

Neural Induction with Neurogenin1 Increases the Therapeutic Effects of Mesenchymal Stem Cells in the Ischemic Brain

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ABSTRACT

Mesenchymal stem cells (MSCs) have been shown to ameliorate a variety of neurological dysfunctions. This effect is believed to be mediated by their paracrine functions, since these cells rarely differentiate into neuronal cells. It is of clinical interest whether neural induction of MSCs is beneficial for the replacement therapy of neurological diseases. Here we report that expression of Neurogenin1 (*Ngn1*), a proneural gene that directs neuronal differentiation of progenitor cells during development, is sufficient to convert the mesodermal cell fate of MSCs into a neuronal one. Ngn1-expressing MSCs expressed neuron-specific proteins, including NeuroD and voltage-gated Ca²⁺ and Na⁺ channels that were absent in parental MSCs. Most importantly, transplantation of Ngn1-expressing MSCs in the animal stroke model dramatically improved motor func-

tions compared with the parental MSCs. MSCs with Ngn1 populated the ischemic brain, where they expressed mature neuronal markers, including microtubule associated protein 2, neurofilament 200, and vesicular glutamate transporter 2, and functionally connected to host neurons. MSCs with and without Ngn1 were indistinguishable in reducing the numbers of Iba1⁺, ED1⁺ inflammatory cells, and terminal deoxynucleotidyl transferase dUTP nick-end labeling⁺ apoptotic cells and in increasing the numbers of proliferating Ki67⁺ cells. The data indicate that in addition to the intrinsic paracrine functions of MSCs, motor dysfunctions were remarkably improved by MSCs able to transdifferentiate into neuronal cells. Thus, neural induction of MSCs is advantageous for the treatment of neurological dysfunctions. *STEM CELLS* 2008;26:2217–2228

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stem cells (MSCs) are of clinical interest because of their potential use in autologous transplantation, which is attributable to their ability to improve a variety of dysfunctions of a non-neurological, as well as neurological, nature [1]. The critical question that remains unanswered is whether MSCs should be genetically modified and tailored to be more efficacious for specific target organs and diseases. MSCs easily differentiate into mesodermal tissues such as fat, bone, cartilage, and muscle [2–4] but rarely into neuronal cells [5–7]. There have been many attempts to induce trans-differentiation of MSCs into neuronal cells [8, 9]. Unfortunately, most of these

studies suffered from artifacts created by in vitro chemical stress [10, 11] or from misinterpretation of the in vivo transfer of donor antigens from dying MSCs to host cells [12]. Therefore, it still remains unclear whether neural-induced MSCs can serve as better sources for replacement cell therapy of neurological diseases. To address this, it will be necessary to develop a novel strategy with minimal cellular stress and without loss of proliferation capability.

Neurogenin1 (*Ngn1*) is a member of the basic helix-loop-helix (bHLH) transcription factor family [13]. It is expressed in early neuronal progenitor cells during development of the nervous system [14–16]. Forced expression of *Ngn1* has been shown to increase the number of neuronal cells in *Xenopus* embryos [13] and in uncommitted pluripotent stem cells, such as

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embryonic carcinoma P19 cells [17, 18], by activation of downstream proneural bHLH transcription factors, including NeuroD [13]. Once NeuroD is expressed, the cells exit the cell cycle and terminally differentiate into neurons. Meanwhile, Ngn1 suppresses astroglial differentiation by sequestering CREB binding protein/p300 and Smad1 away from signal transducers and activators of transcription-containing complexes that are required for glial fibrillary acidic protein (GFAP) expression [19].

In the present study, we show the neurogenic potential of Ngn1 in converting the committed progenitor cells of non-neural lineage, such as MSCs, into neuronal progenitor cells without causing cellular stresses. We also show that neural induction with Ngn1 dramatically enhances the therapeutic effects of MSCs for the treatment of stroke using an animal model. Finally we provide evidence that higher motor recovery in animals grafted with neural-induced MSCs is partly due to transdifferentiation into neuronal cells *in vivo*.

MATERIALS AND METHODS

Preparation of MSC-Ngn1 Cells and In Vitro Differentiation

Human mesenchymal stem cells were isolated from bone marrow with approval of the Institutional Review Board of Ajou University Medical Center. Cells were grown as adherent cultures in growth medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum [FBS] and 10 ng/ml basic fibroblast growth factor [bFGF]). Differentiation into mesoderm cells was induced as described by Pittenger et al. [2]. The full-length mouse Ngn1 cDNA (GenBank U63841) was inserted into the EcoRI site of pMSCV-puro retroviral vector (Clontech, Palo Alto, CA, <http://www.clontech.com>) with a FLAG epitope at the N terminus to yield the retroviral vector pMSCV-puro/Ngn1. Retroviruses were produced using a standard method by cotransfecting 293T cells with pMSCV-puro/Ngn1 or pMSCV-puro/ β -galactosidase (β -gal) (Clontech) and gag- and env-expression vectors. For infections, the MSCs were exposed three times to 1×10^6 particles of vesicular stomatitis virus-glycoprotein pseudotyped retrovirus with 8 μ g/ml polybrene (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) for 6 hours each time. After a 2-day incubation in growth medium, the cells were split 1:20 into selection medium containing puromycin (2 μ g/ml; Sigma-Aldrich). After 14 days, the surviving cells were subcultured up to three or four passages for use in experiments. To induce neuronal differentiation, cells were grown on poly-D-lysine/collagen-coated culture dishes or coverslips in growth medium containing 10 μ M 5-aza-deoxycytidine (Aza-dC; Sigma-Aldrich) for 3 days and then in DMEM/F12 medium (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) containing 10% FBS and 10 μ M forskolin (Sigma-Aldrich) for 14 days.

Immunocytochemistry

Cells were induced to differentiate on coverslips and fixed with 4% paraformaldehyde (PFA) for 10 minutes. After blocking in normal serum, the cells were incubated in 0.1% Triton X-100 in phosphate-buffered saline (PBS) containing a primary antibody against microtubule associated protein 2 ([MAP2]; monoclonal, 1:100; Sigma-Aldrich), neurofilament 200 (NF200) (polyclonal, 1:200; Chemicon, Temecula, CA, <http://www.chemicon.com>), and neuronal nuclei (NeuN) (monoclonal, 1:100; Chemicon) at 4°C overnight. Antibody reaction was visualized with Alexa Fluor 488-conjugated anti-mouse or anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR, <http://probes.invitrogen.com>), and the cells were counterstained with bis-benzimide (Molecular Probes). Epifluorescent images were acquired with a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany, <http://www.zeiss.com>).

Western Blot Analysis

Cells were lysed in SDS buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 50 mM dithiothreitol). Soluble proteins (30 μ g) were subjected to Western blot analysis using antibodies against MAP2 (1:500) or GFAP (1:500) (Sigma-Aldrich), NF200 (1:1,000) or NeuN (1:500) (Chemicon), or β -tubulin III (1:500) (Covance, Richmond, CA, <http://www.covance.com>). The proteins were probed with horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>) and visualized using an enhanced chemiluminescence kit (Pierce, Rockford, IL, <http://www.piercenet.com>). Mouse brain extracts (4 μ g) were used as controls.

Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated using RNeasy B (Tel-Test, Friendswood, TX, <http://www.isotexdiagnostics.com>), and 1 μ g was reverse-transcribed using a first-strand cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland, <http://www.roche-applied-science.com>). The polymerase chain reaction (PCR) primers were designed to span separate exons to eliminate possible DNA contamination. Forward and reverse primers were GGCAGCGTTGGAACAGAGGTTGGA and CTCTAAACTGG-AGTGGTCAGGGCT for Nestin, GCCCAGGGTTATGAGACTATCACT and GACAGAGCCAGATGTAGTTCTT for NeuroD, and CCACAGTCCATGCCACT and GAGCTTG-ACAAAGTGGTCGT for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR was performed for 28 cycles for nestin (60°C) and GAPDH (56°C) and for 33 cycles for NeuroD (58°C).

