Neural Stem Cells Genetically Modified to Overexpress Cu/Zn-Superoxide Dismutase Enhance Amelioration of Ischemic Stroke in Mice

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**Background and Purpose**—The harsh host brain microenvironment caused by production of reactive oxygen species after ischemic reperfusion injury offers a significant challenge to survival of transplanted neural stem cells (NSCs) after ischemic stroke. Copper/zinc-superoxide dismutase (SOD1) is a specific antioxidant enzyme that counteracts superoxide anions. We have investigated whether genetic manipulation to overexpress SOD1 enhances survival of grafted stem cells and accelerates amelioration of ischemic stroke.

**Methods**—NSCs genetically modified to overexpress or downexpress SOD1 were administered intracerebrally 2 days after transient middle cerebral artery occlusion. Histological and behavioral tests were examined from Days 0 to 28 after stroke.

**Results**—Overexpression of SOD1 suppressed production of superoxide anions after ischemic reperfusion injury and reduced NSC death after transplantation. In contrast, downexpression of SOD1 promoted superoxide generation and increased oxidative stress-mediated NSC death. Transplantation of SOD1-overexpressing NSCs enhanced angiogenesis in the ischemic border zone through upregulation of vascular endothelial growth factor. Moreover, grafted SOD1-overexpressing NSCs reduced infarct size and improved behavioral performance compared with NSCs that were not genetically modified.

**Conclusions**—Our findings reveal a strong involvement of SOD1 expression in NSC survival after ischemic reperfusion injury. We propose that conferring antioxidant properties on NSCs by genetic manipulation of SOD1 is a potential approach for enhancing the effectiveness of cell transplantation therapy in ischemic stroke. (Stroke. 2012;43:2423-2429.)

**Key Words:** copper/zinc-superoxide dismutase ▪ ischemic stroke ▪ neural stem cell ▪ neuroprotection

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**Materials and Methods**

**Isolation and Culture of Fetal NSCs**

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.
NSCs were isolated from the subventricular zones of postnatal Day 1 green fluorescent protein (GFP) Tg mice, SOD1/GFP double Tg mice, and SOD1 KO/GFP Tg mice as described.11

**Induction of Oxygen–Glucose Deprivation**

We used oxygen–glucose deprivation (OGD) and reoxygenation (RO), an in vitro model that best mimics in vivo cerebral ischemia and reperfusion. NSCs were subjected to OGD and RO by replacing the medium with a buffered salt solution without glucose. The plates were placed in an anaerobic chamber at 37°C. After 8 hours, the medium was replaced with culture medium with glucose and the plates were returned to a 5% CO2/95% air incubator for various RO periods.

**In Situ Detection of Superoxide Anion Production**

Hydroethidine was used for the detection of early production of superoxide anions as previously described.12 Production of superoxide anions was shown by oxidized hydroethidine as diffuse signals and small particles in the cytosol.

**Focal Cerebral Ischemia**

Adult male C57BL/6 mice (26–30 g) were subjected to transient focal cerebral ischemia by 45 minutes of intraluminal middle cerebral artery blockade with a suture as described previously.13

**Cell Transplantation**

Three 1.0-μL deposits of NSCs (1×10⁵ cells/μL) were transplanted into the peri-infarct cortex 2 days after stroke.

**Statistical Analysis**

Behavioral data were assessed using repeated-measures analysis of variance. We used Schefé post hoc analysis of a Rotorod test and analyzed modified neurological severity scores using the Steel-Dwass test. For other experimental data, comparisons among multiple groups were performed with one-way analysis of variance followed by Schefé post hoc analysis. Data are expressed as median for the modified neurological severity scores and mean±SD for the other experiments. Significance was accepted with P<0.05.

**Results**

**Characterization of NSCs**

We isolated the NSCs from fetal GFP Tg mice (WTNSCs), SOD1/GFP double Tg mice (TgNSCs), or SOD1 KO/GFP Tg mice (KONSCs). These NSCs, grown as adherent cultures, were self-renewing and multipotent (Figure IA–E in the online-only Data Supplement). The percentage of neurons (10.4%±2.6%, 10.7%±2.2%, and 10.2%±2.4%) and astrocytes (72.2%±9.9%, 70.2%±11.1%, and 69.2%±8.3%) differentiated from the WTNSCs, TgNSCs, and KONSCs was similar. Moreover, no difference was observed in the percentage of Ki-67-positive cells after 8 hours of OGD and 48 hours of RO among the 3 groups (Figure IF in the online-only Data Supplement).

