*. The proportion of grafted cells was also determined by staining with Ki-67 or MAP2 and with GFAP and 4',6 diamidino-2-phenylindole as described above.

Measurement of Striatum Atrophy

The brain sections were stained with cresyl violet. We estimated the striatum size as a percentage of the ipsilateral striatum. The area of both sides of the striatum was measured on nine serial coronal sections per brain (0.25 mm apart) and the size of the striatum was analyzed by an observer masked to the experimental cohort.

Quantification of Neuronal Survival

The brain sections were stained with NeuN (a neuronal marker) and 4',6 diamidino-2-phenylindole as described above. Surviving neurons were analyzed in a masked manner by counting the number of cells or profiles in six defined regions of interest per striatum, measuring 62,500 μm^2 , both ipsilateral and contralateral to the ICH using unbiased computational stereology.²⁴

Behavioral Analysis

A cylinder test and a corner turn test were used for behavioral analysis.^{22,25} To obtain three groups with an equal post-ICH behavior score, the animals were ranked based on their post-day-1 results and assigned to one of the three groups. The animals were retested just before cell transplantation and 7, 14, 21, 28, and 35 days after ICH (Supplementary Figure 1C). Forelimb use during exploratory activity was analyzed in a transparent cylinder (12 cm in diameter). The behavior was scored according to the following criteria: independent use of the left or right forelimb for

contacting the wall while the animals were standing on their hindlimbs to initiate a weight-shifting movement and simultaneous use of both the left and right forelimbs to contact the wall. Behavior was quantified by determining the number of times the ipsilateral (unimpaired) forelimb (I), contralateral forelimb (C), and both forelimbs (B) were used as a percentage of total number of limb usage. A single, overall limb-use asymmetry score was calculated as follows: forelimb-use asymmetry score ($I/(I+C+B) - C/(I+C+B)$). The second behavioral analysis involved a corner turn test. The mouse was allowed to proceed into a corner, the angle of which was 30°. To exit the corner, the animal could turn either to the left or right. When the mouse turned, its choice of direction was recorded. This was repeated 10 to 15 times and the percentage of right turns was calculated.

Statistical Analysis

Behavioral data were assessed using repeated measures of ANOVA followed by the Tukey test. For the paracrine factor experiment, measurement of striatum atrophy, and neuronal survival, comparisons among multiple groups were performed with one-way ANOVA, followed by the Tukey test. For other experiments, comparisons between two groups were achieved with Student's unpaired *t*-test. Data are expressed as the mean \pm s.d. Significance was accepted with $P < 0.05$.

RESULTS

Characterization of Neural Stem Cells

We isolated NSCs from fetal GFP Tg mice (WT NSCs) or SOD1/GFP double Tg mice (Tg NSCs). These NSCs, grown as adherent cultures, were self-renewing and multipotent (Figure 1A). The percentage of neurons ($9.9 \pm 3.2\%$ and $10.4 \pm 2.5\%$, $n = 4$) and astrocytes ($66.3 \pm 8.9\%$ and $68.7 \pm 10.1\%$, $n = 4$) differentiated from the WT and Tg NSCs was similar after culturing in differentiation medium containing 1% fetal bovine serum for 5 days (Figure 1A). Moreover, no difference was observed in the percentage of Ki-67-positive cells ($n = 4$) (Figure 1B).

Neural Stem Cell Death after Exposure to Hemoglobin

We investigated whether the levels of SOD1 expression could rescue NSCs from death after exposure to Hb. After 24 hours of exposure to Hb, cell viability decreased depending on the concentration of Hb, using the WST-1 assay ($n=6$) (Figure 2A). Cell viability was significantly higher in the Tg NSCs compared with the WT NSCs ($n=6$) (Figure 2A). This cytoprotective effect in the Tg NSCs was confirmed by a LIVE/DEAD assay, where there was a significant decrease in death in the Tg NSCs ($11.9 \pm 7.0\%$) compared with the WT NSCs ($32.9 \pm 5.9\%$) ($n=5$, $P<0.001$) after 12 hours of exposure to $20 \mu\text{mol/L}$ Hb ($n=5$, $P<0.01$) (Figure 2B). After 4 hours of exposure to $20 \mu\text{mol/L}$ Hb, the WT NSCs showed a significant increase in hydroethidine staining, which indicates production of superoxide anions (Figure 2C). This signal was significantly reduced in the Tg NSCs compared with the WT NSCs ($n=5$, $P<0.001$).

