Bystander Effect Fuels Human Induced Pluripotent Stem Cell-Derived Neural Stem Cells to Quickly Attenuate Early Stage Neurological Deficits After Stroke

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ABSTRACT

Present therapies for stroke rest with tissue plasminogen activator (tPA), the sole licensed antithrombotic on the market; however, tPA’s effectiveness is limited in that the drug not only must be administered less than 3–5 hours after stroke but often exacerbates blood-brain barrier (BBB) leakage and increases hemorrhagic incidence. A potentially promising therapy for stroke is transplantation of human induced pluripotent stem cell-derived neural stem cells (hiPSC-NSCs). To date, the effects of iPSCs on injuries that take place during early stage ischemic stroke have not been well studied. Consequently, we engrafted iPSC-NSCs into the ipsilesional hippocampus, a natural niche of NSCs, at 24 hours after stroke (prior to secondary BBB opening and when inflammatory signature is abundant). At 48 hours after stroke (24 hours after transplant), hiPSC-NSCs had migrated to the stroke lesion and subsequently, we engrafted iPSC-NSCs into the ipsilesional hippocampus, a natural niche of NSCs, at 24 hours after stroke (prior to secondary BBB opening and when inflammatory signature is abundant). At 48 hours after stroke (24 hours after transplant), hiPSC-NSCs had migrated to the stroke lesion and consequently, we engrafted iPSC-NSCs rapidly improved neurological function (less than 24 hours after transplantation). Rapid hiPSC-NSC therapeutic activity is mainly due to a bystander effect that elicits reduced inflammation and BBB damage.

SIGNIFICANCE

Clinically, cerebral vessel occlusion is rarely permanent because of spontaneous or thrombolytic therapy-mediated reperfusion. These results have clinical implications indicating a much extended therapeutic window for transplantation of human induced pluripotent stem cell-derived neural stem cells (hiPSC-NSCs; 24 hours after stroke as opposed to the 5-hour window with tissue plasminogen activator [tPA]). In addition, there is potential for a synergistic effect by combining hiPSC-NSC transplantation with tPA to attenuate stroke’s adverse effects.

INTRODUCTION

Stroke causes long-term neurological disability. Transient ischemic reperfusion (IR), not permanent occlusion, is more relevant because, from a clinical standpoint, a good deal of stroke injury results from reperfusion following ischemia. The blood-brain barrier (BBB), which separates the brain from the circulatory system, is a key target of the ischemic reperfusion insult. IR causes biphasic opening of the BBB [1] that initiates within several hours. If unchecked, an irreversible second opening occurs within the following 24–72 hours. This second episode contributes significantly to cell death.

Reducing these early stage injuries could extend the therapeutic window and lead to opportunities for better clinical outcome. Presently, the only available drug approved by the U.S. Food and Drug Administration is thrombolytic recombinant tissue plasminogen activator (tPA), which dissolves clots and salvages cells in the ischemic penumbra. The downside of tPA is its short (5-hour) therapeutic window and greater risk of hemorrhage due to exacerbated damage to the BBB [2].

Human induced pluripotent stem cell-derived neural stem cells (hiPSC-NSCs) could be an ideal transplantation cell type for stroke therapy [3]. These cells potentially possess multiple therapeutic
actions, including functional neural replacement and bystander effects (e.g., anti-inflammatory action, enhancement of endogenous repair mechanisms by delivery of genetically engineered or inherently synthesized gene products) [4, 5]. We hypothesized that reducing inflammation and curtailing BBB leakage during stroke’s initial stage could mitigate further neuronal damage and hemorrhage.

We previously reported that human NSCs derived from pluripotent embryonic stem cells have both a bystander effect (e.g., anti-inflammatory effect) and a neural cell replacement function in vivo without tumor formation [4]. Others have reported engraftment of exogenous iPSC-derived NSCs after ischemia in to various regions of the brain at various time points in rodent stroke models [6–11]. However, we and others demonstrated in previous studies that when stem cells are transplanted into both intracranial and intravascular regions 4–12 hours after middle cerebral artery occlusion (MCAO), cell migration into the ischemic territory of parenchyma is severely limited.

Early stage BBB damage is linked with increased endothelial-leukocyte adhesion molecules, inflammation, matrix metalloprotei-nases, and disruption of tight junctions [12–17]. In this study, we tested the bystander or “chaperone” effect of iPSC-NSCs on repairing the BBB between 24 and 48 hours after IR. This time frame (24–48 hours) coincides with the second phase of BBB opening and the peak of proinflammatory and proapoptotic factors [18]. The goal of this study was to limit early stage BBB injuries, an outcome that would protect against the second stage of stroke damage.

To investigate the impact of iPSC-NSCs between 24 to 48 hours after stroke, we used a well-defined rodent experimental stroke model: filamentous middle cerebral artery occlusion (60-minute MCAO) with subsequent reperfusion (MCAO/R) [19]. We used well-characterized iPSC-derived NSCs; iPSCs are reprogrammed from foreskin fibroblasts by ectopic expression of chromatin-remodeling transcription factors (OCT4, KLF4, SOX2, and c-MYC) and differentiated into NSCs. We transplanted hiPSC-NSCs into the ipsilateral hippocampus, where neurogenesis is ongoing and signals for migration, proliferation, differentiation, and integration are widespread.