**SOD1 Expression in NSCs**

We next investigated the expression of SOD1 in the WTNSCs, TgNSCs, and KONSCs. Western blot analysis revealed that SOD1 expression was significantly lower in the KONSCs compared with the WTNSCs under both normal conditions and after 8 hours of OGD and 3 hours of RO. No changes were observed in manganese–superoxide dismutase (SOD2) expression. Beta-actin was used as an internal control (n=4). OD indicates optical density. B, Fluorescent staining with 4’,6 diamidino-2-phenylindole (DAPI; blue), GFP (green), total SOD1 (mouse±human; red), and human SOD1 (magenta) in NSCs under normal conditions. The KONSCs showed increased cytosolic total SOD1 signals together with human SOD1 signals, whereas there were less total SOD1 signals observed in the KONSCs compared with the WTNSCs. Scale bar, 20 μm. *P<0.05; §P<0.001. SOD1 indicates copper/zinc-superoxide dismutase; NSCs, neural stem cells; WT, wild-type; Tg, transgenic; KO, knockout; OGD, oxygen–glucose deprivation; RO, reoxygenation; GFP, green fluorescent protein.

**Figure 1**

SOD1 expression in NSCs in vitro. A, Western blot analysis of WTNSCs, TgNSCs, and KONSCs. SOD1 expression significantly increased in the TgNSCs and decreased in the KONSCs compared with the WTNSCs under both normal conditions and after 8 hours of OGD and 3 hours of RO. No changes were observed in manganese–superoxide dismutase (SOD2) expression. Beta-actin was used as an internal control (n=4). OD indicates optical density. B, Fluorescent staining with 4’,6 diamidino-2-phenylindole (DAPI; blue), GFP (green), total SOD1 (mouse±human; red), and human SOD1 (magenta) in NSCs under normal conditions. The KONSCs showed increased cytosolic total SOD1 signals together with human SOD1 signals, whereas there were less total SOD1 signals observed in the KONSCs compared with the WTNSCs. Scale bar, 20 μm. *P<0.05; §P<0.001. SOD1 indicates copper/zinc-superoxide dismutase; NSCs, neural stem cells; WT, wild-type; Tg, transgenic; KO, knockout; OGD, oxygen–glucose deprivation; RO, reoxygenation; GFP, green fluorescent protein.

**Full Methods**

The full methods are detailed in the online-only Data Supplement.
Involvement of SOD1 Expression in NSC Death

In Vitro

We next asked if the levels of SOD1 expression were associated with NSC death after ischemic reperfusion injury. After 8 hours of OGD and 15 minutes of RO, WTNSCs showed a significant increase in hydroethidine signals in the cytosol, which represents production of superoxide anions (Figure 2A). This signal increase was significantly reduced in the TgNSCs, whereas they were enhanced in the KO NSCs (n=4). Scale bar, 20 μm. B, NSCs analyzed by TUNEL staining (red) and DAPI (blue) after 8 hours of OGD and 24 hours of RO. The cell-counting study revealed a significant decrease in TUNEL positivity in the TgNSCs as well as a significant increase in the KO NSCs (n=4). Scale bar, 50 μm. LDH (n=4; C) and WST-1 (n=4; D) assays showed a significant reduction in NSC death and increased viability in the TgNSCs after 8 hours of OGD and 24 hours of RO. In contrast, LDH release was increased in the KO NSCs compared with the WT NSCs. *P<0.05; †P<0.01; §P<0.001. SOD1 indicates copper/zinc-superoxide dismutase; NSC, neural stem cell; HEt, hydroethidine; WT, wild-type; OGD, oxygen–glucose deprivation; RO, reoxygenation; Tg, transgenic; KO, knock-out; TUNEL, terminal deoxynucleotidyl transferase-mediated uridine 5’-triphosphate-biotin nick end labeling; LDH, lactate dehydrogenase.

SOD1 Overexpression Reduced Grafted Cell Death

In Vivo

We transplanted WTNSCs, TgNSCs, or KO NSCs into the peri-infarct cortex 2 days after stroke (Figure III in the online-only Data Supplement). When the WT NSCs were transplanted into the ischemic brains, hydroethidine signals drastically increased 1 hour after transplantation compared with those in the intact brains (Figures 3A and 3C). SOD1 overexpression significantly diminished this increase, whereas hydroethidine signals were significantly elevated in the KO NSC group compared with the WT NSC group. We next counted the number of TUNEL-positive cells that were also GFP-positive 2 days after transplantation. When the WT NSCs were transplanted into the peri-infarct cortex, the number of TUNEL-positive grafted cells increased significantly com-
pared with those in the intact brains. However, this increased TUNEL positivity was significantly reduced by 43% in the TgNSC group (Figures 3B and 3D).

