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Human Bone Marrow Stem Cells Exhibit Neural Phenotypes and Ameliorate Neurological Deficits after Grafting into the Ischemic Brain of Rats

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There is now evidence to suggest that bone marrow mesenchymal stem cells (MSCs) not only differentiate into mesodermal cells, but can also adopt the fate of endodermal and ectodermal cell types. In this study, we addressed the hypotheses that human MSCs can differentiate into neural cells when implanted in the brain and restore sensorimotor function after experimental stroke. Purified human MSCs were grafted into the cortex surrounding the area of infarction 1 week after cortical brain ischemia in rats. Two and 6 weeks after transplantation animals were assessed for sensorimotor function and then sacrificed for histological examination. Ischemic rats that received human MSCs exhibited significantly improved functional performance in limb placement test. Histological analyses revealed that transplanted human MSCs expressed markers for astrocytes (GFAP$^+$), oligodendroglia (GalC$^+$), and neurons (βIII$^+$, NF160$^+$, NF200$^+$, hNSE$^+$, and hNF70$^+$). The morphological features of the grafted cells, however, were spherical in nature with few processes. Therefore, it is unlikely that the functional recovery observed by the ischemic rats with human MSC grafts was mediated by the integration of new "neuronal" cells into the circuitry of the host brain. The observed functional improvement might have been mediated by proteins secreted by transplanted hMSCs, which could have upregulated host brain plasticity in response to experimental stroke.

Key Words: **bone marrow; stem cell; transplantation; brain ischemia; stroke; middle cerebral artery occlusion; human; rat.**

INTRODUCTION

Stem cells have the capacity for self-renewal and differentiation into diverse cell types. During embryogenesis, totipotent stem cells give rise to ectoderm, mesoderm, and endoderm (16, 56). However, once stem cells are committed to a given tissue or organ, their phenotypic potentiality is restricted to the tissue of origin. Hematopoietic stem cells, for instance, are thought to differentiate only into blood cell lineages (36), while neural stem cells are thought to be limited to neural progeny (6). A number of recent publications have challenged this notion. Neural stem cells are capable of restoring the hematopoietic system into blood cells (6) and differentiate into muscle (17). Bone marrow stromal cells or mesenchymal stem cells (MSCs) not only give rise to mesodermal lineage cells such as osteoblasts, chondrocytes, adipocytes, and muscle cells (4, 13, 29, 55, 33), but also adopt neuroectodermal cell fate. It has been shown that donor-derived neurons and astrocytes are found in host rodent brain following transplantation of rodent bone marrow (8, 14, 29, 35). However, it is not known if transplantation of MSCs into the brain can be beneficial in treating neurological disorders.

Stroke is a leading cause of disability worldwide (43, 47, 54). Effective therapeutics for the rehabilitation of stroke survivors have yet to be developed. Recently Chopp and coworkers reported that rodent bone marrow cells grafted into the ischemic rat brain resulted in functional improvement (32). In this study, however, bone marrow cells were not purified to isolate stem cells. Our laboratory has isolated human bone marrow mesenchymal stem cells with multipotent properties. The aims of the present study, therefore, were to determine if purified human MSCs could differentiate into neural cell types when transplanted into the brain, and whether they ameliorate neurological deficits in rats with ischemic brain injury.

MATERIALS AND METHODS

Human Bone Marrow Stem Cell Preparation

Human bone marrow was harvested from the iliac crest of 10 to 35-year-old healthy volunteers. MSCs