This study is the first to show that, following stroke, behavioral functions recover quickly (within 24 hours after transplantation), and pathophysiology is attenuated when iPSC-NSCs are transplanted intracranially (hippocampus) during the acute phase after stroke.

### MATERIALS AND METHODS

#### Middle Cerebral Artery Occlusion

All experiments were approved by the institutional animal care and use committee of Tulane University and conducted according to the guidelines of the American Veterinary Medical Association and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals and procedures include 24- to 26-g male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) that were treated with either a sham operation or MCAO/R with or without transplantation.

We induced focal ischemia via intraluminal MCA occlusion, as described previously [20]. Briefly, mice were anesthetized with 1% isoflurane in 30% oxygen, with body temperature continuously maintained at 37 ± 0.5°C. An incision was made to the midline of the neck to expose both the left common and external carotid arteries. To the left internal carotid artery, we introduced a 6-0 nylon monofilament coated with silicon rubber (Doccol Corporation, Sharon, MA, http://www.doccol.com) through the external carotid stump to block the origin of the middle cerebral artery (MCA). After 60-minute occlusion, reperfusion was introduced by withdrawing the filament. Sham-operated control mice underwent a similar surgery, but filament removal was carried out immediately after insertion (no occlusion, no reperfusion). We assessed regional cerebral blood flow (rCBF) using the MCA using a transcranial laser Doppler (Perimed, Stockholm, Sweden, http://www.perimed-instruments.com); severely reduced rCBF (>80%) was established after successful MCAO, and reperfusion resulted in recovery of blood flow (>90%). Brains were collected 48 hours after MCAO/R (24 hours after transplantation) for quantitative analyses.

### Human iPSC-NSC Culture

We used two different types of iPSC-NSCs. One type was iPSC-NSCs derived by EMD Millipore (Billerica, MA, http://www.emdmillipore.com). We purchased and used viral transgene-free human iPSC-derived neural progenitor cells (SCC035). Specifically, iPSCs were generated by EMD Millipore using the kit containing STEMCCA Cre-excisable constitutive polycistronic (OCT4, KLF4, SOX2, and c-MYC) lentivirus. These hiPSC-NPCs proliferate as an adherent cell monolayer, and more than 80% of the cells express neural stem cell markers (e.g., Nestin and Sox-2). We purchased passage 3 cells and expanded the cells using our well-defined NSC media. The other type was human iPSCs (IMR90-1, IMR90 clone 1) purchased from WiCell Research Institute (Madison, WI, http://www.wicell.org). The iPSC line was generated by WiCell from IMR90 fetal lung fibroblasts through viral transduction by combining the OCT4, SOX2, NANOG, and LIN28 genes. Using a previously described protocol [21] and a well-established protocol in our laboratory, we derived NSCs from these iPSCs in two steps, first, producing neuroepithelial cells (NEPs) from these iPSCs and, second, producing NSCs from NEPs. Specifically, we generated NEPs in N2B27 medium containing Dulbecco’s modified Eagle’s medium nutrient mixture F12, two supplements (N2 and B27), and 0.55 mM β-mercaptoethanol (all from Invitrogen; Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com); human recombinant noggin (300 ng/ml; PeproTech, Rocky Hill, NJ, https://www.peprotech.com) and SB431542 (10 μM; Tocris Bioscience, Bristol, U.K., http://www.tocris.com) were also added. This medium was changed daily. After 8–10 days of differentiation, neural rosettes containing NEPs appeared. At this point, the medium was changed to N2B27 medium, which was supplemented with human epidermal growth factor (20 ng/ml; R&D Systems, Minneapolis, MN, http://www.rndsystems.com) and human basic fibroblast growth factor (20 ng/ml; PeproTech). These NSCs were cultured for 3–5 days until confluent, then passaged (dilution factor: approximately 1:3 to 1:5) using Accutase. Prior to confluence, the hiPSC-NSCs were transferred enzymatically during refedding once per week or within 48–72 hours prior to transplantation. The cells proliferated as an adherent cell monolayer, and more than 80% of the cells expressed appropriate NSC markers (e.g., Nestin and Sox-2), as confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and immunostaining during the expansion phase.

It has been previously documented that hNSCs can be successfully xenografted and integrated into the mouse brain [6, 11]. Previous studies by others showed naive iPSCs (not derived to NSCs) to be tumorigenic when intracranially transplanted into a rodent brain.
model of MCAO [22]. We found no non-neural cell types or tumors after the hiPSC-NSCs were engrafted.

**hiPSC-NSC Transplantation**

At 24 hours after MCAO/R, we transplanted hiPSC-NSCs into the hippocampus. Adult C57BL/6J mice were anesthetized with 1% isoflurane in 30% oxygen using a face mask and placed in a stereotaxic frame (Stoelting Co., Wood Dale, IL, http://www.stoeltingco.com). To prevent the eyes from drying, ocular ointment was applied, animal heads were wiped with 70% ethanol, and the skin on the head was cut open at the midline. A small burr hole (0.5-mm diameter, F.S.T.) was drilled in the skull (2-mm posterior to the bregma, 1.5-mm lateral to the sagittal suture; 2 μl of phosphate-buffered saline (PBS) containing ~100,000 viable cells were injected over a 3-minute period into the ipsilesional hemisphere (depth of 2- to 2.5-mm dorsal). Sham controls were injected with 2 μl PBS alone. The wound was sealed with cyanoacrylate glue (Vector Laboratories, Burlingame, CA, https://www.vectorlabs.com), and the brains were collected for analysis 48 hours after MCAO/R (24 hours after transplantation).