**Increased Survival of Grafted NSCs With SOD1 Overexpression In Vivo**

Twenty-eight days after stroke, staining with GFP revealed an extensive migration of grafted cells toward the ischemic lesion border in the WTNSC, TgNSC, and KO NSC groups (Figure 4A). GFP-positive cells were not found in the striatum. No mice had signs of tumor formation caused by the transplanted NSCs 28 days, or even 3 months, after stroke. The number of surviving grafted cells in the ischemic brains was significantly large in the TgNSC group compared with the WTNSC group (Figure 4B). In contrast, contrasted cell survival was less in the KO NSC group than in the WTNSC group. We next examined the differentiation profiles of the NSCs. Fluorescent double staining of the lineage specific phenotype markers and GFP demonstrated that GFP-positive cells exhibited a neuronal marker, β-tubulin, and an astrocytic marker, glial fibrillary acidic protein, 28 days after stroke (Figure 4C). The percentage of neurons (8.9% ± 1.0%, 8.8% ± 1.1%, and 9.3% ± 1.4%) and astrocytes (40.1% ± 4.3%, 37.1% ± 5.9%, and 38.3% ± 8.0%) differentiated from the grafted NSCs was similar among the WTNSC, TgNSC, and KO NSC groups.
Transplantation of TgNSCs Enhanced Angiogenesis In Vivo

Secretion of vascular endothelial growth factor by transplanted NSCs is involved in functional recovery after ischemic stroke.14 Therefore, vascular endothelial growth factor levels in the cortex were measured by a sandwich enzyme-linked immunosorbent assay 4 days after stroke. These levels significantly increased in the TgNSC group compared with the nontransplanted control and WTNSC groups (Figure 5A). To analyze the effects of the NSCs on angiogenesis, we examined the density of lectin-perfused vessels in the peri-infarct cortex 14 days after stroke, which revealed a significantly higher blood vessel density in the TgNSC group than in the nontransplanted control and WTNSC groups (Figures 5B and 5C). However, this enhanced angiogenesis was not observed in the WTNSC or KO-NSC groups.

Mice Transplanted With TgNSCs Exhibited Amelioration of Ischemic Stroke

To investigate whether transplantation of NSCs could promote amelioration of ischemic stroke, infarct size was measured by hematoxylin and eosin staining 28 days after stroke. Transplantation of TgNSCs significantly reduced the cortical infarct size by 19.8% and 13.1% compared with the nontransplanted control and WTNSC groups (Figure 6A). No changes were observed in striatal infarct size among the 4 groups (data not shown). In addition, a significantly larger number of neurons was observed in the peri-infarct cortex in the TgNSC group than the nontransplanted control and WTNSC groups (Figure IV in the online-only Data Supplement). We next analyzed behavioral performance using the Rotorod test and modified neurological severity scores. The mice that received TgNSCs showed significant functional improvement from Day 7 compared with the nontransplanted control group and from Day 21 compared with the WTNSC group according to the Rotorod test (Figure 6B). Although a statistical significance was not observed, the TgNSC group showed behavioral improvement as indicated by the modified neurological severity scores (Figure 6C). Significant behavioral improvement was not observed in the WTNSC and KO-NSC groups at any time point according to either test compared with the nontransplanted control group.

Discussion

We have demonstrated in this study that SOD1 played a pivotal role in protecting NSCs from ischemic reperfusion injury. The major findings are: (1) SOD1 overexpression increased survival of NSCs after ischemic reperfusion injury by enhancing the antioxidant capacity; (2) downexpression of SOD1 accelerated oxidative stress-mediated NSC death; and (3) transplantation of TgNSCs attenuated infarct size and promoted functional recovery. These findings provide evidence that conferring antioxidant properties on grafted stem cells by genetically manipulating SOD1 expression enhances the effectiveness of stem cell therapy in ischemic stroke.