Copper/Zinc-Superoxide Dismutase and Phosphorylated Serine Threonine Kinase Expression in Neural Stem Cells after Exposure to Hemoglobin

We next investigated whether SOD1 expression is changed by exposure to Hb. Western blot analysis revealed that, as expected, SOD1 expression was significantly higher in the untreated Tg NSCs (2.46 ± 0.16 -fold). Hemoglobin exposure did not change SOD1 expression in the WT and Tg NSCs ($n=4$) (Figures 3A and 3B). Phosphorylated serine threonine kinase levels were significantly reduced after exposure to Hb for 4 and 8 hours in the WT NSCs ($n=4$, $P<0.05$) (Figures 3C and 3D). There were no differences in pAkt expression between the WT and SOD1 Tg NSCs before exposure to Hb or after exposure to Hb for 4 hours ($n=4$). In contrast, pAkt was significantly higher in the Tg NSCs compared with the WT NSCs after exposure to H_2O_2 ($n=4$, $P<0.05$) (Figures 3E and 3F).

Protein Oxidation in Grafted Neural Stem Cells

We transplanted WT and Tg NSCs into the perihematomal striatum 3 days after ICH. In the WT NSC transplantation, fluorescence intensity of 2,4-dinitrophenyl drastically increased 2 days afterwards compared with WT NSC transplantation in intact brains (Figures 4A and 4B). This increase was significantly diminished in the SOD1 Tg NSCs ($n=5$, $P<0.01$) (Figures 4A and 4B).

Survival of Grafted Neural Stem Cells

Green fluorescent protein staining revealed that grafted cells surrounded the ICH lesion in the WT and Tg NSC groups 14 days (Figure 5A) and 35 days (Figure 5B) after ICH. The number of surviving grafted cells was significantly higher in the Tg NSC group compared with the WT NSC group both 14 and 35 days after ICH ($n=7$, 14 days: $P<0.05$; 35 days: $P<0.01$) (Figure 5C). There were no signs of tumor formation caused by the transplanted NSCs 35 days after ICH in either group.

Cell Differentiation after Transplantation

Fluorescent staining with GFP, MAP2, and GFAP revealed that the grafted NSCs differentiated into neurons (MAP2) and astrocytes (GFAP) 35 days after ICH (Figure 5D). The percentage of neurons ($3.8 \pm 3.6\%$ versus $5.3 \pm 4.2\%$, $n=4$) and astrocytes ($88.2 \pm 5.3\%$ versus $86.9 \pm 7.6\%$, $n=4$) differentiated from the grafted cells was similar between the WT and Tg NSCs. There were no cells positive for Ki-67 (cell proliferation marker) 35 days after ICH (Figure 5E).

Paracrine Factor Expression in the Striatum

We measured VEGF and GDNF levels in the striatum using ELISA. These paracrine factors significantly increased in both the WT and Tg NSC groups compared with the PBS control group 2 days after transplantation ($n=5$) (Figures 6A and 6B), and significantly

