Supplementary Data

Supplementary Materials and Methods

Cell culture

Human mesenchymal stem cells (hMSCs) were isolated from human placenta as described previously [1]. Briefly, term (38–40 weeks' gestation) placentas from healthy donors were harvested with written informed consent and the procedure was approved by the Ethics Committee of Xili Hospital. The placental tissue was washed several times with cold phosphate-buffered saline (PBS) and then mechanically minced and enzymatically digested with 0.25% trypsin-ethylenediaminetetraaceticacid (EDTA) for 30 min at 37°C in a water bath. The digest was subsequently filtered, pelleted, and resuspended in a growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen), 10% fetal bovine serum (Gibco-Invitrogen), and antibiotics. Cells were seeded on uncoated polystyrene dishes and medium was replaced every 2 days to reach 80% confluence. Cells were subcultured after trypsinization.

To form spheroids, passage 5–7 hMSCs were cultured by a hanging-drop method as described previously [2] with modifications. Briefly, 3,000 hMSCs in 35 μL growth medium per drop were plated in hanging drops and incubated for 36 h to form spheroids. Then, the spheroids were transferred to a suspension culture with fresh growth medium and incubated for 24 h. To obtain single cells from spheroids, spheroids were incubated with 0.25% trypsin/EDTA for 6–10 min (depending on size of spheroids) with gentle pipetting every 2–3 min.

Rat middle cerebral artery occlusion model

Female Sprague–Dawley rats (250–270 g; purchased from the Laboratory Animal Centre) were maintained in a temperature-controlled environment (20 ± 1°C) with access to food and water throughout the experiment. All procedures were performed with the approval of the Animal Ethics Committee of Tsinghua University.

After anesthesia with 10% chloral hydrate (Sigma-Aldrich) at 400 mg/kg, intraperitoneal injection, rats were subjected to transient cerebral focal ischemia for 120 min by the intraluminal suture technique as described previously [3] with modifications. Briefly, the left common carotid artery, external carotid artery, and internal carotid artery were carefully exposed via a midline cervical incision. The external carotid artery and the common carotid artery were ligated with a 4-0 silk. A silicon-rubber-coated round-tip nylon surgical thread was inserted into the internal carotid artery (~18 mm from the bifurcation) from the common carotid artery to occlude the origin of the middle cerebral artery. A silk suture around the origin of internal carotid artery was tightened to prevent bleeding from the puncture site. After 120 min of middle cerebral artery occlusion (MCAO), reperfusion was performed by withdrawing the suture.

Behavioral tests

Each rat was subjected to a series of behavioral tests [4,5] to evaluate neurologic functions before MCAO and 1, 3, 5, 7, 9, 11, and 14 days after MCAO. The modified neurological severity score test was performed according to a method reported previously [4]. The score was determined by parameters of motor (muscle status and abnormal movement), sensory (visual, tactile placement, and proprioceptive), and reflex tests (pinna, corneal, and startle reflex) [6]. One point was awarded for the inability to correctly perform a task or for the lack of a tested reflex.

Real-time polymerase chain reaction

Total RNA was extracted from hMSCs with TRIzol (Invitrogen) following the manufacturer's instructions. First-strand cDNA was prepared by reverse transcription with Superscript II reverse transcriptase (Invitrogen) and oligo(dT) primers and stored at 20°C. Real-time polymerase chain reaction (real-time PCR) was performed using SYBR® Premix Ex Taq™ II on an ABI 7300 QPCR System. As an internal control, levels of glyceraldehyde-3-phosphate dehydrogenase were quantified in parallel with target genes. Normalization and fold changes were calculated using the ΔΔCt method. Primer sets are listed in Supplementary Table S1.

Enzyme-linked immunosorbent assay

Monolayer and spheroid hMSCs were seeded at equal density in six-well tissue culture dishes in DMEM for 24 h, and media were collected, centrifuged, and stored at −80°C. Human vascular endothelial growth factor and basic fibroblast growth factor in the media were measured by enzyme-linked immunosorbent assay (4A Biotech Co. Ltd) following the manufacturer's instructions.

Immunofluorescence stain

For immunofluorescence analysis, rats were transcardially perfused with 4% paraformaldehyde (PFA) in PBS. The whole brain was dissected and again fixed in 4% PFA in PBS overnight at 4°C and dehydrated in 30% sucrose. Ten-micrometer-thick cryostat sections were stained with primary antibodies at appropriate concentrations at 4°C overnight: mouse anti-human nuclei monoclonal antibody (M1281, 1:100; Millipore), caspase-3 (1:50; Cell Signaling Technology), glial fibrillary acidic protein (GFAP, 1:80; Sigma–Aldrich), nestin (1:50; Santa Cruz), microtubule-associated protein (MAP)-2 (1:80; Millipore), Von Willebrand factor (vWF, 1:50; Santa Cruz), α-smooth muscle actin (α-SMA)-Cy3 (1:200; Santa Cruz), and Sox2 (1:100; Santa Cruz). Sections were then stained with fluorescein isothiocyanate- or Cy3-conjugated secondary antibodies (Jackson Immunoresearch). For 5-bromo-2′-deoxyuridine (BrdU) immunostaining, DNA was denatured by incubating tissue sections in 2 N HCl for 45 min at 37°C, followed by a 10-min wash in 0.1 M boric acid in PBS (pH 8.5). BrdU was detected with an anti-BrdU antibody (1:200; Sigma-Aldrich). Nuclei of cells were stained with 4′,6-diamidino-2-phenylindole. Samples were examined under confocal laser scanning microscope (FV1000; Olympus).

For measurement of vascular density, eight fields of view (magnification: anti-vWF, 200×; anti-α-SMA, 100×) in the infarct boundary zone per tissue section were analyzed after staining with anti-vWF (tissues at 3 days) or anti-α-SMA (tissue at 7 days) antibody. The total area of vessels per macroscopic field area was measured using Image J [6].

For quantification of $GFAP^+$ cells in the ischemic boundary zone, the numbers of cells positive for GFAP alone or double positive for GFAP and BrdU or Sox2 per microscopic field (magnification 200×) was counted, and 10 fields per tissue section were randomly selected. Three tissue sections per sample were examined. The same method was used for the quantification of M1281-positive hMSCs engrafted into the cerebral lesion and their differentiation into MAP-2-positive cells [7].

Supplementary References

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