Although little information exists about the effects of ROS on NSCs, recent reports have shown that NSCs use ROS to regulate proliferation, self-renewal, and neurogenesis under physiological conditions.15 Although it is controversial whether NSCs generally have lower or higher endogenous ROS levels than their differentiated progeny,15–17 these cells seem to possess an enhanced antioxidant capacity against oxidative stress compared with more differentiated cells because of the high expression of antioxidant enzymes, including uncoupling protein 2 and glutathione peroxidase.16 This activity may be a protective mechanism in stem cell populations with active oxidant-mediated signaling to prevent an excess of or toxic levels of ROS from being generated. Regardless of these reports, we have found in this study that a large number of NSCs suffered oxidative stress-mediated death after ischemic reperfusion injury. In line with our data, excessive oxidative stress generated under pathophysiological conditions is reported to be toxic for survival of NSCs.18–21 These findings lead us to surmise that genetic modification of NSCs to overexpress antioxidant enzymes might be beneficial for promoting survival of these grafted cells.

Because the proliferation and differentiation capacity of the NSCs are highly dependent on endogenous ROS levels under physiological conditions, manipulating the expression of antioxidant enzymes may unexpectedly change their pro-
For example, conditional deletion of phosphatase and tensin homolog on chromosome 10 in nestin-expressing NSCs in the developing brain and in glial fibrillary acidic protein-expressing stem cells in the subventricular zone of the adult brain promoted and sustained NSC self-renewal and neurogenesis, contributing to brain overgrowth. In contrast, TgNSCs as well as KONSCs demonstrated similar proliferation and differentiation capacities in our study compared with WTNSCs. In addition, neither the SOD1 Tg nor the KO mice had any difference from the WT mice in phenotypes, including brain size. This discrepancy might be because the basal expression of antioxidant or pro-oxidant enzymes in TgNSCs and KO NSCs demonstrated similar proliferation and differentiation capacities in our study compared with WT NSCs. In addition, neither the SOD1 Tg nor the KO mice had any difference from the WT mice in phenotypes, including brain size. This discrepancy might be because the basal expression of antioxidant or pro-oxidant enzymes in TgNSCs and KO 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.

In this study, we were able to identify the cell type in less than half of the grafted cells in vivo. However, because tumor formation was not observed even 3 months after transplantation indicates that the grafted cells did not transform into tumorigenic cells.

In this study, we focused on the cytoprotective effects of SOD1 in NSCs. First, SOD1 overexpression reduced ROS levels and increased NSC survival after ischemic reperfusion injury. This is consistent with our previous studies showing that SOD1 overexpression exhibited neuroprotection in rodent brains after transient focal cerebral ischemia and transient global cerebral ischemia. Second, SOD1 down-expression contributed to enhanced oxidative stress and increased NSC death. This agrees with our previous reports showing that a reduction in SOD1 activity led to larger infarct size and brain swelling after ischemic stroke. These findings indicate a strong relationship between SOD1 expression and NSC survival under pathophysiological conditions. Thus, we propose that genetic manipulation of SOD1 is a potential molecular target for stem cell therapy in ischemic stroke.

Despite many preclinical studies showing that cell transplantation can ameliorate ischemic stroke, the mechanisms mediating recovery are not well known. The majority of exogenous stem cells under investigation exert so-called “nursing functions” to the injured brain such as cytoprotection or stimulation of endogenous repair mechanisms. Considering the reduced cortical infarct size and increased blood vessel density in the ischemic brain, neuroprotection and angiogenesis might be among the main therapeutic actions of transplanted NSCs in the present study. Reduced grafted cell death with SOD1 overexpression might contribute to the enhanced accumulation of neuroprotective trophic factors, including proangiogenic vascular endothelial growth factor, that are secreted from NSCs. In this study, we performed subacute delivery of NSCs, which has more clinical relevance than acute delivery. However, subacute delivery might limit the neuroprotective action of the NSCs. We conclude that this is why the behavioral improvement

**Figure 6.** Effects of NSCs on infarct size and behavioral performance. A, Measurement of the infarct size by H&E staining 28 days after stroke. Cortical infarct size was significantly decreased in the TgNSC group compared with the nontransplanted control and WTNSC groups (n=12). Behavioral performance using the Rotorod test (B) and mNSS (C). Transplantation of the TgNSCs showed the greatest functional improvement in the Rotorod test (n=12). Despite the tendency toward enhanced recovery, the TgNSC group did not show a significant behavioral improvement compared with the nontransplanted control and WTNSC groups according to the mNSS (n=12). Black bars denote nontransplanted control group; yellow bars denote WTNSC group; blue bars denote TgNSC group; red bars denote KONSC group. *P<0.05; †P<0.01, ‡P<0.005, §P<0.001. NSCs indicates neural stem cells; H&E, hematoxylin and eosin; Tg, transgenic; WT, wild-type; mNSS, modified neurological severity score; KO, knockout.
observed in the present study was relatively small and occurred at a later time point.