Electrophysiological Analyses

For Ca²⁺ currents, the cells were differentiated as described above. The bath solution was 150 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 10 mM HEPES, and the pipette solution was 90 mM cesium aspartate, 55 mM CsCl, 3 mM Na₂ATP, 3 mM Na₂-creatine phosphate, 1 mM MgCl₂, 10 mM HEPES, and 10 mM EGTA, pH 7.4. For Na⁺ currents, the cells were incubated in the presence of bFGF and epidermal growth factor for 2 weeks. The bath solution was 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, and the pipette solution was 140 mM KCl, 5 mM NaCl, 1 mM CaCl₂, 10 mM HEPES, 5 mM EGTA, and 2 mM Mg-ATP, pH 7.3. The electric currents were amplified using either an Axopatch-1C patch-clamp amplifier or Axopatch 200B patch-clamp amplifier (Axon Instruments/Molecular Devices Corp., Foster City, CA, <http://www.moleculardevices.com>). The signals were amplified with and digitized by an analog-to-digital interface (Gould 1425 [Gould Instruments, Hainault, U.K., <http://www.lds-group.com>] or Digidata 1320 [Axon Instruments]) and analyzed with the Clampfit version 6.0.3 software package or pClamp8.2 (Axon Instruments).

Ischemic Animal Model and Cell Transplantation

All animal protocols were approved by the institutional animal care and use committee of Ajou University Medical School. Male Sprague-Dawley rats (~250 g) were anesthetized with intraperitoneal (i.p.) administration of ketamine (75 mg/kg) and xylazine hydrochloride (5 mg/kg). Transient middle cerebral artery occlusion (MCAo) was induced using a method of intraluminal vascular occlusion [20]. Rectal temperature was maintained at 37°C throughout the surgical procedure, using an electronic temperature controller linked to a heating pad (FHC, Bowdoinham, ME, <http://www.fh-co.com>). Animals showing similar scores (e.g., remaining on the Rotarod [Ugo Basile, Comerio, Italy, <http://www.ugobasile.com>] for more than 300 seconds during pretraining but less than 10 seconds after surgery) and comparable infarct volumes in magnetic resonance imaging (MRI) were selected and randomly grouped. This way, we were able to scrupulously control the quality of animals and minimize variations among experimental groups. Three days after MCAo, 1.0×10^6 cells in a total fluid volume of 10 μ l were intracranially transplanted into the striatum (anteroposterior [AP], 0.5; mediolateral [ML], 2.5; dorsoventral [DV], 5.0) and cortex (AP, -0.5; ML, 2.0; DV, 2.5) in the ipsilateral hemisphere

using a stereotactic apparatus (David Kopf Instruments, Tujunga, CA, <http://www.kopfstruments.com>).

Sixty rats in total were directly used to obtain the final data shown in this study: PBS ($n = 19$), MSC-LacZ cells ($n = 19$), and MSC-Ngn1 cells ($n = 22$) (supplemental online Table 1). Eight animals in each group were used in monitoring the infarct volume with MRI and the behavioral outcome (Fig. 2) and finally sacrificed to determine transdifferentiation after 8 weeks (Fig. 6C–6H; supplemental online Figs. 1, 2). Six animals per group were sacrificed at week 2 to verify the survival of grafted cells and cell type-specific differentiation (Fig. 6A, 6B). To count the immunoreactive cells to antibodies against 5-bromo-2'-deoxyuridine (BrdU), Ki67, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), or ED1, five animals per group were injected daily with BrdU (50 mg/kg in saline, i.p.) for 4 days and analyzed on day 7. The remaining rats were used for neuronal tracing with fluorogold. Three normal rats were used for transplantation of MSC-Ngn1 cells expressing wheat germ agglutinin (WGA).

Fluorescent Immunohistochemistry

Rats were anesthetized with ketamine and perfused transcardially with ice-cold 0.9% saline followed by 4% PFA at indicated time after transplantation. Brains were removed, postfixed in 4% PFA overnight, and then embedded in paraffin. Serial 6- μ m-thick paraffin sections were deparaffinized and then placed in boiled citrate buffer (pH 6.0) for 10 minutes. After blocking in 1% bovine serum albumin (BSA) and 5% normal serum, the sections were incubated with antibodies against FLAG epitope (1:100), MAP2 (1:100), NF200 (1:200), and GFAP (1:200) (Sigma-Aldrich); NeuN (1:100), human mitochondria (1:100), and vesicular glutamate transporter 2 (VGLUT2; 1:100) (Chemicon); β -galactosidase (1:100) (AbD Serotec, Raleigh, NC, <http://www.ab-direct.com>); human Tau (1:50) (Pierce); or synapsin I (1:50) (Abcam, Cambridge, U.K., <http://www.abcam.com>) at 4°C overnight. Then, the sections were incubated with Alexa Fluor 488- or 594-conjugated anti-IgG secondary antibodies and counterstained with bis-benzamide (Molecular Probes) to visualize the entire population of cells. Fluorescent images were acquired using a Zeiss LSM510 confocal microscope (Carl Zeiss). To increase the confidence in the colocalization analysis, care was taken to collect potentially overlapping emissions separately using the "multitrack" function. For orthogonal projections, a stack of 20–30 confocal images that were 0.25 μ m apart were typically collected and analyzed. To verify the survival of grafted cells and cell type-specific differentiation, the numbers of β -galactosidase-expressing MSCs-LacZ or FLAG-tagged MSC-Ngn1 cells were analyzed in the ischemic boundary zone. In each animal, approximately 100 grafted cells were analyzed for cell type-specific differentiation (NeuN or GFAP) by confocal microscopy. All counts were performed using split-panel and z -axis analyses, and multichannel configuration was performed with a $\times 40$ objective. Each cell was examined in its full z -dimension, and only cells with a grafted cell-specific marker unambiguously associated with a lineage-specific marker were scored as positive.

Stereological Quantification

Five animals in each group were daily injected with BrdU (50 mg/kg in saline, i.p.) for 4 days after transplantation at day 3 until sacrifice at day 7. To determine the total numbers of the BrdU-, Ki67-, TUNEL-, ED1-positive cells in the subventricular zone (SVZ) or ischemic territory, 50- μ m-thick frozen sections were prepared from ischemic central lesion (anteroposterior, +1.2 mm \sim -0.8 mm from bregma). To detect BrdU incorporation, sections were pretreated with 2 N HCl at 37°C for 30 minutes and neutralized with 0.1 M sodium borate (pH 9.0) for 10 minutes. The sections were incubated in PBS containing 1% H₂O₂ for 20 minutes and then in 1% BSA and 5% normal serum for 1 hour at room temperature. The sections were probed with a mouse monoclonal anti-BrdU antibody (1:200; Sigma-Aldrich) at 4°C overnight. To further verify proliferating cells, sections were probed with anti-Ki67 antibody (1:200; Chemicon). Apoptotic cells were analyzed with the In Situ Cell Death Detection Kit, peroxidase (Roche Diagnostics), according to the manufacturer's instructions. To determine the infiltration of inflammatory cells, the sections were probed with an anti-ED1

antibody (1:200; Serotec Ltd., Kidlington, U.K., <http://www.serotec.com>). Then the antibody reactions were visualized using an ABC kit (Vector Laboratories). Briefly, after washing the primary antibody, the sections were sequentially incubated with biotinylated goat anti-mouse secondary antibody (1:200 dilution; Vector Laboratories) for 1 hour and avidin-peroxidase conjugate (Vector Laboratories) solution for 30 minutes. The horseradish peroxidase reaction was detected with 0.05% diaminobenzidine and 0.03% H₂O₂ in 50 mM Tris-HCl (pH 7.0). The sections were dehydrated through graded alcohols, cleared in xylene, and coverslipped in permanent mounting medium (Vector Laboratories).

After staining with each indicated antibodies, the sections were analyzed with the Computer-Assisted Stereological Toolbox system, version 2.1.4 (Olympus, Ballerup, Denmark, <http://www.olympus-global.com>), equipped with an Olympus BX51 microscope, a motorized microscope stage (Prior Scientific, Rockland, MA, <http://www.prior.com>) run by an IBM-compatible computer, and a microcator (ND 281B; Heidenhain, Traunreut, Germany, <http://www.heidenhain.com>) connected to the stage and feeding the computer with the distance information in the z -axis as previously described [21]. The SVZ or ischemic territory was delineated using a $\times 1.25$ objective and generated counting grid of 100 \times 100 μ m or 300 \times 300 μ m. An unbiased counting frame of known area (48.6 \times 36.1 μ m = 1,754 μ m²) superimposed on the image was placed randomly on the first counting area and systemically moved through all counting areas until the entire delineated area was sampled. Actual counting was performed using a $\times 100$ oil objective. The estimate of the total number of cells was calculated according to the optical fractionator formula [22]. More than total 200 points over all sections of each specimen were analyzed.