**Behavioral Tests**

Behavioral tests for each mouse included adhesive removal, beam walk, and rotarod, which were carried out 3 days consecutively before MCAO/R (as training) and from 2 to 30 days after MCAO/R. For the adhesive removal test, we attached a small piece (3 x 4 mm) of adhesive tape onto the contralateral forepaw of each mouse using equal pressure [23]. The mouse was then transferred to a transparent Perspex box (VetEquip, Inc., Pleasanton, CA, http://www.vetequip.com), and we recorded the time it took for the mouse to remove the tape (to 120 seconds maximum).

The beam walk test required that the mice be trained to traverse an elevated beam (700 x 5 mm) to reach an enclosed box. For the study, we chose only the mice that reached the box in 20 seconds without stopping. After MCAO/R, latency to traverse the beam (to 60 seconds maximum) was recorded.

The rotarod test [24] was performed with minor modifications by using four velocities (10, 15, 20, and 25 rotations per minute [rpm]) with three trials per velocity. The amount of time that a mouse remained on the rod was recorded, but the trial ended if the mouse fell. Speed averages shown are as a percentage relative to the sham mice. Data shown are the total amount of time on the rod as a percentage relative to the sham mice.

**Tissue Processing**

At 48 hours after MCAO/R (24 hours after transplantation), mice were deeply anesthetized and transcardially perfused with normal saline and then with phosphate-buffered 4% paraformaldehyde (PFA). Brains were postfixed in PFA for 24 hours and cryoprotected in 30% sucrose; free-floating coronal sections (30 μm) were prepared using a vibratome (VT1000 S; Leica Biosystems, Wetzlar, Germany, http://www.leica-biosystems.com); frozen sections were embedded in Tissue-Tek O.C.T. compound (catalog no. 4583; Andwin Scientific, Schaumburg, IL, https://www.andwinci.com), and then cut coronally at a 20-μm thickness.

**Quantification of Infarct Volume**

Triphenyl tetrazolium chloride (TTC) staining was used to measure infarct (lesion) volume. Fresh mouse brains were cut into 1-mm coronal sections at 48 hours after MCAO/R, then incubated in 2% TTC solution (Sigma-Aldrich, St. Louis, MO, https://www.sigmaaldrich.com). When brain tissue is viable, mitochondria convert the TTC to a red substance, and the ischemic area remains colorless. ImageJ analysis (NIH, Bethesda, MD, http://imagej.nih.gov/ij/) was used to measure infarct size. Lesion volume was calculated as a percentage volume of the contralateral hemisphere to compensate for edema, using the following formula: 

\[ \text{infarct volume} = \left( \frac{\text{volume of total ipsilateral hemisphere} - \text{volume of infarct area}}{\text{volume of contralateral hemisphere}} \right) \]

**Immunohistochemistry**

To block nonspecific binding to the 30-μm coronal free-floating sections, brain sections were first incubated in 10% goat serum in PBS, pH 7.4 (containing 0.1% Tween 20, 0.3% Triton X-100), and then incubated with primary antibodies at 4°C overnight. Primary antibodies (and dilutions) were used as follows: rabbit anti-Iba-1 (1:300; Wako Pure Chemical Industries, Wako, Japan, http://www.wako-chem.co.jp/english/); mouse anti-human cytoplasm STEM121 (1:500; StemCells, Inc., Palo Alto, CA, http://www.stemcellsinc.com); mouse anti-human mitochondria (1:300; EMD Millipore); mouse anti-human Nestin (1:300; Abcam, Cambridge, MA, http://www.abcam.com) for neural stem cells; mouse anti-beta-tubulin (Tuj-1, 1:300; Abcam) for neuron; rabbit anti-S100-β for astroglia; rat anti-mouse CD31 (1:400; Abcam); and rabbit anti-brain-derived neurotrophic factor (anti-BDNF; 1:400; Abcam). For immunofluorescent staining, we used the following secondary antibodies: goat anti-rabbit IgG (Alexa Fluor 594, 1:1500; Invitrogen; Thermo Fisher Scientific), goat anti-mouse IgG (Alexa Fluor 488, 1:1500; Invitrogen; Thermo Fisher Scientific), and goat anti-rat IgG (Alexa Fluor 594, 1:1500; Invitrogen; Thermo Fisher Scientific).