Conclusions
We have shown the strong involvement of SOD1 expression in NSC survival after ischemic reperfusion injury. Our findings indicate that conferring antioxidant properties on NSCs by genetic manipulation of SOD1 is a potential approach for enhancing the effectiveness of cell transplantation therapy in ischemic stroke.

Acknowledgments
We thank Liza Reola and Bernard Calagui for technical assistance, Cheryl Christensen for editorial assistance, and Elizabeth Hoyte for assistance with the figures.

Sources of Funding
This work was supported by National Institutes of Health grants PO1 NS014543, RO1 NS038653, and RO1 NS025372 and by the James R. Doty Endowment.

Disclosures
None.

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Stroke. 2012;43:2423-2429; originally published online June 19, 2012; doi: 10.1161/STROKEAHA.112.656900

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Neural Stem Cells Genetically Modified to Overexpress Cu/Zn-Superoxide Dismutase Enhance Amelioration of Ischemic Stroke in Mice

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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 GFP Tg mice [C57BL/6-Tg(UBC-GFP)30Scha/J; The Jackson Laboratory, Bar Harbor, ME] were bred with heterozygous SOD1 Tg mice (C57BL/6 background, backcrossed with C57BL/6 for more than 10 generations) or heterozygous SOD1 KO mice (C57BL/6 background, backcrossed with C57BL/6 for more than 10 generations) to generate heterozygous GFP Tg mice, heterozygous SOD1/GFP double Tg mice, and heterozygous SOD1 KO/GFP Tg mice. These animals were used for isolation of NSCs. We also used WT C57BL/6 mice (The Jackson Laboratory) for middle cerebral artery occlusion models. Animals were randomized into groups. All methods and assessments described below were carried out by individuals blinded to the groups.

Isolation and Culture of Fetal NSCs
NSCs were isolated from the heterozygous GFP Tg mice, heterozygous SOD1/GFP double Tg mice, and heterozygous SOD1 KO/GFP Tg mice as described, with some modification. In brief, bilateral subventricular zones from postnatal Day 1 mouse brains were dissected in Dulbecco’s PBS (14040-182; Invitrogen, Carlsbad, CA) and mechanically dissociated. The cells were collected and re-suspended in NEUROBASALTM-A medium (10888-022; Invitrogen) containing B-27 supplement (12587-010; Invitrogen), l-glutamine (25030-081; Invitrogen), 20 ng/ml murine fibroblast growth factor-basic (450-33; PeproTech, Rocky Hill, NJ), and 10 ng/ml murine epidermal growth factor (315-09; PeproTech). Cells were cultured on a 10-cm plastic dish pre-coated with poly-L-ornithine hydrobromide (P3655-100MG; Sigma-Aldrich, St. Louis, MO) and laminin (L2020-1MG; Sigma-Aldrich) at 37°C and 5% CO2 as adherent monolayers. The medium was changed every 2 days and the cells were passaged once a week. Cells that had been passaged 4 to 10 times were used for the experiments. Because we isolated WT NSCs, Tg NSCs, and KO NSCs from the littermates, we predicted that the recipients' inflammatory responses to the grafted NSCs would not differ among these groups after transplantation.

Western Blot Analysis
The NSCs were treated with cell lysis buffer (9803; Cell Signaling Technology, Beverly, MA) and used as whole cell lysate samples. Protein concentrations were examined by comparison with a known concentration of bovine serum albumin (BSA) using a kit (23225; Thermo Fisher Scientific, Waltham, MA). Equal amounts of the samples (10 μg) were loaded per lane and analyzed by SDS-PAGE on a 10% NuPAGE Bis-Tris gel (NP0303; Invitrogen) and then
immunoblotted. The primary antibodies were a 1:1000 dilution of a rabbit polyclonal anti-SOD1 antibody (molecular weight: ~ 19 kDa) (SOD-100; Enzo Life Sciences, Plymouth Meeting, MA), a 1:1000 dilution of a rabbit polyclonal anti-manganese-superoxide dismutase antibody (molecular weight: ~ 25 kDa) (SOD-110; Enzo Life Sciences), and a 1:100,000 dilution of a mouse monoclonal anti-actin antibody (molecular weight: ~ 42 kDa) (A5441; Sigma-Aldrich). After incubation with horseradish peroxidase-conjugated anti-mouse IgG (7076; Cell Signaling Technology) or anti-rabbit IgG (7074; Cell Signaling Technology), the antigen was detected by SuperSignal West Pico substrates (1856135/1856136; Thermo Fisher Scientific). Images were captured with a GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA) and the results were quantified using MultiAnalyst software (Bio-Rad).