Neuronal Tracing

MSC-Ngn1 cells were transduced with WGA-expressing adenovirus (multiplicity of infection = 100), and 24 hours later 5×10^5 cells were transplanted into the right hippocampus of the normal rat brain (AP, -3.5; ML, 2.0 mm; DV 3.0). Eight weeks later, the animals were sacrificed and examined for the presence of grafted MSC-Ngn1 cells using antibodies against human mitochondria or WGA (1:100; Vector Laboratories). Double immunofluorescent staining was carried out as described above using Alexa Fluor 488- or 594-conjugated anti-IgG as secondary antibodies and counterstaining with bis-benzamide (Molecular Probes).

Alternatively, 4 weeks after transplantation into the ischemic brain, 1 μ l of fluorogold (FG; 2% solution in sterile saline; Molecular Probes) was stereotactically injected into two sites of the contralateral hemisphere (AP, 0.5; ML, -3.0; DV, 3.0 and AP, 0.5; ML, -5.0; DV, 5.0). One week later, the animals were sacrificed and analyzed for localization of FG using a confocal microscope.

MRI and Measurement of Infarct Volume

MRI scanning was performed using a 3.0-tesla whole-body MRI scanner (Magnus 3.0; Medinus Inc., Yongin, South Korea, <http://www.medinus.co.kr>) equipped with a gradient system capable of 35 milliteslas/m. A fast-spin echo imaging sequence was used to acquire T2-weighted anatomical images of the rat brain in vivo, using the following parameters: repetition time, 4,000 milliseconds; effective echo time, 96 milliseconds; field of view, 55 \times 55 mm²; image matrix, 256 \times 256; slice thickness, 1.5 mm; flip angle, 90°; number of excitations, 2; pixel size, 0.21 \times 0.21 mm². A 300-mm-diameter quadrature 16-rung birdcage coil arrangement was used for RF excitation, and a 40-mm-diameter saddle coil was used for signal detection. A total of 15 slices were scanned to cover the whole rat brain. Ischemic area from each T2-weighted image was marked manually and calculated using the program Osiris (University of Geneva, http://www.sim.hcuge.ch/osiris/01_Osiris_Presentation_EN.htm). The relative infarct volume (RIV) was normalized as described by Neumann-Haefelin et al. [23] using the equation $RIV = [LT - (RT - RI)] \times d$, where LT and RT represent the areas of the left and right hemispheres, respectively; RI is the infarcted area; and d is the slice thickness (1.5 mm). Relative infarct volumes (% HLTV) were expressed as a percentage of the right hemispheric volume.

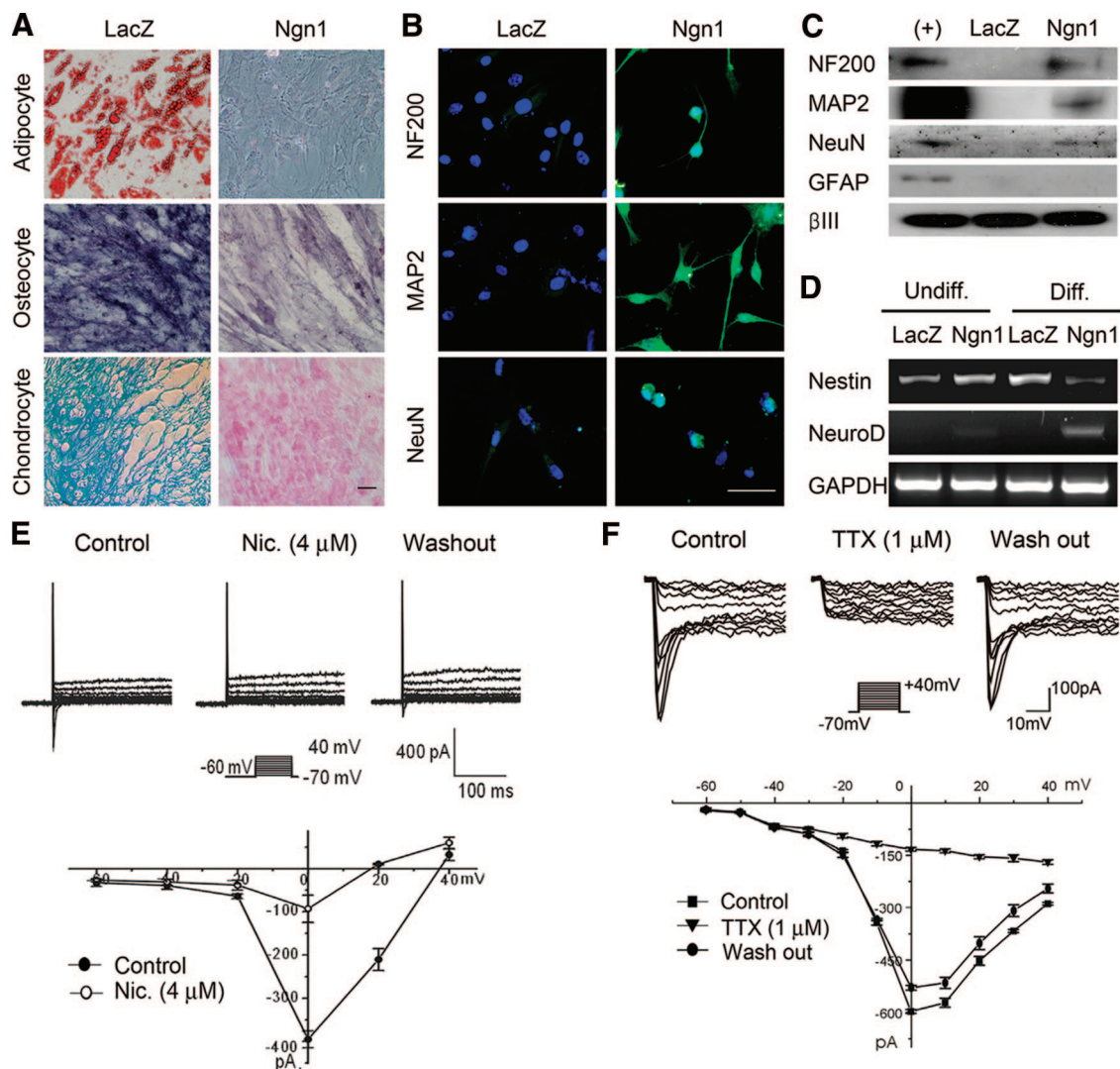


Figure 1. In vitro differentiation of MSC-Ngn1 cells. Cells were induced to differentiate into mesodermal (A) or neural (B–F) cells as described in Materials and Methods. (A): MSC-LacZ cells differentiated into adipocytes (oil red⁺), osteocytes (alkaline phosphatase⁺), and chondrocytes (Alcian Blue⁺). MSC-Ngn1 cells showed only very low levels of alkaline phosphatase activity. (B): Immunocytochemistry revealed expression of NF200, MAP2, and NeuN (green) in MSC-Ngn1 cells but not in MSC-LacZ cells. Nuclei were counterstained with bis-benzimide (blue). Scale bars = 50 μ m (A, B). (C): Western blot analysis showed expression of NF200, MAP2, and NeuN in MSC-Ngn1 cells but absence of those proteins in MSC-LacZ cells. Positive controls (+) were mouse brain extracts. Note that β III was expressed in both MSC-LacZ and MSC-Ngn1 cells, whereas GFAP was not detected at all. (D): Reverse transcription polymerase chain reaction analysis showed that NeuroD was moderately expressed in MSC-Ngn1 cells cultured in growth medium (Undiff.) and further increased after terminal differentiation in the presence of 5-aza-deoxycytidine for 3 days and forskolin for 5 days (Diff.). (E, F): MSC-Ngn1 cells were depolarized by step pulses from -60 to 40 mV at a holding potential of -70 mV. (E): Outward and inward currents were elicited simultaneously, and the inward currents through voltage-gated Ca^{2+} channels could be reversibly blocked by $4 \mu\text{M}$ Nic. The peak inward currents were plotted against the voltages. (F): Voltage-gated Na^{+} currents were activated from a depolarizing step of -40 mV and blocked reversibly by $1 \mu\text{M}$ TTX. Peak current-voltage relationship was plotted against the voltages. Abbreviations: β III, β -tubulin III; Diff., differentiated; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; MAP2, microtubule associated protein 2; ms, milliseconds; MSC, mesenchymal stem cell; NF200, neurofilament 200; Ngn1, Neurogenin1; Nic., nicardipine; TTX, tetrodotoxin; Undiff., undifferentiated.