**Reverse Transcriptase Polymerase Chain Reaction**

To extract total RNA from ipsilesional MCAO/R mouse brains, tissue (~20–30 mg preserved in RNAlater (Ambion; Thermo Fisher Scientific) was immersed in Trizol reagent (Invitrogen; Thermo Fisher Scientific) and homogenized using MagNA Lyser (Roche Diagnostics, Basel, Switzerland, http://www.roche.com). RNA fractions were extracted with chloroform, mixed with an appropriate volume of 70% ethanol, and loaded onto an RNeasy column (Qiagen, Venlo, The Netherlands, https://www.qiagen.com). DNase I (Qiagen) was used to digest the retained material. To prepare first-strand cDNA, 2 μg of total RNA was reverse transcribed with Applied Biosystems high-capacity cDNA reverse transcriptase and random primers (catalog no. 4368814; Invitrogen; Thermo Fisher Scientific). The PCR amplification reaction mixture included 2 μl of reverse transcriptase in 10 μl of SsoFast Probes Supermix with Rox (Bio-Rad, Hercules, CA, http://www.bio-rad.com), which was placed in a CFX96 Real-Time Thermal Cycler (Bio-Rad) under reaction conditions including a 30-second hold at 95°C, 35 cycles of activation for 5 seconds at 95°C, and annealing/extension for 10 seconds at 60°C. The following TaqMan gene expression assays were used (tumor necrosis factor alpha [TNF-a]: Mm00443258_s1; interleukin 6 [IL-6]: Mm00446190_m1; IL-1β: Mm00434228_m1; vascular cell adhesion molecule 1 [VCAM-1]: Mm01320970_m1; intercellular adhesion molecule 1 [ICAM-1]: Mm00516023_m1; Ccl2 [monocyte chemotactic protein 1 [MCP-1]: Mm00441242_m1; Ccl3 [macrophage inflammatory protein 1α [MIP-1α]): Mm00441259_g1; glyceraldehyde-3-phosphate dehydrogenase.
Blood-Brain Barrier Permeability Assay

BBB integrity was assessed by Texas Red-dextran perfusion [26] and IgG Western blots with minor modifications [27]. For Texas Red-dextran perfusion, mice were deeply anesthetized with isoflurane, and Texas Red-dextran 70 kDa (D1864, Invitrogen; Thermo Fisher Scientific) in PBS (50 mg/ml) was injected into the inferior vena cava for 2 minutes of circulation. Mice were immediately killed by decapitation. Brains were quickly extracted and post-fixed in 4% PFA for 24 hours and then cryoprotected with 30% sucrose for 48 hours. After embedding in optimum cutting temperature compound, brains were sliced coronally in 20-μm sections. Slices were directly coverslipped using mounting media with DAPI (4′,6-diamidino-2-phenylindole, H-1200; Vector Laboratories).

Western Blot Analysis

After mice had been deeply anesthetized (48 hours after MCAO/R), we collected the ipsilesional cortex and hippocampal regions (bregma: −1.5 to 3.5 mm) and homogenized them in cold NP-40 lysis buffer (Boston BioProducts Inc., Ashland, MA, http://www.bostonbioproducts.com) to isolate the total protein. The slurries were centrifuged at 12,000 rpm for 15 minutes, and supernatants were collected. We loaded 30-μg protein samples onto 4%–12% bis-Tris NuPAGE Novex gels (Invitrogen; Thermo Fisher Scientific), then transferred them onto nitrocellulose membranes (Invitrogen; Thermo Fisher Scientific). Primary antibodies included matrix metalloproteinase 9 (MMP-9; 1:1,000; Abcam), Iba-1 (1:500; Abcam), CD11b (1:1,000; Abcam), zonula occludens-1 (ZO-1; 1:500; Invitrogen; Thermo Fisher Scientific), BDNF (1:1,000; Abcam), and β-actin (1:2,500; Thermo Fisher Scientific). Horse-radish peroxidase-conjugated goat anti-mouse IgG (1:600, Invitrogen; Thermo Fisher Scientific) was used for detecting mouse IgG in brain tissue. Blots were probed with appropriate horseradish peroxidase-conjugated secondary antibodies (1:3,000, Invitrogen; Thermo Fisher Scientific) and detected using ECL Western Blotting Substrate (Thermo Fisher Scientific). We used ImageJ analysis to quantify protein intensities.

Gelatin Zymography

SDS-polyacrylamide gel electrophoresis zymography was used to detect functional MMP-9 enzyme, as described previously [28]. Brain samples were prepared for Western blotting but without denaturing the supernatants before loading the gels. Protein (30 μg per well) was loaded onto a Novex 10% gelatin zymogram gel (Invitrogen; Thermo Fisher Scientific). Following electrophoresis, renaturing buffer (Invitrogen; Thermo Fisher Scientific) was added to the gel, which was then incubated in developing buffer (Invitrogen; Thermo Fisher Scientific) at 37°C overnight. The gel was stained with 0.5% SimplyBlue SafeStain (Invitrogen; Thermo Fisher Scientific) for 60 minutes, and then destained. MMP-9 protease activity was visualized indirectly as clear bands against a dark background. Images were analyzed using ImageJ.

Confocal Microscopy

Images were generated with a Bio-Rad MRC-1024 confocal laser scanning microscope furnished with a single-photon Kr/Ar laser and a Bio-Rad Radiance 2100 MP confocal microscope furnished with a multiphoton laser. To negate channel cross-talk, images were acquired sequentially. Pixel resolution was 1,024 × 1,024, with negative and positive control images taken at the same settings.

Statistical Analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, CA, http://www.graphpad.com) and SPSS 19 (IBM Corp, Armonk, NY, http://www.ibm.com) were used to carry out statistical analysis. To determine dissimilarities between multiple groups, we performed a one-way analysis of variance with Fisher’s least significant difference post hoc test. Tests were considered statistically significant at p values <.05. Data are presented as mean ± SEM.