**Immunofluorescent Staining**
For immunocytochemistry, the NSCs cultured on eight-well chamber slides (154941; Thermo Fisher Scientific) were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes. They were washed with PBS and incubated for 1 hour in blocking solution (PBS containing 3% BSA and 0.3% Triton X-100). For immunohistochemistry, the animals were anesthetized and perfused with PBS followed by 4% paraformaldehyde in PBS, pH 7.4. The brains were postfixixed overnight in the same fixative at 4°C. For cryosectioning, fixed tissues were 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 (4583; Sakura Finetek USA, Inc., Torrance, CA). Cryostat sections (20 μm) were cut and affixed to glass slides (12-550-15; Thermo Fisher Scientific). 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 (1:100, G10362; Invitrogen), goat anti-GFP (1:100, LS-C67095; LifeSpan BioSciences, Seattle, WA), mouse anti-nestin (1:100, AB353; Millipore, Billerica, MA), rabbit anti-NeuN (a neuronal marker) (1:1000, ABN78; Millipore), mouse anti-Sox2 (a NSC marker) (1:50, 4900; Cell Signaling Technology), rabbit anti-β-tubulin (1:500, PRB-435P; Covance, Princeton, NJ), mouse anti-GFAP (1:100, AB360; Millipore), rabbit anti-NG2 (an oligodendrocytic marker) (1:100, AB5320; Millipore), rabbit anti-Ki-67 (1:50, ab16667; Abcam, Cambridge, MA), rabbit anti-SOD1 (1:50, SOD-100; Enzo Life Sciences), and mouse anti-SOD1 (1:300, ab20926; Abcam). After three washes in PBS, 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 (A21206), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (A21207), Alexa Fluor 488-conjugated donkey anti-goat IgG (A11055), Alexa Fluor 594-conjugated donkey anti-mouse IgG (A21203), and Alexa Fluor 647-conjugated donkey anti-mouse IgG (A31571; all from Invitrogen). After three washes in PBS, the samples were covered with VECTASHIELD
mounting medium with 4',6 diamidino-2-phenylindole (DAPI) (H-1500; Vector Laboratories, Burlingame, CA). The samples were analyzed by confocal microscopy (LSM 510; Carl Zeiss, Thornwood, NY) or fluorescence microscopy (Axioplan 2; Carl Zeiss).

**In Situ Detection of Superoxide Anion Production**

For the in vitro study, 5 μM HEt solution (D23107; Invitrogen) was added to the cell culture medium. The cells were incubated for 5 minutes, followed by fixation with 4% paraformaldehyde in PBS for 15 minutes. For the in vivo study, HEt solution (200 μL of 1 mg/mL in 1% dimethyl sulfoxide with saline) (D11347; Invitrogen) was administered intravenously immediately after transplantation of the NSCs. Animals were killed 1 hour after administration and tissue sections were prepared as described above. For fluorescent double staining of HEt signals and GFP, sections were incubated with rabbit anti-GFP (1:100, G10362; Invitrogen), followed by Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A21206; Invitrogen). Slides were covered with VECTASHIELD mounting medium with DAPI. The sections were observed with a fluorescence microscope, and oxidized HEt 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).

**Assessment of Cell Death and Cell Viability In Vitro**

Cell death was examined by a standard measurement of LDH release using a LDH-cytotoxicity assay kit (K311-400; BioVision, Mountain View, CA). The amount of released LDH was assessed in an aliquot of the cell medium, using the manufacturer’s instructions. Cell viability was analyzed with a cell proliferation reagent using a WST-1 assay kit (05015944001; Roche Diagnostics, Indianapolis, IN). For in situ labeling of DNA fragmentation, the NSCs were prepared as described in the immunofluorescent staining section. An in situ cell death detection kit, TMR red (12156792910; Roche Diagnostics), was used according to the manufacturer’s instructions. Slides were covered with VECTASHIELD mounting medium with DAPI and observed with fluorescence microscopy.

