MATERIALS AND METHODS

Middle Cerebral Artery Occlusion Procedure

MCAO was induced by the intraluminal suture technique as previously described (*1*). Briefly, animals were anesthetized intraperitoneally with 1.5% pentobarbital sodium (50 mg/kg). The rats were shaved, transported to the surgery table in a dedicated surgery room. Rat body temperature was maintained at 37 °C ± 0.5 °C with a warm pad (RWD Life Science Corporation) throughout the procedure. Then the right common carotid artery, external carotid artery, and internal carotid artery were exposed. A 3-0 monofilament nylon suture with a rounded tip was inserted from the right common carotid artery into the internal carotid artery and then advanced approximately 18-20 mm intracranially from the common carotid artery bifurcation in order to block the origin of the middle cerebral artery. Reperfusion was performed by withdrawal of the suture 90 minutes after MCAO. The temporal muscle and skin were sutured with 4-0 nylon threads. Rats were injected with buprenorphine (0.05 mg/kg, subcutaneously) every 8–12 h (*2*) in order to control the pain for the first 24 h after operation.

Neurological Function Test

Animals were subjected to weekly neuro-functional tests over 4 weeks following transplantation using the Garcia neurological grading system (*3*). The neurological evaluation is a composite of Spontaneous Activity (abnormal movement), Symmetry in the Movement of Four Limbs, Forepaw Outstretching, Climbing, Body Proprioception, and Response to Vibrissae Touch tests. The score given to each rat at the completion of

the evaluation is the summation of all six individual test scores. The minimum neurological score is 3 and the maximum is 18. The lower the score, the more severe the behavioral deficits. Rats with scores ranging from 7 to 12 points were used (*4*). Investigators were blinded to the animal group to delete the affection of subjective factors.

Cell Culture and Stem Cell Transplantation Procedure

Green fluorescent protein (GFP)–labeled mouse iPSCs were cultured as described previously (*1*). Briefly, mouse iPSCs were maintained on a mitotically inactivated (mitomycin C) (Roche) mouse embryonic fibroblast feeder layer in a new type of iPSCs cell culture medium. This type of iPSCs cell culture medium composed of 48% DMEM/F12 medium (Gibco), 48% Neurobasal medium (Gibco), 1 mM MEK inhibitor PD0325901 (Selleck Chemicals), 3 mM GSK3 inhibitor CHIR99021 (Selleck Chemicals), β-2-mercaptoethanol (Invitrogen), 50 units of penicillin and a 50 mg/mL dose of streptomycin (Invitrogen), and 1,000 U/mL mouse leukemia inhibitory factor (Invitrogen). Before stem cell transplantation, iPSCs colonies were passaged up to 4 times without feeder cells on 60-mm culture dishes coated with 0.1% gelatin to eliminate contamination of the mouse embryonic fibroblasts.

Humanized mulleri green fluorescent protein (hmGFP)–labeled rat NSCs were cultured as described previously (*5*). Briefly, NSCs were cultured on 60-mm or 100-mm culture dishes coated with poly-L-ornithine and laminin to insure NSCs grew with adherence. In order to coat culture dishes, enough of the poly-L-ornithine solution (10 mg/mL) was used to cover the whole surface of tissue culture-ware at room temperature

for 24 hours. Then move the poly-L-ornithine solution and add enough laminin (5-7 μg/mL) solution to treat the tissue culture-ware overnight at room temperature. The NSC cells were incubated at 37 °C in a 5% CO_2 humidified incubator in Neural Stem Cell Basal Medium (Millipore) (pre-warmed to 37°C) which containing freshly added 20 ng/mL FGF-2 (Millipore) and 1 μg/mL puromycin (Millipore).

Rat was placed in a stereotactic instrument (RWD Life Science Co). A midline skin incision was made in the skull, and then a small burr hole was drilled through the skull. With the use of a Hamilton microsyringe, 1.0×10^6 suspended iPSCs, NSCs, or PBS in a volume of 20 μL was stereotactically injected into the right lateral ventricle (anterior-posterior 0.9 mm to the bregma, mediolateral 1.5 mm and dorsoventral 3.5 mm from dura) in about 15 min (*6*). The needle was left in place for an additional 5 min and then removed out slowly over 5 min.