Motor Function Evaluation

Animals were pretrained for 1 week. In the Rotarod motor test, the Rotarod cylinder was accelerated from 4 to 40 rpm within 5 minutes, and the cutoff time was 300 seconds. For adhesive removal tests, square dots of adhesive-backed paper (100 mm²) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. Animals were given three trials with a cutoff time of 300 seconds. The data are presented as the mean time to remove the left dot. The neurological severity scores (NSS) were calculated according to Li et al. [24], with a slight modification (supplemental online Table 2). NSS tests were composed of the reflex, posture, proprioception, and beam balance tests. Points were

awarded for the inability to perform a given test; thus, higher scores represent more severe injury.

Statistical Analysis

Results were analyzed using one-way or repeated measures analysis of variance with independent variables of treatment groups and days of testing, followed by Scheffé's post hoc test for multiple comparisons at each treatment group. The level of statistical significance was set at $p < .05$. All values are presented as the mean \pm SD.

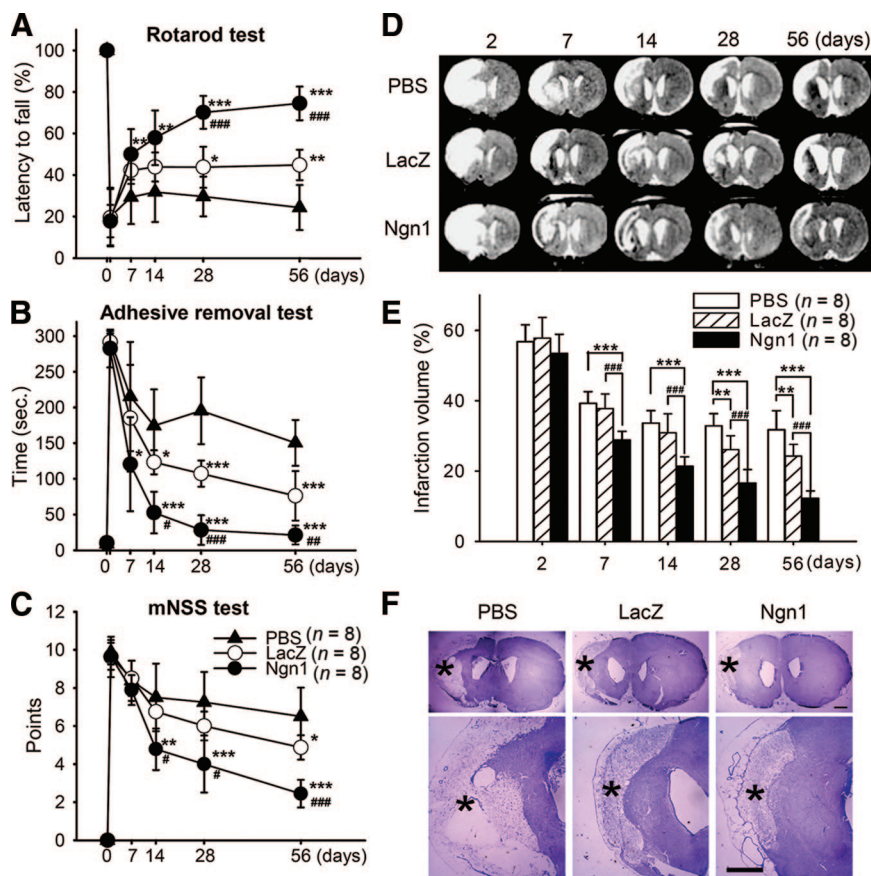


Figure 2. Effects of transplantation on recovery in the middle cerebral artery occlusion animal model. (A–C): Performance in the Rotarod (A), adhesive removal (B), and mNSS (C) tests from 1 to 56 days after ischemia. The data were collected from eight animals per group and are presented as mean values \pm SD. (D): The brain integrity of each animal was monitored by magnetic resonance imaging from 3 to 56 days after ischemia, and results are summarized in (E). (E): The data are presented as mean relative infarct volume \pm SD. (F): Cresyl violet staining at 56 days after ischemia. The ischemic core is marked with *. Scale bar = 100 μ m. Statistically significant differences between the groups were determined by analysis of variance (*, $p < .05$; **, $p < .01$; ***, $p < .001$ compared with the control PBS group; #, $p < .05$; ##, $p < .01$; ###, $p < .001$ compared with the mesenchymal stem cell-LacZ group). Abbreviations: mNSS, modified neurological severity score; Ngn1, Neurogenin1; PBS, phosphate-buffered saline; sec., seconds.

RESULTS

Neural Induction of MSCs with Ngn1

Human bone marrow MSCs stably expressing Ngn1, MSC-Ngn1 cells, were obtained by transducing MSCs with Ngn1-expressing retrovirus. MSC-Ngn1 cells continuously proliferated in growth medium without apparent morphological changes but lost the potential to differentiate into adipocytes, chondrocytes, and osteocytes under conditions in which MSC-LacZ cells, β -galactosidase-expressing MSCs, easily differentiated into these mesodermal cell types (Fig. 1A). Following terminal differentiation with Aza-dC and forskolin, MSC-Ngn1 cells expressed neuron-specific proteins, including NF200, MAP2, and NeuN, as shown by both immunocytochemistry and Western analyses (Fig. 1B, 1C). Importantly, after terminal differentiation for 8 days, MSC-Ngn1 cells expressed NeuroD, a well-known target gene of Ngn1 in terminally differentiating neurons [13] (Fig. 1D). Under the same conditions, these proteins were not detected or very minimally expressed in MSC-LacZ cells. Moreover, 59% of MSC-Ngn1 cells (16 of 27 cells) exhibited voltage-gated Ca^{2+} currents with typical bell shaped current-voltage curves that could be selectively blocked by 4 μ M nifedipine, an L-type Ca^{2+} channel blocker (Fig. 1E), and 75% (15 of 20 cells) exhibited voltage-gated Na^{+} currents, of which 77% could be blocked by 1 μ M tetrodotoxin (Fig. 1F). In contrast, none of the tested MSC-LacZ cells ($n = 53$) exhibited Ca^{2+} currents, or less than 20% exhibited Na^{+} currents that were barely detectable (<10 pA). These results indicate that MSC-Ngn1 cells acquired neuronal cell fate and thereby ex-

pressed neuron-specific phenotypes after being terminally differentiated in vitro.

Therapeutic Effects of MSC-Ngn1 Cells in a Rat Stroke Model

We tested whether neural induction further enhanced the inherent effects of MSCs for the treatment of neurological dysfunctions. After inducing ischemic stroke in rats by MCAO, we transplanted 5×10^5 cells each to two sites of the ischemic boundary zones 3 days after reperfusion and analyzed motor functions with Rotarod, adhesive removal, and NSS tests (Fig. 2A–2C). The control animals that received PBS spontaneously recovered to a limited degree for 2 weeks and then reached a plateau. Animals grafted with MSC-LacZ cells showed higher recoveries than controls over the first 2 weeks, and the performance scores remained constant thereafter. The animals with MSC-Ngn1 cells exhibited the highest recovery among the tested animals and continued to recover for up to 8 weeks even after the other two groups ceased to recover. At 8 weeks, compared with MSC-LacZ cells, MSC-Ngn1 cells showed 1.6-fold higher scores in the Rotarod test ($75\% \pm 8.1\%$ vs. $45\% \pm 7.4\%$), 3.6-fold lower scores in the adhesive removal test (21 ± 13.4 vs. 76 ± 34.7 seconds), and 2.5-fold lower scores in modified neurological severity score test (2 ± 0.7 vs. 5 ± 0.6 points). Our data of moderate functional recovery with MSC-LacZ cells are in good agreement with previous studies [25]. The dramatic enhancement with MSC-Ngn1 cells is due to neural induction.