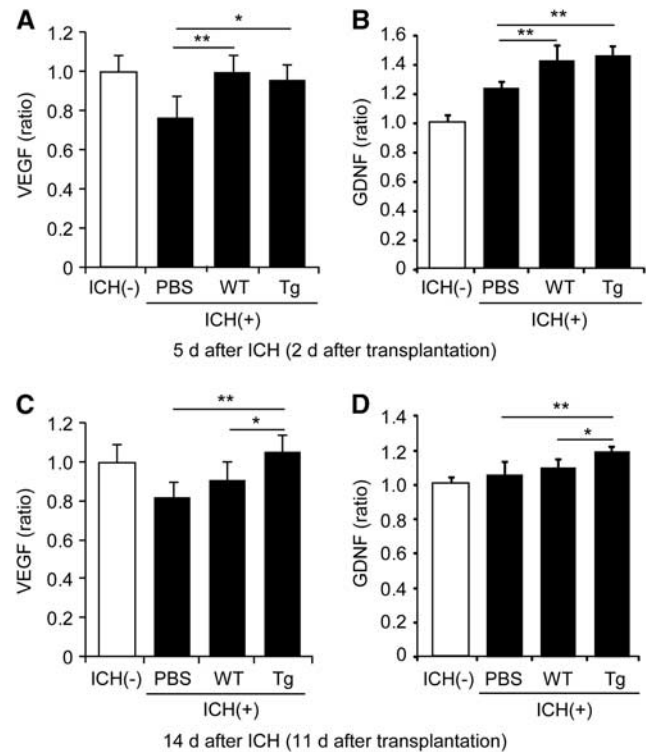


Figure 6. Induction of paracrine factors in the striatum *in vivo*. ELISA of the striatum revealed significant elevations of vascular endothelial growth factor (VEGF) and glial cell-derived neurotrophic factor (GDNF) in both the copper/zinc-superoxide dismutase transgenic (Tg) neural stem cell (NSC) group and the wild-type (WT) NSC group compared with the phosphate-buffered saline (PBS) group 2 days after transplantation (A, B). Significant elevations in VEGF and GDNF in the Tg NSCs compared with the WT NSCs 11 days after transplantation (C, D). * $P<0.05$, ** $P<0.01$. ICH, intracerebral hemorrhage.

increased in the Tg NSC group compared with both the WT NSC and PBS control groups 11 days after transplantation ($n=5$) (Figures 6C and 6D).

Neural Stem Cells Promote Perilesional Tissue Protection

To assess whether SOD1 overexpression in NSC transplantation influences protection of peri-ICH tissue, histochemical studies were performed. NeuN staining revealed that neuronal survival was significantly increased in the Tg NSC group 35 days after ICH compared with the PBS control group ($n=6$, $P<0.01$), although there was no significance between the WT NSC group and the PBS control group ($n=6$) (Figure 7A). Furthermore, NSC transplantation prevented striatal atrophy, and the Tg NSCs significantly attenuated this atrophy compared with the WT NSCs ($n=7$, $P<0.05$) (Figure 7B).

Functional Recovery

We analyzed behavioral performance using the cylinder test and corner turn test. Mice that were transplanted with Tg NSCs showed significant functional improvement from day 21 compared with the PBS control group and at day 35 compared with the WT NSC group according to the cylinder test ($n=9$) (Figure 7C). The corner turn test revealed that although the Tg NSC group showed a statistical significance at day 35 compared with the PBS control group ($n=9$, $P<0.01$), there was no