**Focal Cerebral Ischemia**

Adult male C57BL/6 mice (26 to 30 g) were anesthetized with 2.0% isoflurane in 30% oxygen and 70% nitrous oxide using a face mask. The rectal temperature was controlled at 37°C with a homeothermic blanket. Physiological parameters were monitored throughout the surgeries. After a midline skin incision, the right external carotid artery was exposed, and its branches were electrocoagulated. A 6-0 monofilament nylon suture coated with silicon rubber (6023PK5Re; Doccol Corp., Redlands, CA) was introduced into the right internal carotid artery through the
external carotid artery stump. After 45 minutes of middle cerebral artery occlusion, blood flow was restored by withdrawal of the suture. The animals were maintained in an air-conditioned room at 20°C with ad libitum access to food and water before and after surgery. Every mouse included in the study showed circling toward the paretic side directly after the surgery, indicating that infarct was successfully produced. A total of four mice died in the first 24 hours after induction of stroke and were excluded from the study (one mouse in the non-transplanted control group, one mouse in the WTNSC group, one mouse in the TgNSC group, and one mouse in the kONSC group.).

**Intracerebral NSC Transplantation**

The mice were anesthetized 2 days after the onset of stroke and the NSCs were transplanted using a 10-μL Hamilton syringe with a 33 G needle attached to a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The mice were given three 1.0-μL deposits of single cell suspension in Dulbecco’s PBS (1×10^5 cells per μL) along the anterior-posterior axis into the cortex at these coordinates: (1) anterior–posterior (A–P), +1.0; medial–lateral (M–L), +2.0; dorsal–ventral (D–V), -1.0; (2) A–P, -0.5; M–L, +2.5; D–V, -1.0; (3) A–P, -2.0; M–L, +2.5; D–V, -1.0. These targets approximated the penumbra area in the cortex. Deposits were delivered at 0.5 μL/minute and the needle was left in situ for 5 minutes post-injection before being slowly removed.

**In Situ Labeling of DNA Fragmentation in the Transplanted NSCs**

The tissue sections were prepared as described above. Every eighth section (160 μm apart) containing the graft region (A–P, -0.5; M–L, +2.5; D–V, -1.0) was chosen for staining using the in situ cell death detection kit, TMR red. The sections were then incubated with rabbit anti-GFP (1:100; G10362; Invitrogen) and mouse anti-nestin (1:100; AB353; Millipore), followed by Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A21206; Invitrogen) and Alexa Fluor 647-conjugated donkey anti-mouse IgG (A31571; Invitrogen). Slides were covered with VECTASHIELD mounting medium with DAPI. TUNEL-positive cells, also stained with GFP, were counted using unbiased computational stereology (fractionator method, using STEREOL7NEGTIGATOR software [MicroBrightfield, Inc., Williston, VT]), as described.³

**Quantification of Survival of the Transplanted NSCs**

The transplanted GFP-positive cells were counted using unbiased computational stereology as described above. The sections were stained with GFP and DAPI. All the GFP-positive cells were counted on six serial coronal sections per brain (1 mm apart).
Assessment of NSC Differentiation Profiles
The proportion of GFP-positive cells, also stained with lineage-specific phenotype markers (β-tubulin and GFAP), was determined by confocal microscopy. Split panel and z axis analyses were used for all counting. One hundred or more GFP-positive cells were scored for both markers per animal.

Detection of VEGF
Fresh brain tissue was removed 2 days after transplantation. The rectangular cuboid tissue block of the cortex, 1 mm on either side of the NSC-transplanted regions, was dissected (width 2 mm × length 5 mm) and used as a sample. Protein was extracted as described in the Western blot analysis section. Commercial VEGF ELISA kits (MMV00; R&D Systems, Minneapolis, MN) were used to quantify the concentration of VEGF-A in each of the samples.

BVD Analysis
BVD was assessed 14 days after stroke as described previously,4 with some modifications. Briefly, vessels were labeled by jugular vein injection of DyLight 594-labeled Lycopersicon esculentum lectin (50 mL) (DL-1177; Vector Laboratories) 30 minutes before the animals were killed. The tissue sections (40 μm) were prepared as described in the immunofluorescent staining section. The images were captured by confocal microscopy at these six coordinates: (1) A–P, +1.0; M–L, ±2.5; D–V, -0.5; (2) A–P, -0.5; M–L, ±3.0; D–V, -0.5; (3) A–P, -2.0; M–L, ±3.0; D–V, -0.5. BVD was measured using ImageJ software to determine pixel number/image, and the average of BVD ratio (ipsilateral/contralateral) was calculated.