RESULTS

Transplanted hiPSC-NSCs Ameliorate Neurological Dysfunction

We transplanted hiPSC-NSCs into the ipsilesional hippocampus 24 hours after stroke, and evaluated the behavioral deficits 24 hours after transplantation. The three tests [29, 30] were chosen to determine sensorimotor, balance, and motor function beginning 1 day after hiPSC-NSC transplantation (Fig. 1A–1C). Each mouse was subjected to the three tests: before surgery 3 days consecutively (pretraining) and from 2 to 30 days after surgery. Improved neurological dysfunction was clearly evident 2–8 days after injury; the efficacy of the transplanted hiPSC-NSCs for behavioral dysfunction was maintained over 1 month. Consequently, we assessed the impact of hiPSC-NSCs at early time points and found that the hiPSC-NSCs attenuated the impaired behavior.

For the adhesive removal test, adhesive tape was applied to the contralateral forepaw of mice for the sham-operated and for MCAO/R mice with or without transplanted hiPSC-NSCs. The MCAO/R mice took much longer to remove the tape than the sham-operated mice (p < .0001 at days 2, 4, and 6, and p < .001 at day 8) (Fig. 1A). However, mean removal time was significantly shorter for the hiPSC-NSC-transplanted mice compared with the nontransplanted control MCAO/R mice (p < .001 at day 2; p < .001 at day 4) (Fig. 1A), demonstrating improvement in sensorimotor deficits.

To determine behavioral balance, mice were subjected to the beam walk test [31], in which we measured the time it took for the animal to walk across an elevated narrow beam to a platform. MCAO/R mice were significantly slower in mean walk time compared with sham controls (p < .0001). However, the mean walk time of transplanted mice was significantly faster for hiPSC-NSC-transplanted mice compared with the nontransplanted control MCAO/R mice, demonstrating improved balance (p < .0001) (Fig. 1B).

For motor coordination, we used the rotarod test to evaluate how long an animal remained on a rotating rod. MCAO/R control mice demonstrated extreme impairment in the ability to stay on the rod compared with sham-operated mice, starting at 2 days after MCAO/R and remaining low for the entire test period (p < .0001) (Fig. 1C). However, hiPSC-NSC-transplanted mice remained on the rod significantly longer, and this improved neurological outcome persisted for the entire test period. Sham-operated mice showed no behavioral dysfunction. This suggests that hiPSC-NSC transplantation into the hippocampus at 24 hours after MCAO/R can provide long-term benefits.

As a negative control, we transplanted heat-killed hNSCs. These engrafted cells failed to migrate, and behavioral dysfunction remained on the rod significantly longer, and this improved neurological outcome persisted for the entire test period. Sham-operated mice showed no behavioral dysfunction. This suggests that hiPSC-NSC transplantation into the hippocampus at 24 hours after MCAO/R can provide long-term benefits.
was not distinguishable from or worse than that of nontransplanted MCAO/R mice. These results validated that the effects of the engrafted hiPSC-NSCs were not due to any transplant-induced inflammatory response (data not shown). To validate that hiPSC-NSC xenotransplantation was optimal in the mouse brain, we compared the effect of hiPSC-NSCs with that of C17.2 [4, 32], well-characterized mouse NSCs (mNSCs). We found that the mNSC-engrafted stroke mouse performance was similar to hiPSC-NSC-engrafted mice, demonstrating that hiPSC-NSC xenotransplantation is not suboptimal and allowing us to confidently use hiPSC-NSCs for this study. To validate the behavioral findings that we obtained from human NSCs derived from IMR-90 iPSCs, we transplanted another source of well-characterized hiPSC-NSCs (SCC035; EMD Millipore). We obtained similar results: the stroked mice engrafted with these hiPSC-NSCs performed significantly better than nontransplanted MCAO/R control mice, thus showing a positive outcome (data not shown). Based on these results, we continued to carry out subsequent experiments with IMR-90 hiPSC-NSCs in this study.

**hiPSC-NSCs Rapidly Migrated Into the Site of Stroke Injury**

To explore the potential mechanisms involved in improved behavioral outcomes, we investigated the effects of hiPSC-NSCs by measuring lesion volumes at 24 hours after transplantation (48 hours after MCAO/R). In vitro, hiPSC-NSCs uniformly expressed the NSC-associated markers Sox2 and Nestin (Fig. 2B), which verifies their characteristics. In viable brain tissue, TTC is converted by mitochondria to a red substance but remains colorless in the ischemic area (TTC staining: white, infarct) (Fig. 2C). Previous reports show similar results to ours: 60-minute MCAO and 48-hour reperfusion-induced ischemic damage in both the cortex and striatum but not the hippocampus [33]. Although overall infarct volume measured by TTC staining was not significantly reduced in hiPSC-NSC-transplanted mice (Fig. 2C, 2D), cresyl violet staining patterns showed reduced infarct volume in the cortex, suggesting a positive impact (data not shown).

We next examined the distribution of engrafted (donor) human iPSC-NSCs by immunostaining with the human-specific antibody (STEM121) against a human cytoplasmic protein (Fig. 2E–2H; STEM121, green, arrowhead). Engrafted hiPSC-NSCs are positive for the human-specific neural stem cell marker hNestin (Fig. 2I; red, arrow). By 24 hours after transplant, the hiPSC-NSCs had migrated to the lesion, including the cortex, but hiPSC-NSCs migrated less extensively to the caudoputamen lesion.