We monitored the structural integrity of the brains using MRI analysis over the 8-week experimental period. The hyperintense areas in T2-weighted images over the central eight

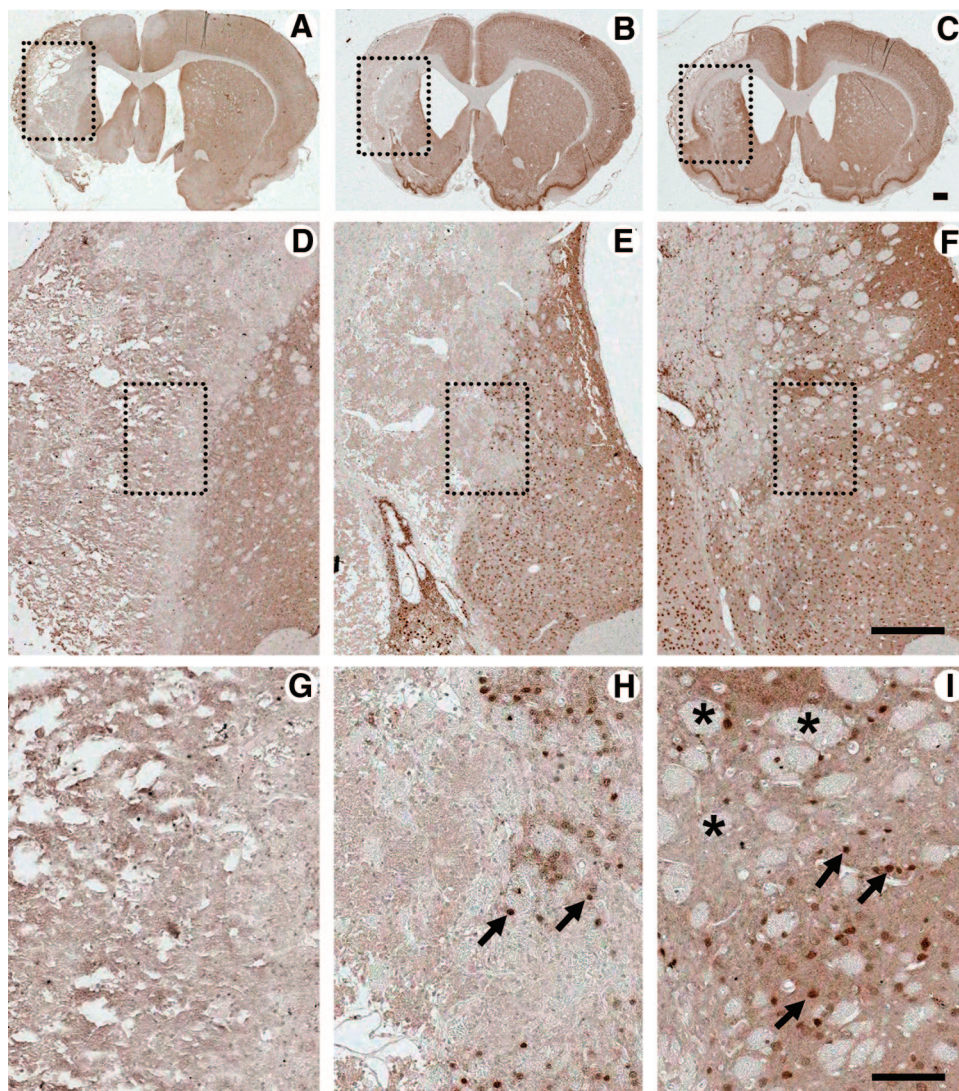


Figure 3. Effects of cell transplantation against ischemic brain damages. Coronal sections of brain at the level of the striatum were immunostained with anti-NeuN antibody to visualize the ischemic neuronal loss 8 weeks after middle cerebral artery occlusion. Loss of NeuN-positive cells in the ipsilateral striatum was dramatically reduced by transplantation of mesenchymal stem cell (MSC)-Neurogenin1 cells (C, F, I) compared with MSC-LacZ (B, E, H) or phosphate-buffered saline-treated control (A, D, G). (D–F): Higher magnification of areas marked in (A–C), respectively. (G–I): Higher magnification of areas marked in (D–F), respectively. NeuN⁺ cells are indicated with arrows; striatal axon bundles are marked with asterisks. Scale bars = 500 μ m (A–F) and 100 μ m (G–I).

images (1.5-mm-thick section) were combined to obtain the infarct volume (Fig. 2D, 2E). The original infarct volume was $57\% \pm 4.8\%$ of the intact contralateral hemisphere. In the PBS group, the infarct decreased spontaneously to $32\% \pm 5.4\%$ after 2 weeks and then remained constant up to 8 weeks. Transplantation of MSC-Ngn1 cells reduced the infarct to $21\% \pm 2.7\%$ at 2 weeks; it continued to decrease thereafter and reached $12.4\% \pm 2.2\%$ at 8 weeks. MSC-LacZ cells reduced the infarct to a lesser degree compared with MSC-Ngn1 cells ($24\% \pm 3.3\%$ at 8 weeks). In PBS-injected animals, Nissl staining showed the massive degeneration 8 weeks after ischemia (Fig. 2F), and most NeuN⁺ cells and axon bundles in cortex and striatum were lost (Fig. 3D, 3G). In the MSC group, degeneration of striatum was less severe, and some NeuN⁺ cells were found in the remaining medioventral part of the striatum (Fig. 3E, 3H). Importantly, degeneration was the smallest in the MSC-Ngn1 group. In particular, overall structures of ventrolateral cortex and lateral striatum were greatly preserved, NeuN⁺ cells were found more frequently, and striatal axon bundles appeared less

degenerated (Fig. 3F, 3I). The data collectively indicate that the higher recovery of motor functions observed in the MSC-Ngn1 group is possibly correlated to the higher tissue integrity of the host brain.

Paracrine Effects of MSC-Ngn1 Cells on Surrounding Tissues

It is known that MSCs produce bioactive cytokines that promote neurogenesis of resident stem cells in the SVZ [26, 27] and protect delayed cell death in the detrimental environment of damaged tissues [28, 29]. Since both neurogenesis and apoptosis are known to be prominent 1 week after ischemic injury [27, 30], we administered BrdU to animals daily for 4 days beginning immediately after transplantation and sacrificed them at day 7. As previously reported, the control group showed moderate proliferation of progenitor cells in SVZ in response to ischemic injury (Fig. 4, compare the numbers of BrdU⁺ cells in the ipsilateral and contralateral SVZs). Compared with the control, transplantation of MSC-LacZ and MSC-

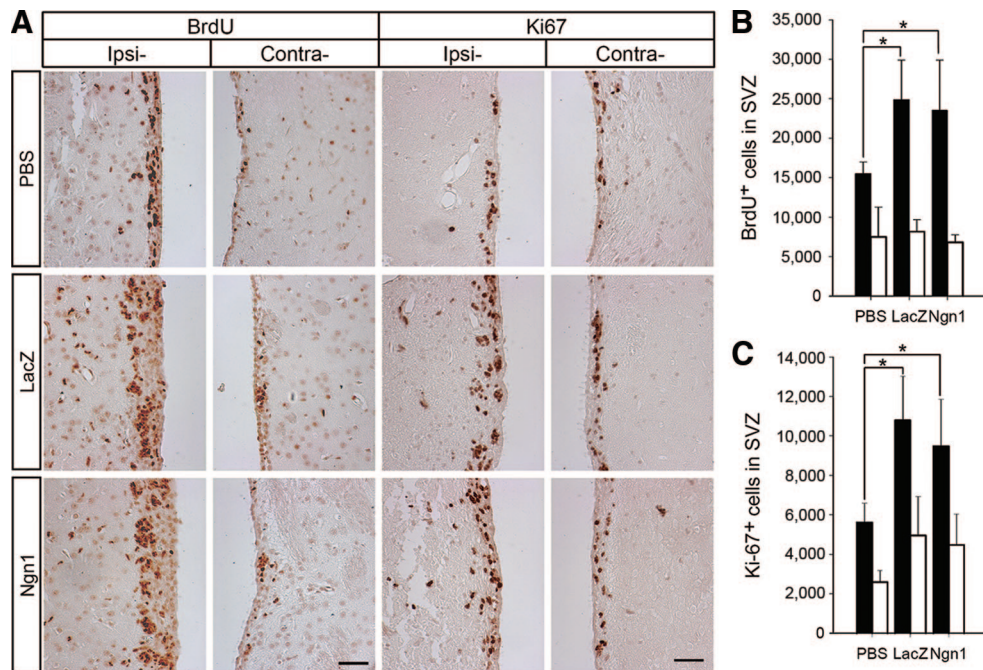


Figure 4. Effects of grafted cells on endogenous neurogenesis. (A): BrdU⁺ or Ki67⁺ neural progenitor cells in the SVZ were increased by transplantation of mesenchymal stem cell (MSC)-LacZ or MSC-Ngn1 cells compared with PBS-treated controls. (B, C): Quantitative analysis of BrdU⁺ (B) or Ki67⁺ (C) cells in the SVZ of Ipsi hemisphere (filled) and Contra hemisphere (open). Data from five animals are presented as mean values \pm SD. Both MSC-LacZ and MSC-Ngn1 groups showed significant differences from the control PBS group (analysis of variance; *, $p < .05$). Scale bars = 100 μ m. Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; Contra, contralateral; Ipsi, ipsilateral; Ngn1, Neurogenin1; PBS, phosphate-buffered saline; SVZ, subventricular zone.