Measurement of Infarct Size
The brain sections were stained with H&E. We estimated the cortical/striatal infarct size as a percentage of the ipsilateral cortex/striatum using the following: [(area of contralateral cortex/striatum) - (area of remaining ipsilateral cortex/striatum)/(area of contralateral cortex/striatum) × 100]. The area of both sides of the cortex and striatum was measured on six serial coronal sections per brain (1 mm apart), and the area of the infarct was quantified over these six levels using Adobe Photoshop (Adobe Systems, San Jose, CA).

Quantification of Neuronal Survival
The brain sections were stained with NeuN and DAPI as described above. The images were captured by a fluorescence microscope at these coordinates: (1) A–P, +1.0; M–L, +2.5; D–V, -0.5; (2) A–P, -0.5; M–L, +3.0; D–V, -0.5; (3) A–P, -2.0; M–L, +3.0; D–V, -0.5. NeuN-positive cells were counted using unbiased computational stereology by individuals blinded to the animal.
Behavioral Analysis
A rotarod test and mNSS were examined by two individuals, blinded to the mouse-treatment status, on the day of the stroke surgery (baseline), and at 2, 7, 14, 21, and 28 days after the onset of stroke. The mNSS are the result of a battery of motor and coordination tests assessing the severity of neurological deficits on a graded scale ranging from 0 to 14, where 0 represents normal function and 14 represents maximal deficits as described.\textsuperscript{5}

For the rotarod test, after 3 days of training prior to the stroke surgery, the mice were placed on the cylinder (ENV-577M; Med Associates Inc., St. Albans, VT) and the time the animals remained on the rotarod was recorded. The speed was slowly increased from 4 to 40 rpm within a period of 5 minutes. The trial was ended if the animal fell off the rungs or gripped the device and spun around for two consecutive revolutions. The maximum duration on the device was recorded with three rotarod measurements on the day of transplantation prior to the stroke surgery. Rotarod test data are presented as percentages of the maximal duration, compared with the internal baseline control.
Figure S1. Characterization of GFP-positive NSCs in vitro. The NSCs grown as adherent cultures were examined by immunocytochemistry for GFP (green) and the NSC markers nestin (A) and Sox2 (red) (B). Nearly all the GFP-positive cells colocalized with both nestin and Sox2. Nuclei were counterstained with DAPI (blue). Scale bars, 50 μm. After culturing in differentiation medium containing 1 μM retinoic acid and 1% fetal bovine serum for 5 days, GFP-positive cells (green) colocalized with the neuronal marker β-tubulin (C), the astrocytic marker GFAP (D), and the oligodendrocytic marker NG2 (red) (E). Nuclei were counterstained with DAPI (blue). Scale bars, 20 μm. F, Fluorescent staining of the NSCs with Ki-67 (red) and DAPI (blue) 48 hours after OGD in vitro. The percentage of Ki-67-positive cells was similar among the WTNSCs, TgNSCs, and KO NSCs. Scale bar, 20 μm.
Figure S2. Overexpression of SOD1 reduced NSC death under oxidative stress. NSCs were subjected to the oxidative stimuli H$_2$O$_2$ (200 μM) (A) and diethylenetriamine/nitric oxide (DETA/NO) (250 μM) (B). SOD1 overexpression significantly reduced the release of LDH from the NSCs under both stimuli, whereas downexpression of SOD1 increased LDH release after H$_2$O$_2$ stimulation (n=4). *P<0.05; §P<0.001.
Figure S3. Focal cerebral ischemia and transplantation of NSCs. A schematic diagram illustrates the three transplanted sites in the peri-infarct cortex (A). Study designs for in vivo experiments are outlined in B.
**Figure S4.** Enhanced neuronal survival with transplantation of TgNSCs. Fluorescent staining of NeuN (red) and DAPI (blue) and quantification of NeuN-positive cells in the peri-infarct cortex revealed that neuronal survival was significantly increased in the TgNSC group 28 days after stroke, compared with the non-transplanted control and WTNSC groups (n=8). Scale bar, 20 μm. *P<0.05; §P<0.001.
Supplemental References