It is predicted that the sooner the intervention to prevent BBB damage, the better the clinical outcome after stroke; however, we found that hiPSC-NSCs injected at other than 24 hours after MCAO/R (i.e., 6 or 12 hours after MCAO/R) migrated less extensively to the injured site, and this was associated with a lack of rapid neurological improvement (data not shown). In addition, compared with the hippocampal injection site, hiPSC-NSCs transplanted into the ipsilateral striatum or lateral ventricle 24 hours after MCAO/R showed less extensive migration to the injured sites (data not shown). Based on these findings, we focused on the impact of hiPSC-NSCs transplanted into the hippocampus 24 hours after MCAO/R.

**Transplanted hiPSC-NSCs Reduce Proinflammatory Cytokine Levels**

Ischemia and reperfusion prompt upregulation of proinflammatory cytokines and chemokines [12–17]. We assessed the effect of transplanted hiPSC-NSCs on proinflammatory cytokines using RT-PCR. We found that at 48 hours after MCAO/R in the ipsilesional hemisphere, proinflammatory cytokines TNF-α, IL-6, and...
IL-1β were increased by 30.6 ± 7.8-fold, 12.7 ± 3.9-fold, and 5.2 ± 0.8-fold, respectively (Fig. 3A). In contrast, hiPSC-NSC engraftment reduced TNF-α, IL-6, and IL-1β expression (Fig. 3A). Compared with the sham group, the MCAO/R group showed upregulation of cell adhesion molecules ICAM-1 and VCAM-1 by 10.8 ± 3.5-fold and 2.7 ± 0.5-fold, respectively; however, transplanted hiPSC-NSCs reduced ICAM-1 and VCAM-1 gene expression compared with the MCAO/R group (Fig. 3A). Levels of mRNAs encoding chemokines MCP-1 and MIP-1α (indicators of microglial/macrophage activation) were also significantly increased in the MCAO/R mice by 249.5 ± 71.7-fold and 49.1 ± 4.7-fold, respectively, compared with sham mice (Fig. 3B). Engrafted hiPSC-NSCs decreased gene expression associated with inflammation and microglial/macrophage activation.

hiPSC-NSC Engraftment Ameliorates BBB Damage
Upregulation of proinflammatory cytokines and chemokines after stroke are associated with increased BBB permeability [34, 35].
Loss of BBB integrity then promotes extravasation of fluids and intravascular proteins into the brain parenchyma. To determine the effect of hiPSC-NSCs on BBB permeability, we assessed the level of the blood-derived substance IgG into the brain parenchyma. Western blots showed significantly increased IgG levels in the ipsilesional cortex 48 hours after injury, indicating BBB leakage in MCAO/R brains and significantly downregulated in transplanted brains compared with MCAO/R control brains. *p < .05, **p < .01, ***p < .001 vs. sham mice; #p < .05, ##p < .01, ###p < .001 vs. MCAO/R mice. (B): Chemokines MCP-1 and MIP-1α are dramatically elevated in MCAO/R brains and significantly downregulated in hiPSC-NSC-transplanted brains. *p < .05, **p < .01, ***p < .001, ****p < .0001 vs. sham; #p < .05, ##p < .01, ###p < .001, ####p < .0001 vs. MCAO/R. Data are expressed as mean ± SEM (n = 4). Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hiPSC-NSC, human induced pluripotent stem cell-derived neural stem cell; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; MCAO/R, middle cerebral artery occlusion with subsequent reperfusion; MCP-1, monocyte chemotactic protein 1; MIP-1α, macrophage inflammatory protein 1α; TNF-α, tumor necrosis factor α; Tx, transplantation; VCAM-1, vascular cell adhesion molecule 1.

Figure 3. Proinflammatory gene expression reduced by hiPSC-NSC transplantation. (A): Reduced inflammatory marker expression in the ipsilesional hemisphere of MCAO/R-transplanted MCAO/R brains. Reverse transcriptase-polymerase chain reaction was used to measure inflammatory gene expression, then normalized to that of GAPDH. Transcript levels of proinflammatory cytokines TNF-α, IL-6, IL-1β, and ICAM-1 and VCA1-1 are elevated in the MCAO/R brains and downregulated in transplanted brains compared with MCAO/R control brains. *p < .05, **p < .01, ***p < .001 vs. sham mice. Reduced inflammatory marker expression in the hiPSC-NSC-transplanted group compared with the MCAO/R group increased with the sham group and significantly downregulated MMP-9 activity. (Fig. 4H, 4I). Consistent with previous studies by others, MMP-2 activity was low at 48 hours after MCAO/R during the second BBB opening period (Fig. 4H).

Reduced inflammatory marker expression in the hiPSC-NSC-transplanted group compared with the MCAO/R group increased with the sham group and significantly downregulated MMP-9 activity (Fig. 4H, 4I). Consistent with previous studies by others, MMP-2 activity was low at 48 hours after MCAO/R during the second BBB opening period (Fig. 4H).

Upregulated MMPs are linked to dysfunction of tight junctions between endothelial cells (tight junction proteins are key to maintaining BBB integrity) [38]. It has been reported that, in vivo, BBB leakage occurs despite a structurally intact occludin and claudin-5 network, transmembrane proteins integral to tight junction integrity 24 hours after MCAO [26, 39]. Expression of MMP-9 [40] during this time reportedly degrades the tight junction protein ZO-1 [41]. Western blots showed decreased ZO-1 levels in MCAO/R mice (Fig. 4J, 4K), but engrafted hiPSC-NSCs showed that ZO-1 degradation was significantly prevented (Fig. 4J, 4K). Consistent with previous findings by others [26, 39], no significant differences in claudin-5 and occludin levels were found among all three groups (data not shown). These findings indicate a role of hiPSC-NSCs in maintaining BBB integrity.