Ngn1 cells further increased the number of BrdU⁺ cells by 1.6- and 1.5-fold, respectively, and the number of Ki67⁺ cells by 1.9- and 1.7-fold (Fig. 4). The differences between MSC-LacZ and MSC-Ngn1 cells were statistically insignificant, suggesting that both types of cells are equivalent in promoting a neurogenesis in SVZ.

To determine the effect on the delayed cell death in the penumbra regions such as ischemic boundary zone (IBZ) and dorsolateral striatum (ST) (Fig. 5A), we carried out TUNEL assays on the same animal brains. The control animals showed massive apoptosis 1 week after ischemic injury. Transplantation of MSC-LacZ and MSC-Ngn1 cells reduced the number of TUNEL⁺ cells by 45% and 37% in IBZ, respectively, and by 48%–49% in ST (Fig. 5B, 5C). Again, the differences between animals grafted with MSC-LacZ and MSC-Ngn1 cells were insignificant.

Pathological changes following ischemia trigger inflammatory responses, including activation of resident microglia and infiltration of leukocytes into microvessels and ischemic cerebral parenchyma, which may cause subsequent damages to the brain tissue [29]. Thus, we tested for differences in inflammatory cells 4 days after transplantation using ED1 antibody (Fig. 5D–5E). ED1 recognizes CD68, a phagocytic cell marker for active microglia, monocytes, and macrophages, and indicates ongoing inflammation. The numbers of ED1⁺ cells were reduced by 34% in IBZ and 41%–50% in ST in the animals with MSC-LacZ and MSC-Ngn1 cells compared with the control animals. Again, the differences between MSC-LacZ and MSC-Ngn1 cells were insignificant. Taken together, our data indicate that the immediate effects of parental MSCs and MSC-Ngn1 cells on the host brain are indistinguishable and suggest that neural induction of MSCs with Ngn1 may further increase the functional recovery through a mechanism that is yet unknown.

Transdifferentiation of MSC-Ngn1 Cells In Vivo

We sought evidence that the dramatic functional recovery in animals with MSC-Ngn1 cells was due, at least in part, to neuronal differentiation of the transplanted cells. Evaluation of neuronal differentiation of grafted cells in vivo was performed carefully, since small chemicals such as Hoechst or BrdU can be transferred from donor to host cells [12, 31] and autofluorescence can be emitted from dying cells [12]. We identified MSC-LacZ cells with anti- β -gal antibody and MSC-Ngn1 cells with anti-FLAG antibody in addition to anti-human mitochondrial antibody to avoid possible misinterpretation of donor-to-host cell transfer of labeling. Two weeks after transplantation, MSC-LacZ cells were found in IBZ, of which 21% were GFAP⁺, whereas 79% remained undifferentiated. NeuN⁺ cells were scarcely found (Fig. 6A). Thereafter, these cells gradually disappeared and were barely detectable 4 weeks after transplantation. At 2 weeks, MSC-Ngn1 cells could be found in both cortical core and IBZ, where more than 45% of them differentiated NeuN⁺ cells, whereas 55% remained undifferentiated. GFAP⁺ cells were seldom found (Fig. 6B). Importantly, unlike MSCs, many of the MSC-Ngn1 cells survived through the full 8-week experimental period and became NeuN⁺ and MAP2⁺ (Fig. 6C–6H). These cells exhibited morphology of mature neurons with long processes and expressed functional neuronal proteins such as VGLUT2 (Fig. 6E), as well as NF200 (an intermediate filament protein found in thick and thin axons of neuron) and tau (a neural microtubule-associated protein), which are found in mature neurons (supplemental online Figs. 1, 2). Moreover, expression of synapsin I (a presynaptic neuron-specific phosphoprotein) was colocalized with axon-like structures of these cells, suggesting synapse formation between host neurons (presynaptic) and MSC-Ngn1 cells (postsynaptic).

To determine whether these cells were functionally connected to host neural networks, we performed tracing experiments with two independent approaches. First, we injected a retrograde tracer,

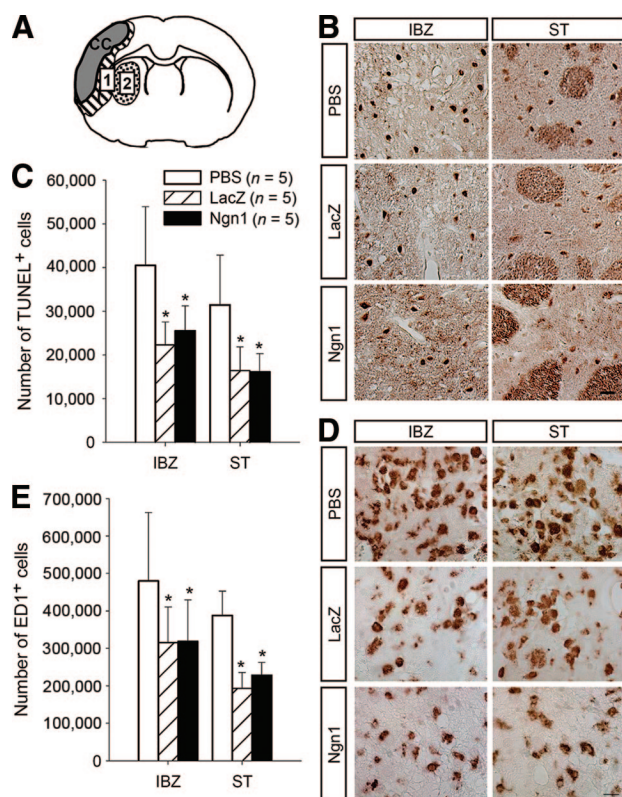


Figure 5. Effects of grafted cells on cell death and inflammation. (A): The ischemic brain, schematically illustrated: CC, IBZ (1), and ST (2). The numbers of TUNEL⁺ cells (B) and ED1⁺ cells (D) in the ipsilateral IBZ, and ST are illustrated. (C, E): Data are presented as mean numbers of positive cells \pm SD. Note that the numbers of TUNEL⁺ or ED1⁺ cells were decreased by transplantation of mesenchymal stem cell (MSC)-LacZ or MSC-Ngn1 cells compared with PBS-injected control. Both MSC-LacZ and MSC-Ngn1 groups showed significant differences from the control PBS group in the IBZ and ST (analysis of variance; *, $p < .05$). Scale bars = 20 μ m. Abbreviations: CC, cortical core; IBZ, ischemic boundary zone; Ngn1, Neurogenin1; PBS, phosphate-buffered saline; ST, striatum; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

FG [32–34], into the contralateral corpus callosum of the ischemic brain 4 weeks after transplantation and sacrificed the animals at 5 weeks. The grafted cells with a human mitochondria antigen in the ischemic hemisphere were found to contain the FG label (Fig. 7A–7C), indicating that the FG had been transported from the host neurons through the synapses and taken up by MSC-Ngn1 cells. Second, we engineered MSC-Ngn1 cells to express WGA in vitro and then transplanted them in the hippocampus of normal rat brains. WGA is known to be transported bidirectionally to axons and dendrites and taken up by pre- and postsynaptic neurons [35]. Eight weeks after transplantation, we found WGA not only in the grafted MSC-Ngn1 cells (Fig. 7E, 7F) but also in the soma and axons of the host hippocampal neurons in the contralateral hemisphere (Fig. 7G, 7H) of all tested animals ($n = 3$). The data clearly indicate that MSC-Ngn1 cells terminally differentiate into mature neurons in vivo and functionally connected to the host's neuronal networks.