PET Imaging and Image Analysis

At 3 day after MCAO and at 1, 2, 3, and 4 week after stem cells transplantation, rats were anesthetized with isoflurane (2%) and administered approximately 18.5 MBq (500 μCi) of ^{18}F-FDG via tail vein. Then rats were put back in a quiet room for an ^{18}F-FDG uptake period of 40 min. The images were acquired in a high-resolution microPET R4 scanner (Siemens Medical Solutions) in 10 min (static acquisition) with the mid-skull in the center of the field of view, and isoflurane anesthesia was maintained during data acquisition. The images were reconstructed with a modified back-projection (MBP) algorithm. With using the AMIDE software package (version 9.2; Stanford University),

^{18}F-FDG uptake was calculated as the percentage injected dose per gram of tissue. To assess changes of metabolism after MCAO, regions of interest (ROIs) 2 mm in diameter in the infarction area and the cerebellum normal area were identified in images of the transversal brain sections, and the mean percentage injected dose per gram of tissue was averaged. The average radioactivity concentration within the infarction was obtained from the mean pixel values (*7*), normalized to that of nonischemic cerebellum area (*8*). The Lesion-to-Cerebellum (L/C) ratio was used for semiquantitative analysis and it was calculated abide by the following formula: L/C ratio = mean counts per pixel of lesion region of interest / mean counts per pixel of cerebellum area.

Autoradiographic Imaging

To verify the image data obtained by PET, 6 animals (1 per group) were sacrificed under deep anesthesia immediately after the final small-animal PET imaging. The brains were removed rapidly into a rodent brain matrix and stay at -20 °C for about 40 min. Coronal brain slices (1 mm thick) were cut and exposed to an imaging plate (BAS-IP MS 2040, Fujifilm) for 15 min. The exposed plate was scanned with high-resolution imaging plate reader (FLA-7000, Fujifilm) at a resolution of 50 μm, and then the images were displayed with a Dell computer (Image Reader FLA-7000, version 1.12, Fujifilm) for qualitative evaluation.

Immunohistochemical and Immunofluorescent Staining

Immunohistochemical studies were performed to determine whether transplanted

stem cells could induce neurogenesis and/or angiogenesis. Rats (n = 4 to 5 per group) were deeply anesthetized and transcardially perfused with 0.9% saline followed by 4% chilled paraformaldehyde in PBS (pH 7.4) after the final small-animal PET scan. The brains were immediately removed, dissected and immersed in the same fixative for 24 hours. After that, the 5-10 mm sections were washed in dH_2O for 30 minutes. And they were rinsed in 80% ethanol about 1 hour, rinsed twice in 95% ethanol for 1 hour each, rinsed twice in 100% ethanol for1 hour each, rinsed twice in absolute xylene for 20 min each for dehydration. Then samples were immersed in paraffin at 58-60 °C for 3 h. After the tissue was paraffin embedded, 4 μm coronal serial sections were processed with a sliding microtome (Microm HM-340 E microtome, Microm, Walldorf, Germany) throughout the entire ischemic damaged brain. The specimens were stained with EnVision™ two-step strategy and high-temperature antigen retrieval. Afterward, the slides were incubated with 3% hydrogen peroxide for 10 minutes and rinsed in PBS about 5 min for three times. For immunohistochemical staining, the slides were permeabilized with 0.04% Triton X-100 and blocked by 10% normal goat serum in PBS for 1 h and then incubated overnight in a moist chamber at 4 °C with primary antibodies: Mouse monoclonal antibody against neuronal nuclei (NeuN) (1:200 dilution; clone: A60, Millipore), Rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) (1:300 dilution; Abcam), rabbit polyclonal antibody against von Willebrand factor (vWF) (1:200 dilution; DAKO), Mouse monoclonal antibody against glucose transporter 1 (GLUT-1) (1:150 dilution; Clone:5B 12.3, Millipore), and Rabbit polyclonal antibody against glucose transporter 3 (GLUT-3) (1:150 dilution; Abcam). The sections were rinsed 3

times with PBS for 10 min each and treated in HRP-lated secondary antiserum (DAKO EnVision™ kit) for 30 min at 37°C. Then they were washed thoroughly and treated in 0.05% diaminobenzidine (DAB) with 0.03% H_2O_2 for 3–5 min until a brown reaction product was observed. The number of positively stained cells with NeuN or vWF in 3 different microscopic areas (468.54μm × 351.17μm) (×200 magnifications) was counted. To evaluate the average integral optical density (IOD) of GFAP, GLUT-1 and GLUT-3, hot spots of the section were selected by using a microscope (×100 magnification). Individual counts were then made in ×200 areas (BX60, Olympus). Sections (5 microscopic areas per slice, 1 slice per animal) were digitized with the use of a camera. The IOD values of GFAP, GLUT-1 and GLUT-3 were obtained by using Image-ProPlus 5.0 software.