We then studied whether engrafted hiPSC-NSCs after MCAO/R would reduce the number of activated inflammatory cells. Resting microglia (via a ramified shape) were identified in sham brains by immunostaining for the microglial/macrophage marker Iba-1 (Fig. 5A). The number of activated inflammatory cells (Iba-1-positive and amoeboid shape) after MCAO/R were dramatically increased throughout the lesion compared with the sham group (Fig. 5B, 5D) but reduced in hiPSC-NSC-engrafted brains (Fig. 5C, 5D). BDNF is central for functional recovery and neuroprotection after stroke [42]. At 2 days after surgery, we found reduced BDNF levels in nontransplanted MCAO/R brains (Fig. 5E), whereas mice transplanted with hiPSC-NSCs showed increased BDNF protein levels in the ipsilesional cortex (Fig. 5F).

Although it is beyond the scope of this study, as we mainly analyzed the effect of hiPSC-NSCs at 24 hours after transplantation, we examined whether engrafted hiPSC-NSCs could differentiate into multiple neural cell types at 30 days after MCAO/R and how they affected proinflammatory cytokines. Proinflammatory cytokines are highly expressed in the acute and subacute stages of MCAO but return to normal baseline levels 12 days after MCAO [43]. We analyzed the expression of inflammatory markers in the ipsilesional hemisphere of hiPSC-NSC-transplanted brains 30 days after MCAO/R. As expected, in the nontransplanted brains 30 days after MCAO/R (unlike at 48 hours after MCAO/R), there was no significant increase of these inflammatory cytokines and adhesion molecules, which suggests no deleterious role of these factors 30 days after MCAO/R (supplemental online Fig. 1). We found no significant differences in MMP-2 and MMP-9 levels among groups (data not shown). The number of activated microglia were highly increased 48 hours after MCAO and still exist 30 days after MCAO [44]. We also found increases in MCP-1 and MIP-1α in the nontransplanted MCAO/R brains. Our results showed no reduction of these genes in the transplanted MCAO/R group. The mechanism of increased MIP-1α expression in hiPSC-NSC transplanted
Figure 4. Blood-brain barrier leakage lessened by hiPSC-NSC transplantation. (A): Western blot shows mouse IgG levels in ipsilesional cortex in sham, MCAO/R, and transplanted mice. (B): Quantification of panel A ($n = 3$; *, $p < .05$, ***, $p < .001$ vs. sham group; #, $p < .05$ vs. MCAO/R group). (C): Brain diagram. Two sampling sites are shown (rectangles). (D): Spatial distribution of intravenously administered Texas Red-dextran 70 kDa (red) in stroke's contralateral (Di, Diii) and ipsilateral (Dii, Div) cortical regions with or without hiPSC-NSC transplantation. Reduced extravasation was observed in hiPSC-NSC-transplanted brains. Scale bar = 10 μm (10 μm, inset). (E): Distribution of engrafted hiPSC-NSCs (STEM121-positive, green) around the blood vessels (CD31-positive endothelial cells in red). Scale bar = 10 μm. (F): Western blot analysis of MMP-9 protein levels in ipsilesional cortex. (G): Quantification of panel F ($n = 3$; ***, $p < .001$ vs. sham group; ##, $p < .01$ vs. MCAO/R group). (H): Zymography assay shows MMP-9 activity in the ipsilesional cortex. (I): Quantification of panel H ($n = 3$; *, $p < .05$, ****, $p < .0001$ vs. sham group; ###, $p < .001$ vs. MCAO/R group). (J): Western blot analysis of ZO-1. (K): Quantification of panel J ($n = 3$; *, $p < .05$ vs. sham group; #, $p < .05$ vs. MCAO/R group). Data are expressed as mean ± SEM. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; hiPSC-NSC, human induced pluripotent stem cell-derived neural stem cell; MCAO/R, middle cerebral artery occlusion with subsequent reperfusion; MMP, matrix metalloproteinase; Tx, transplantation; ZO-1, zonula occludens-1.
brains remains to be elucidated. Next, we analyzed engrafted donor cells and found that hiPSC-NSCs are stably engrafted (supplemental online Fig. 2) in brains 30 days after MCAO/R. The vast majority of donor cells (90% ± 3%) remained Nestin-positive neural stem cells. Only a small percentage of donor-derived cells coexpressed the neuronal marker (TuJ-1) (supplemental online Fig. 2B; hMito, green; TuJ-1, red; merged, yellow) and glial marker (S-100β) (supplemental online Fig. 2D; hMito, green; S-100β, red; merged, yellow). These findings support the paracrine effect of hiPSC-NSCs in our experimental conditions (transplanting cells 24 hours after MCAO/R).