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were enriched by sequential Ficoll density gradient purification followed by removal of CD45 and Glycophorin A positive cells using the Super MACS magnet (Miltenyi Biotec, Sunnyvale, CA). The cells were plated and expanded in fibronectin (5 ng/mL)-coated flasks with MSC expansion medium: 56% DMEM-LG, 40% MCDB-201 (Sigma), 2% FCS, 1 × ITS Media Supplement (Sigma), 1 × linoleic acid–BSA (Sigma), 10^{-8} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 100 U penicillin/1000 U streptomycin (Gibco-BRL), 10 ng/mL EGF (R&D Systems), and 10 ng/mL PDGF-BB (R&D Systems). After culture expansion, undifferentiated MSCs were negative for CD34, CD36, CD38, CD45, HLA-DR, HLA-type I, Muc 18, and E-selectin/P-selectin, expressed low levels of Flkl, CD44, and β2 microglobulin, and were positive for CD10, CD13, CD49b, and CD49d. The undifferentiated MSCs were then transduced with an eGFP (enhanced green fluorescent protein) containing retroviral vector (MFG-eGFP packaged in the PA317 or PG13 cell line and 10 μg/mL protamine). After FACS selection, eGFP-positive cells (Fig. 1) were expanded in fibronectin-coated flasks with expansion medium until they were used for cell transplantation. Approximately 26-passaged hMSCs were grafted into the rat brain. The multipotent nature of hMSCs was determined in *in vitro* studies. Human MSCs can differentiate into osteoblasts, chondrocytes, adipocytes, fibroblasts, skeletal myoblasts, smooth muscle myoblasts, endothelium, and cells with neuroectodermal features (unpublished data).

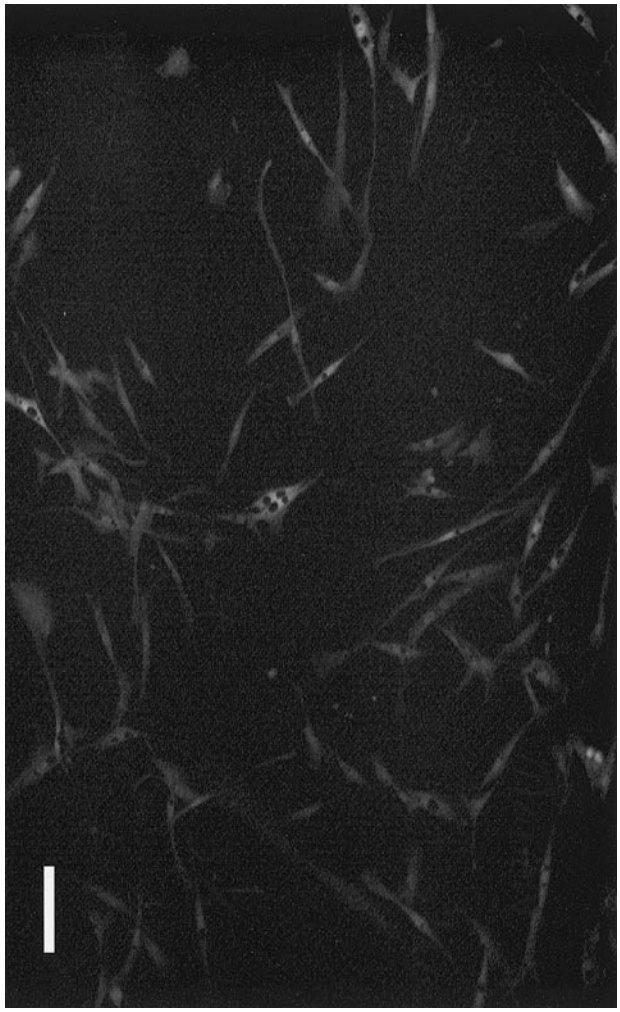

FIG. 1. Purified human MSCs labeled with eGFP in tissue culture. Note the spindle shape morphology of the cells in culture. Scale bar, 200 μm.

Experimental Groups

Adult male spontaneously hypertensive rats (SHRs, Charles River Laboratories), weighing 230–250 g, were used in this study. Animals were randomly divided into four groups: ischemia + MSC transplantation + systemic injection of Cyclosporin A (CsA) (n = 15, MSCs), ischemia + intracerebral PBS injections + CsA (n = 8, CsA), ischemia + intracerebral PBS injections + systemic injection of saline (n = 14, Saline), and a sham-operated group (n = 4, Sham).