Double-label immunofluorescence was used to follow up on the fate of GFP-labeled transplanted stem cells. At 31 days after the last small-animal PET scan, rats (n = 1 from each group of iPSCs, NSCs, iPSCs+QKL, and NSCs+QKL) were deeply anesthetized and the brains were obtained as described above. They were dissected and post fixed in 4% paraformaldehyde overnight and then transferred to 30% sucrose in 0.1 M PBS at 4 °C for dehydration over 2 days. Brains were frozen on powdered dry ice, and frozen coronal sections of the brains were cut with a sliding microtome set to 20 μm. Sections were blocked and incubated at 4 °C overnight with primary antibodies (NeuN, GFAP and vWF) as mentioned above; the dilution of all primary antibodies was 1:200. After washes three times in PBS, sections were incubated with fluorescence-conjugated secondary antibodies (Alexa Fluor 568, 1:500; Invitrogen) for 1 h at room temperature. Then

sections were washed three times in PBS and counterstained with the nuclear dye 4, 6-diamino-2-phenylindole. Fluorescence-labeled sections were viewed with use of a confocal microscope (LSM710; Zeiss).

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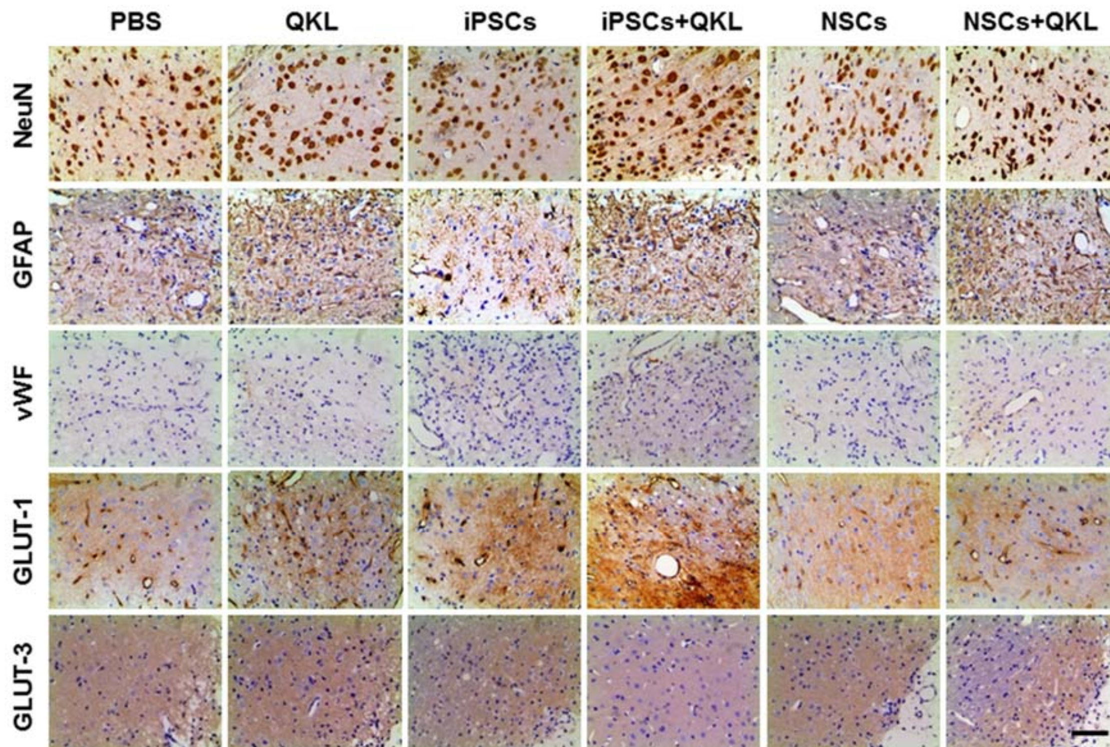

Supplemental Figure 1. Photomicrograph (×200) of immunohistochemical of NeuN positive cells, GFAP positive cells, vWF positive cells, GLUT-1 positive cells, and GLUT-3 positive cells in cerebral ischemic region in animals of six groups. Scale bar = 100μm.