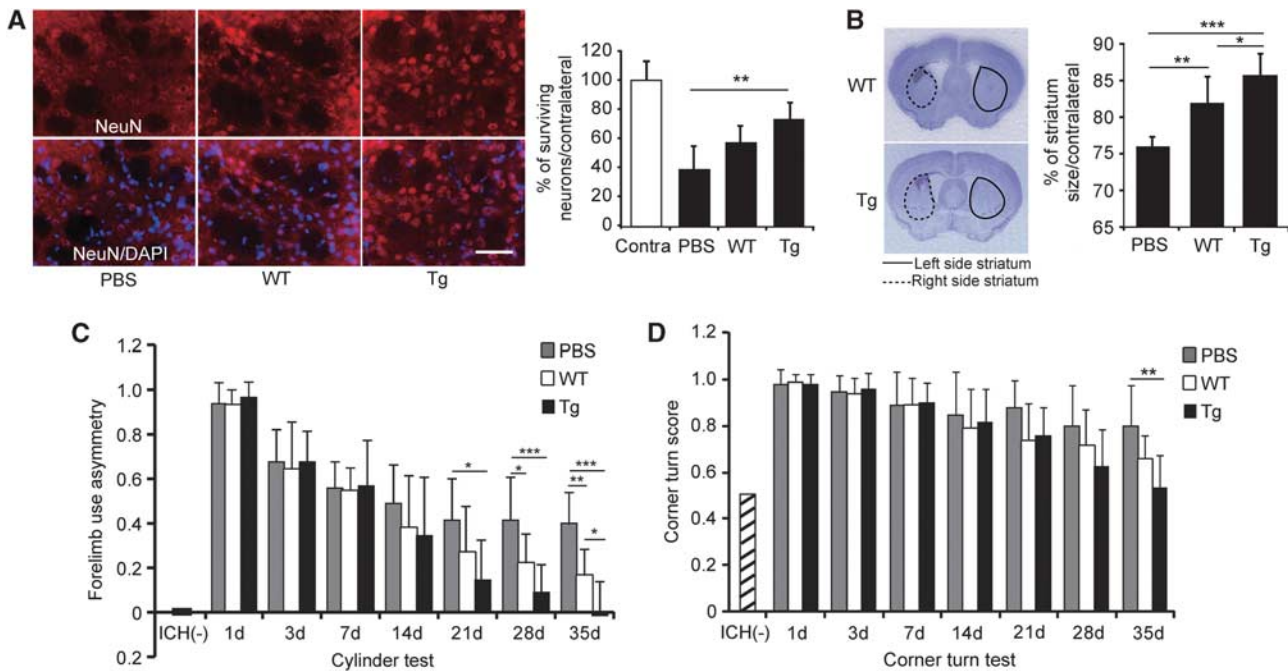


Figure 7. Copper/zinc-superoxide dismutase transgenic (Tg) neural stem cells (NSCs) promote perilesional tissue protection and enhance functional recovery. **(A)** Enhanced neuronal survival with transplantation of Tg NSCs. Fluorescent staining of NeuN (a neuronal marker) (red) and 4',6 diamidino-2-phenylindole (DAPI) (blue) and quantification of NeuN-positive cells in the striatum. Scale bar, 50 μ m. **(B)** Striatal atrophy 35 days after intracerebral hemorrhage (ICH). The Tg NSCs significantly reduced atrophy compared with the wild-type (WT) NSC and phosphate-buffered saline (PBS) groups 35 days after ICH. Behavioral performance using the cylinder test **(C)** and the corner turn test **(D)**. The Tg NSC group showed significant functional improvement compared with the WT NSC and PBS groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Contra, contralateral striatum.

significant behavioral improvement at any time point between these two groups ($n = 9$) (Figure 7D).

DISCUSSION

Reactive oxygen species mediate oxidative brain damage after ICH. Cell culture studies have shown that Hb-induced neurotoxicity is mediated by iron released from Hb as free radical scavengers, and that an iron chelator blocked Hb-induced neurotoxicity.^{26,27} Injection of autologous lysed erythrocytes, Hb, and iron into the brain produces ROS, causing brain damage in animal models.^{14,28,29} In this study, we demonstrated that (i) Hb led to NSC death via ROS production, which may also mediate grafted cell death in ICH; (ii) overexpression of SOD1 in NSCs reduced ROS and increased survival of these cells after exposure to Hb and after transplantation in the ICH brain; and (iii) transplantation of SOD1 Tg NSCs enhanced neuroprotection and facilitated behavioral recovery in mice with ICH.

Although the vulnerability and response of NSCs to Hb cytotoxicity have not yet been examined, several studies have shown a rapid and massive loss of NSCs under oxidative stress.^{30,31} We recently showed that ROS production resulted in a reduction in grafted cells and diminishment of cell transplantation therapy for ischemic stroke.^{15,19,21} Similarly, in the present study, we showed that ROS induced by Hb accelerated NSC death. Hydroethidine staining was enhanced in WT NSCs that were treated with Hb, whereas it was reduced in SOD1 Tg NSCs exposed to Hb. This demonstrates that ROS have an important role and are affected by exposure to Hb. Furthermore, we showed that ROS accelerated death of grafted NSCs after ICH, where carbonyl protein increased in WT NSCs after transplantation whereas it was reduced in SOD1 Tg NSCs. These findings suggest that ROS not only cause host tissue damage but also hinder survival of grafted NSCs in ICH.

Research on ROS has focused on their effect on toxicity in cells, but recent studies suggest that low levels of ROS contribute to stem cell proliferation, self-renewal, and neurogenesis under physiologic conditions.^{32,33} However, in our study, SOD1 Tg NSCs demonstrated similar proliferation and differentiation capacities compared with WT NSCs *in vitro* and similar differentiation *in vivo*. This discrepancy might be because the basal expression of antioxidant or pro-oxidant enzymes in Tg NSCs is altered in association with SOD1 expression and compensates for the regulation of ROS levels. Further study is needed to clarify this important issue.