DISCUSSION

Stem cell therapy has emerged as an exciting candidate for the treatment of neurological diseases. Among the various types of stem cells, MSCs are of particular clinical interest because they

can be isolated from bone marrow, adipose tissues, and umbilical cord blood. MSCs were shown to improve the neurological dysfunctions in stroke patients [36]. The present study presents the scientific basis for a requirement of neural induction of MSCs for better treatment of neurological dysfunctions.

During development of the nervous system, Ngn1 directs neural precursor cells to acquire neuronal cell fates by activating a series of downstream proneural bHLH transcription factors, such as Nex1, NeuroM, and NeuroD/BETA2 [13], while suppressing astroglial differentiation [19]. Surprisingly, overexpression of Ngn1 in MSCs is sufficient to convert their mesodermal cell fate to a neuronal one. The expression of NeuroD was increased when MSC-Ngn1 cells were challenged to exit the cell cycle and terminally differentiate by treatment with forskolin and Aza-dC (Fig. 1D), which is in good agreement with previous report that NeuroD causes cell cycle exit in terminally differentiating neurons [37, 38].

We have previously shown that treatment of forskolin alone in the presence of low serum may facilitate neuronal differentiation, which, however, is not sufficient to induce expression of voltage-gated ion channels [39], nor can cotreatment of forskolin with Aza-dC in the presence of high serum induce expression of voltage-gated Ca²⁺ or Na⁺ channels in MSCs as in this study. In contrast, these channels were easily detected in terminally differentiated MSC-Ngn1 cells (Fig. 1E, 1F) along with other neuronal markers, such as MAP2, NF200, and NeuN (Fig. 1B, 1C). Voltage-gated L-type Ca²⁺ channels and tetrodotoxin-insensitive voltage-gated Na⁺ channels are critical elements for initiation and propagation of action potential in neurons [40]. Thus, expression of these channels indicates that MSCs acquire neuron-like membrane excitability under the influence of Ngn1. Taken together, our results indicate that MSC-Ngn1 cells transdifferentiate into neuronal cells in vitro and that the emergence of neuronal characteristics in MSC-Ngn1 cells is not the result of the simple cytoskeletal rearrangement caused by cellular stress [10, 11] but rather is due to acquisition of neuronal cell fates.

After being transplanted, both MSC-LacZ and MSC-Ngn1 cells survived to a similar extent for 2 weeks in the brain parenchyma without treatment of immune suppressants. Transplanted human MSCs can survive preferentially in injury sites following MCAo [24, 41–43] and brain trauma [44] without any immune suppressants. Escape from the immune surveillance during the early period is partly due to the fact that MSCs lack MHC class II or costimulatory molecules such as CD40, CD80, and CD86 [45–47] or secrete inhibitory molecules against xenograft rejection [48, 49]. In a 2-week period, surviving MSCs either took GFAP⁺ astrocytic cell fates or remained undifferentiated. MSCs grown in our culture conditions seldom became NeuN⁺ cells in vivo and almost disappeared in 4 weeks. Our result of short-term survival of MSCs is in good agreement with the previous reports that transplanted MSCs cannot survive for an extended period of time, and only a very limited number of them, as low as 1%–2%, typically differentiate into neuronal cells [50, 51]. In contrast, MSC-Ngn1 cells survived up to 8 weeks and probably even longer in the pathogenic environment. These cells expressed functional neuronal proteins such as VGLUT2 (Fig. 6E), NF200, synapsin I, and tau, which are found in mature neurons (supplemental online Figs. 1, 2). The prolonged survival of MSC-Ngn1 cells may be partly due to the consequence of transdifferentiation into neurons and their connectivity to the host neurons. During development, target cells send neurotrophic signals retrogradely over long distances to presynaptic neuronal cell bodies and then selectively promote the survival of presynaptic neurons and maintenance of proper neural connectivity

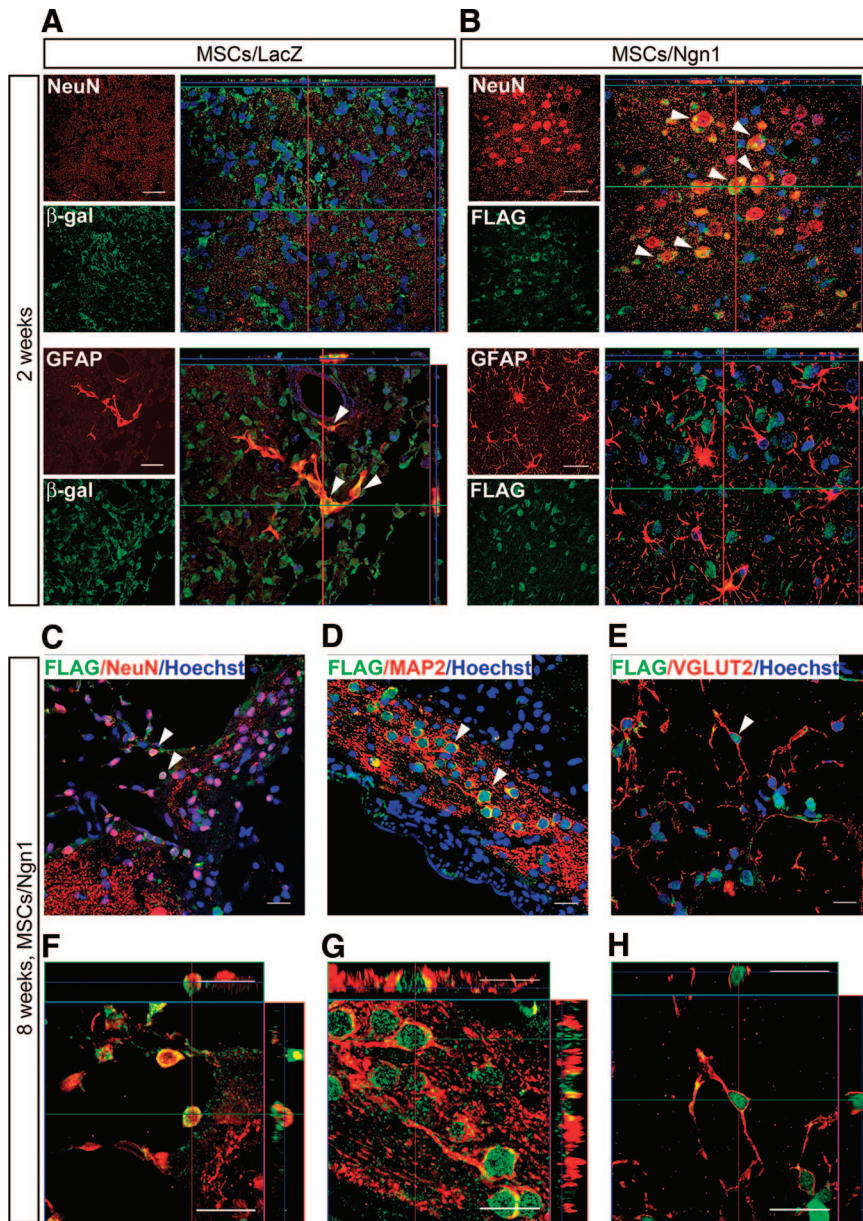


Figure 6. In vivo differentiation of grafted cells in the ischemic brain. (A, B): Confocal images of the grafted cells at 2 weeks after ischemia. MSC-LacZ and MSC-Ngn1 cells were identified by the expression of cytosolic β -gal (A) (green) and nuclear FLAG (B) (green), respectively. Glial fibrillary acidic protein (red) was expressed in MSC-LacZ cells (A), whereas NeuN⁺ (red) was expressed in MSC-Ngn1 cells (B). Arrowheads denote the donor-derived cells (green) colocalized with cell type-specific antigens (red) in enlarged orthographic images. (C–E): Confocal images of the MSC-Ngn1 cells at 8 weeks after ischemia. Nuclei staining (blue) shows scarce distribution of cells in the infarct region. MSC-Ngn1 cells (nuclear FLAG, green) survived for more than 8 weeks and expressed NeuN (C) (red), MAP2 (D) (red), and VGLUT2 (E) (red). (F–H): Enlarged orthographic images show colocalization of NeuN (F) (perinucleus), MAP2 (G) (dendrite), or VGLUT2 (H) (axon) in MSC-Ngn1 cells (green). Scale bars = 20 μ m. Abbreviations: β -gal, β -galactosidase; MAP2, microtubule associated protein 2; MSC, mesenchymal stem cell; Ngn1, Neurogenin1; VGLUT2, vesicular glutamate transporter 2.

while excess neurons that fail to form functional synapse are pruned out [52]. Indeed, MSC-Ngn1 cells established a connection to the host neurons located in the contralateral hemisphere, as shown by transport of WGA or FG (Fig. 7). Collectively, these results suggest that MSC-Ngn1 cells terminally differentiate into functional neurons in vivo and, in return, survive for an extended period of time.