Cortical Brain Ischemia

The experimental protocols were approved by the Animal Care Committee at the University of Minnesota. The experiment was conducted under the auspices of Research Animal Resources, a facility approved by the American Association for the Accreditation of Laboratory Animal Care. The rats were anesthetized with methohexital sodium (50 mg/kg, ip, Jones Pharma Inc., St. Louis, MO). Cortical brain ischemia was produced by permanently ligating the right middle cerebral artery (MCA) distal to the striatal branch as described by Coyle (12). Briefly, an incision was made between the right ear and eye. Using a microscope, a 2 × 2-mm craniotomy was drilled in the landmark where the zygoma fuses to the squamosal bone. The right middle cerebral artery was ligated with a 10-0 monofilament nylon suture. The body temperature during the operation was kept close to 37°C. The procedure for the sham operation was as the same as above except for the ligation of MCA.

MSC Transplantation

eGFP-labeled MSCs were harvested with 0.25% trypsin/EDTA, washed twice, and resuspended in phosphate-buffered saline (PBS). The viability and cell density of the MSCs were evaluated using a trypan blue exclusion method. The viability of the MSCs was more than 90%. Three sites in the cortex surrounding the infarction were targeted for transplantation at the following coordinates: (i) 1.0 mm rostral to the bregma, 2.0 mm lateral to the midline, 1.2 mm ventral to the

dura; (ii) 3.0 mm caudal to the bregma, 1.5 mm lateral to the midline, 1.2 mm ventral to the dura; (iii) 6.0 mm caudal to the bregma, 2.0 mm lateral to the midline, 1.2 mm ventral to the dura. The tooth bar was set at zero. One week after brain ischemia, the rats were anesthetized with equithesin (3 mL/kg, ip) and 3 μL of MSCs (25,000 cells/μL) was stereotaxically transplanted into each site by using a 10-μL Hamilton microsyringe (Hamilton, Reno, NV). The injection was performed over 3 min and the cannula was kept *in situ* for an additional 3–5 min before it was withdrawn. An equal volume of sterile PBS was injected into the same sites in the control rats. The sham rats received only craniectomies. Cyclosporin A (10 mg/kg, ip) injections were given daily to immunosuppress rats that received MSC implants, and an equal volume of CsA or saline was injected to the CsA-control rats and the saline-controls, respectively.

Neurological Deficit Evaluation

Limb placement test and tactile stimulation test were blindly assessed 1 week before brain ischemia, 1 day before transplantation, and at 2 and 6 weeks after grafting. The limb placement test included eight subtests described by Johansson and coworkers (38). Briefly, the four limbs of a rat were tested on a counter top surface and at its edges. For each subtest, an animal received a score of 0 if it was unable to place its limbs, a score of 1 if there was a partial and/or delayed (more than 2 s) placement of its limbs, or a score of 2 if the animal exhibited an immediate and correct placement of its limbs. In a tactile stimulation test (51, 52), a small piece of adhesive tape (113.1 mm^2) was rapidly applied to the radial aspect of each forepaw. The rats were then returned to their home cages, and the order of the tape removal (i.e., left versus right) was recorded. Three to five trials were conducted on each test day. Each trail was terminated when the tapes were removed from both forepaws or after 3 min.