**DISCUSSION**

We found that when hiPSC-NSCs were transplanted after stroke into mice, they quickly migrated to the site of injury and rapidly improved neurological and pathophysiological functions. Because proinflammatory signals recruit transplanted stem cells to the injured site [45], we timed our transplantations 24 hours after MCAO/R, when cell migration signals are abundant. In addition, we assessed the pathophysiological effects of hiPSC-NSCs that lead to swift behavioral improvement 24 hours after transplantation (48 hours after MCAO/R).

Specifically, we transplanted hiPSC-NSCs into the ipsilateral hippocampus, the brain region in which neurogenesis is ongoing, even under normal circumstances and that is rife with signals for migration, proliferation, differentiation, and integration. Others have previously shown only limited migration when stem cells are injected into other areas of the brain [44, 46]. We also did not find extensive migration of hiPSC-NSCs elsewhere, although the subventricular zone (SVZ) is the other area of ongoing adult neurogenesis. It is suggested that rodent SVZ harbors neural precursors that migrate mainly to the olfactory bulb, but it is controversial whether the human SVZ can give rise to neurons; however, it is well established that neurons arise from the human hippocampus, thus we transplanted hiPSC-NSCs into the hippocampus 24 hours after MCAO/R.

We previously reported the anti-inflammatory action of NSCs in central nervous system disorders [4, 5]. Early phase inflammatory responses in cerebral ischemia/reperfusion accompany upregulation of proinflammatory cytokines, which alter endothelial cell matrix interactions [12, 13]. Overproduced proinflammatory mediators after MCAO/R are associated with (a) upregulation of vascular endothelial adhesion molecules (mediates leukocyte adhesion) [14], (b) factors that mediate transendothelial migration of leukocytes [14, 15], and (c) increase in vascular permeability.
In this study, we showed that engrafted hiPSC-NSCs downregulate expression of proinflammatory cytokines (TNF-α, IL-6, and IL-1β), adhesion molecules (ICAM-1 and VCAM-1), and MCP-1 (CCL2) and MIP-1α [15], which mediate the infiltration process and contribute to ischemic stroke injury. Downregulating these factors after stroke would likely reduce neutrophil and monocyte passage into the brain. In fact, we found decreased numbers of Iba-1-positive, activated myeloid cells in hiPSC-NSC-engrafted brains compared with nontransplanted MCAO/R brains.

MMPs, a family of more than 20 zinc-binding proteases, are crucial to the process that results in damage to the BBB during ischemic injury, thus the role of MMPs has been investigated extensively. Elevated MMP-9 activity is associated with degradation of ZO-1 [48] (tight junction proteins in vascular endothelial cells) and BBB breakdown with subsequent hemorrhagic transformation [16, 40]. In this study, we showed that engrafted hiPSC-NSCs significantly reduced MMP-9 levels and increased ZO-1 expression, demonstrating that hiPSC-NSCs ameliorate BBB disruption by reducing MMP-9 activity and protecting ZO-1 from degradation. BBB leakage can be demonstrated with extravasation of injected fluorescence Texas Red-dextran as early as 4 hours after stroke in mice [26]. We confirmed recovery of BBB integrity by reduced extravasation of both IgG and fluorescent dextran (injected) in hiPSC-NSC-engrafted brains. Because MMP-9 upregulation is linked with tPA-induced hemorrhage in stroke patients [49] and animal models [50, 51], MMP-9 reduction by hiPSC-NSCs suggests that a combination treatment of tPA and hiPSC-NSCs could help reduce hemorrhagic events caused by tPA in the early stage of stroke.

Our results showed that engrafted hiPSC-NSCs can ameliorate secondary inflammatory brain damage by reducing cytokine production and ameliorating later phase BBB leakage. Furthermore, because neurotrophins promote functional recovery after stroke [42], Western blot analysis showed significantly increased BDNF expression, suggesting that BDNF upregulation may add a protective mechanism to this system. Neurological function rapidly improved in mice 24 hours after transplantation; recovery of behavioral function persisted throughout the month-long monitoring period. Consequently, we assessed the multiple actions of hiPSC-NSCs that underlie their ability to promote beneficial function 24 hours after transplantation (48 hours after stroke). Although it is beyond the scope of this study, it would be interesting to determine whether early intervention (bystander effect) by engrafted cells could enhance neurogenesis, thus achieving even more enduring positive outcomes in stroke patients. Our immunostaining results revealed that engrafted neural stem cells survived 30 days after transplantation. Furthermore, most engrafted cells remained neural stem cells, although a small percentage of cells expressed neural and astroglial markers. Moreover, our findings support the potential role of hiPSC-NSC paracrine function against stroke.

**CONCLUSION**

Clinically, cerebral vessel occlusion is rarely permanent because of spontaneous or thrombolytic therapy-mediated reperfusion. Our results have clinical implications indicating a much extended therapeutic window for hiPSC-NSC transplantation (24 hours after stroke as opposed to the 5-hour window with tPA). Moreover, there is potential for a synergistic effect by combining transplanted hiPSC-NSCs with tPA to attenuate the adverse effects of stroke.

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**AUTHOR CONTRIBUTIONS**

A.E.: collection and assembly of data, data interpretation and manuscript writing, revision of the manuscript; L.H. and R.G.: collection and/or assembly of data; H.-S.K.: data analysis and interpretation; M.H.H.: data analysis and interpretation, manuscript writing; J.-P.L.: conception and design, data analysis and interpretation, manuscript writing, financial support, final manuscript approval.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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Eckert, Huang, Gonzalez et al. 851