During cortical development, Ngn1 and Ngn2 are expressed in dorsal progenitors in the telencephalon, which give rise to cortical glutamatergic pyramidal neurons [53]. A forced expression of Ngn2 specifies neural stem cells of the adult subependymal zone to differentiate into glutamatergic neurons, whereas overexpression of *Mash1* causes cells to differentiate into GABAergic neurons [54], suggesting that bHLH proneural genes function to instruct cell type specification, as well as directing cell fate determination. In our study, MSC-Ngn1 cells spontaneously differentiated into VGLUT2⁺ glutamatergic neurons in vivo (Fig. 6E) but rarely became GFAP⁺ astrocytes (Fig. 6), suggesting that Ngn1 directs MSCs to acquire the glutamatergic neuronal cell fate,

as it does during embryonic brain development. Additional studies with more bHLH transcription factors, particularly *Mash1*, will answer whether the bHLH factors, as in neural stem cells, also guide cell type specification in MSCs while instructing neuronal cell fates.

Previously it has been shown that activation of Notch signals together with application of bFGF, forskolin, and ciliary neurotrophic factor could induce neuronal differentiation of MSCs [55]. During development of the nervous system, Notch is known to repress neuronal differentiation of neural progenitor cells [56] and trigger differentiation of glial cells, including radial glia [57], Schwann cells [58], Müller cells in retina [59], and astrocytes [60, 61]. These functions of Notch are mediated by J κ recombination signal-binding protein (RBP-J κ) or C-promoter-binding factor-1, which subsequently downregulates the proneural genes, including Ngn1 [62]. Therefore, one of the issues in need of clarification is how the apparently opposing signals from Notch and Ngn1 can be reconciled with induction of neuronal differentiation of MSCs.

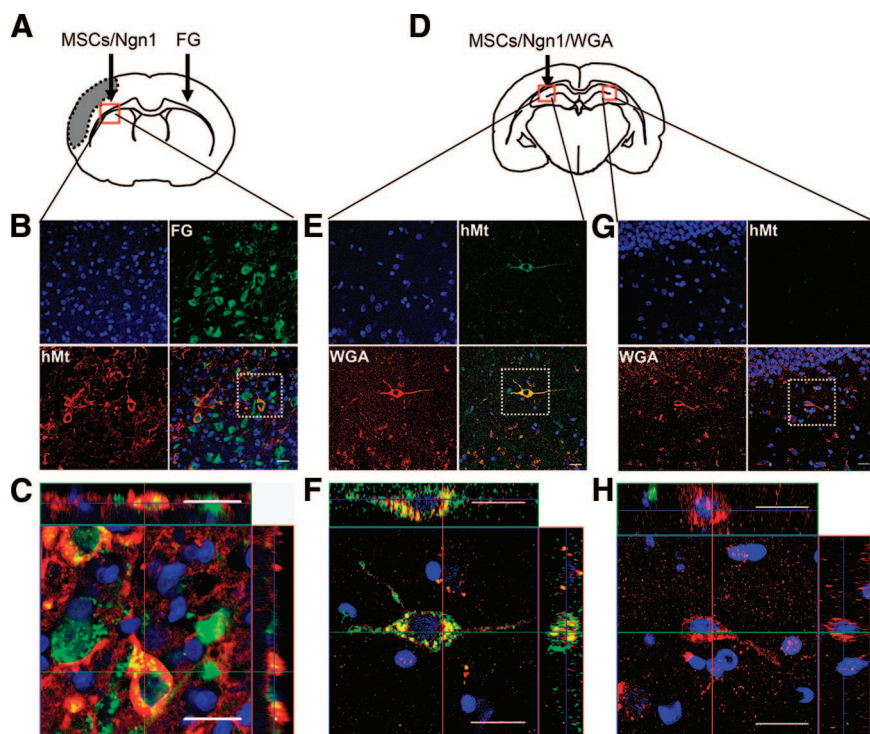


Figure 7. Neural tracing of transplanted MSC-Ngn1 cells. **(A):** A schematic illustration shows transplantation of MSC-Ngn1 in the ischemic brain and injection of FG in the contralateral site (Materials and Methods). **(B):** One week later, FG (green) was found in MSC-Ngn1 cells (red) that were identified with anti-hMt antibody. **(C):** An orthographic image of hMt⁺, FG⁺ cells in **(B)**. **(D):** MSC-Ngn1 cells were prelabeled with WGA and transplanted into hippocampus of the normal brain. **(E):** Eight weeks later, MSC-Ngn1 cells were found to be hMt⁺ (green) and WGA⁺ (red). **(F):** An orthographic image of hMt⁺, WGA⁺ cells in **(E)**. **(G):** Host neurons in the contralateral hippocampus were devoid of hMt and colocalized with WGA (red). Note the prominent WGA⁺ nerve endings (red) in the hippocampus. **(H):** An orthographic image of the WGA⁺ host neuron in **(G)**. Scale bars = 20 μ m. Abbreviations: FG, fluorogold; hMt, human mitochondria; MSC, mesenchymal stem cell; Ngn1, Neurogenin1; WGA, wheat germ agglutinin.

Saving the penumbra, where the functions of neurons are impaired but potentially viable, is the main target of acute stroke therapy. Consistent with the remarkable recovery of motor functions, the tissue integrity of penumbra regions appears to be salvaged more rigorously by the MSC-Ngn1 cells than by MSC-LacZ cells; numerous NeuN⁺ cells were found IBZ and ST, and striatal axon bundles remain less damaged (Figs. 2F, 3). The marvelous rescue of penumbra regions from the ischemic injury is the combinatorial results of several functions: (a) immune suppression; (b) promotion of neurogenesis; (c) protection from the delayed cell death of host neurons and newly generated, migrating neuroblasts; and (d) transdifferentiation. Following ischemic injury, quiescent microglia are activated to have round, enlarged soma and macrophage-like characteristics. This traditional view of microglia has been challenged by recent finding that many Iba1⁺ microglia undergo apoptotic degeneration in the lesion core within 24 hours after brain ischemia [63]. Therefore, the ED1⁺ cells that we observed 1 week after reperfusion are very likely to be infiltrated neutrophils and monocytes [64]. Overall, we could not find significant differences between MSC-LacZ and MSC-Ngn1 cells in promoting neurogenesis, suppressing delayed cell death, and modulating immune responses during the early time period. Therefore, the robust recovery in animals with MSCs-Ngn1 cells is attributed to their transdifferentiation. Directly, transdifferentiated cells are integrated into host neural circuits and replace the damage host neurons. Indirectly, as we mentioned above, transdifferentiated MSCs-Ngn1 cells survive for an extended period of time and secrete beneficial factors in physiologically regulated manners.

MSCs are currently being tested for their potential use in cell therapy of a number of human diseases, in addition to stroke [36], including neurological diseases (such as amyotrophic lateral sclerosis), lysosomal storage diseases, and non-neurological diseases (such as acute myocardial infarction, vascular restenosis, osteogenesis imperfecta, steroid refractory graft-versus-host disease, periodontitis, and bone fracture) [1]. Typical therapeutic

effects of MSCs are attributed to their paracrine function of secreting bioactive substances. The critical question is answered by the present study that these cells are more effective after neural induction.

In summary, we have demonstrated that Ngn1 is sufficient to induce neural cell fates in MSCs. The Ngn1-expressing MSCs are comparable to neural stem cells (NSCs) in that they nestle within the necrotic host parenchyma, become neuronally committed, and provide a source for cellular replacement [65]. As is the case with NSCs, it is possible that the Ngn1-expressing MSCs may secrete neurotrophic factors in a physiologically regulated manner, potentially providing an additional benefit for the reparative processes of host cells. As such, the present study highlights the advantage of neural induction of MSCs, a stem cell with a far greater accessibility than NSCs, in the development of new strategies for treating central nervous system dysfunctions.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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