Serine threonine kinase is a key molecule in cell survival not only in ischemic stroke but also in hemorrhagic stroke.^{34,35} However, the role of Akt signaling in Hb cytotoxicity in NSCs has not been reported. Overexpression of Akt1 enhanced survival of grafted human NSCs transplanted into mouse brains with ICH induced by striatal collagenase injection, as demonstrated by Lee *et al.*¹⁰ Reactive oxygen species contributed to enhanced Akt activity in NSCs,^{32,33} and an increase in Akt activity was induced by intracellular H_2O_2 converted from superoxide by SOD1.³⁶ Although pAkt expression increased in the SOD1 Tg NSCs compared with the WT NSCs after exposure to H_2O_2 , there was no difference in pAkt expression between SOD1 Tg NSCs and WT NSCs after exposure to Hb in this study. An earlier report by our group showed that cortical neurons that overexpress SOD1 had a tolerance against ischemic insults via upregulation of Akt signaling. The increase in pAkt was observed in the penumbral region of the cortex, whereas it was decreased in the ischemic core after transient focal cerebral ischemia.³⁷ These findings suggest that relatively mild insults upregulate Akt phosphorylation, while severe damage downregulates it. Thus, Hb stimulation might be too strong and detrimental for Akt activation in our study.

Many studies have shown that the majority of exogenous, transplanted stem cells exert cytoprotection or stimulate endogenous repair mechanisms in the ICH brain.^{5,6,8,38} The present

study shows that transplantation of SOD1 Tg NSCs in ICH brains reduced their atrophy and death. Moreover, an improvement in the outcome of behavioral tests was observed in these animals, indicating that stem cells facilitate neuroprotection after being grafted to ICH brains. The present study also demonstrates that overexpression of SOD1 enhanced survival of grafted cells and increased paracrine factors in the ICH brain, resulting in enhanced neuroprotection. Levels of VEGF and GDNF in the striatum were significantly increased in both the WT and Tg NSCs compared with the controls 2 days after cell transplantation. There were no significant differences in expression of these paracrine factors between the WT and Tg NSCs at this time point, but higher levels of paracrine factors were detected in the Tg NSCs compared with the WT NSCs 11 days after transplantation. These differences might be caused by a decrease in grafted cells in the WT mice. The number of surviving grafted cells decreased more quickly in the WT mice compared with the Tg mice after transplantation. These results are consistent with previous studies. HB1.F3 gene-modified human NSCs that overexpress VEGF were found to produce a higher level of human VEGF at the transplantation site, and to provide neuroprotection, angiogenesis, and functional recovery in mouse ICH.⁹ Furthermore, HB1.F3 gene-modified human NSCs that overexpress GDNF, which can produce a fourfold higher amount of GDNF compared with parental F3 cells *in vitro*, reduced apoptotic cells around the ICH lesion and induced behavioral improvement after transplantation in mouse ICH.¹¹ These studies show that direct secretion of trophic factors by the grafted cells enhance neuroprotection and neurorepair. In contrast, cell transplantation therapy after stroke can enhance endogenous secretion of trophic factors from host tissue.³⁹

Genetic modification of stem cells generates a number of concerns, including tumor formation after transplantation.⁴⁰ In this study, tumor formation was not observed and grafted cells positive for a proliferation marker were not detected 35 days after ICH. Tumor formation was also not observed 3 months after transplantation of SOD1 Tg NSCs in ischemic mouse brains in our recent report.¹⁵

There are reports of NSCs transplanted at different times and by different routes (intraparenchymal, intraventricular, intravenous, and intraarterial).^{5,8,38} In some reports, NSC transplantation was performed at later time points after ICH compared with this study, and they also describe improved behavioral recovery.^{6,12} However, NSCs were transplanted at the acute stage of ICH in our study for examination of enhanced neuroprotection. As the environment of the host tissue at the acute stage of ICH is worse than at later stages due to edema, acute inflammation, and oxidative stress,¹² we looked at the survivability of modified NSCs. We demonstrated that SOD1 overexpression overcomes this hostile host environment. Andres *et al.*⁵ has reported that notwithstanding the differences in the procedures, including the route, NSCs migrate into the perihematomal region and survive after transplantation in ICH animals, but clinically, surgical evacuation is done either with open craniotomy or stereotactic and endoscopic techniques to prevent expansion, to decrease mass effects, and to block the release of neuropathic products from hematomas at the acute stage of ICH.³ Therefore, intraparenchymal transplantation was used in this present study, because it is possible to graft cells during surgery.

In conclusion, we have shown an important role for SOD1 overexpression in NSC survival after transplantation in the ICH brain. Our findings indicate that conferring antioxidant properties on NSCs is a potential approach for enhancing the effectiveness of cell transplantation therapy in ICH.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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