Immunohistochemistry

Two or 6 weeks after transplantation, the rats were anesthetized with chloral hydrate (350 mg/kg, ip) and the brains were then quickly removed, frozen in powdered dry ice, and stored at −80°C. Coronal cryostat sections of 10 μm thickness were processed for immunohistochemistry. Based on the original design of the study, hMSCs were labeled with eGFP (green) *in vitro* before transplantation, and neural phenotype expressions of the grafts were visualized by using Cy 3 (red fluorescent dye) conjugated secondary antibodies. When unfixed fresh sections were examined under a fluorescent microscope, however, we found some of eGFP labeled grafts became autofluorescent. Therefore, using immunofluorescence to detect the phenotypic fate of the grafted hMSCs was not possible. As an alternative strategy, phenotypes of the grafted cells were identified using antibodies that recognize GFP, human-specific antibodies, and neural cell markers using the immunoperoxidase method. The sections were fixed with 4% formaldehyde in Tris-buffered saline (TBS, 0.05 M, pH 7.6) for 15 min. After rinsing in TBS, the sections were exposed to 0.3% H_2O_2 for 30 min to quench endogenous peroxidase activity. Nonspecific binding was blocked with normal serum from the species in which the secondary antibodies were raised (1:10 in 1% BSA + 0.25% Triton X-100). The sections were incubated with the primary antibodies diluted in 1% BSA + 0.25% Triton X-100 overnight at 4°C. The primary antibodies used in the study were as follows: anti-green fluorescent protein (GFP, 1:50, Clontech), anti-neurofilament 160 (1:100, Sigma), anti-neurofilament 200 (1:400, Sigma), anti-β-tubulin-III (1:400, Sigma), anti-glial fibrillary acidic protein (GFAP, 1:500, Sigma), anti-galactocerebroside (GalC, 1:50, Sigma), anti-human neuron specific enolase (hNSE, 1:100, Chemicon), anti-human neurofilament 70 (hNF-70, 1:50, supplied by Dr. L. Sorriano), anti-human nestin (1:200; a gift from Dr. C. Messam), anti-fibronectin (1:250, transduction), and anti-collagen type I (1:40, Calbiochem). After rinsing in TBS, the sections were incubated with biotinylated secondary antibodies (Vector) for 1 h at room temperature and the reaction products were visualized by the avidin–biotin–peroxidase complex method using 3,3-diaminobenzidine tetrahydrochloride as the chromogen (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA). The sections were then counterstained with hematoxylin. Adjacent sections were used for negative controls. In the control sections all procedures were processed in the same manner as the experimental tissue, but the primary antibodies were omitted.

Determination of Infarction Size

A series of coronal cryostat sections with 20-μm-thick and 900-μm intervals were stained with celestine blue and acid fuchsin. The sections were digitized using a 1× objective lens under a Nikon light microscope (Nikon, Japan) that is connected with a high-resolution digital camera (COOLPIX 950, Nikon, Japan). The images were first collected and stored in a CompactFlash card. The CompactFlash card was then read by using a CompactFlash card reader connected with a Pentium III PC (Dell, Dimension XPS T700r, USA) and the images were analyzed using a software package (Scion Image, Version Beta 4.0.2, Scion Corporation, Frederick, MD). Infarction volume was calculated with infarct areas measured each section and the section interval. According to the time of the termination of this study, secondary tissue loss such as thalamic atrophy has been considered. In order to eliminate an overestimation of infarct size, total tissue volume loss was pre-

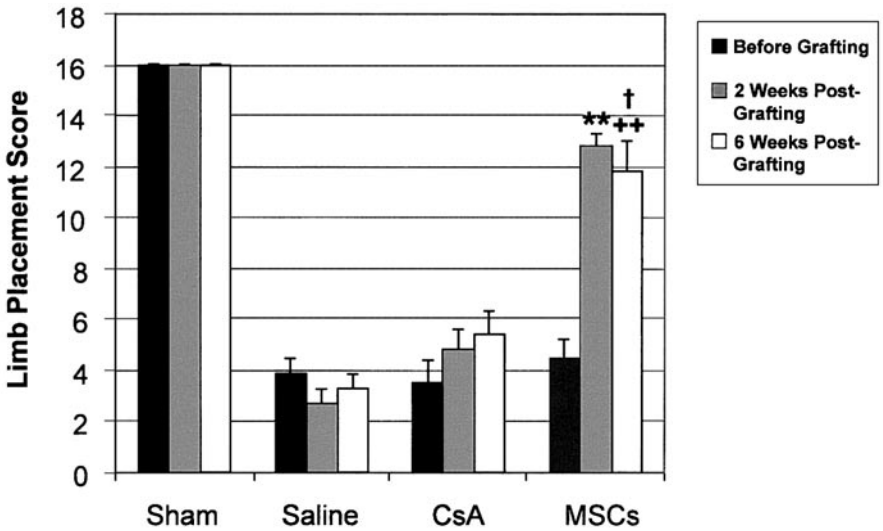

FIG. 2. Assessment of brain ischemia-induced neurological deficits. Deficits are evaluated with a limb placement test (0, severe neurological deficits; 16, no neurological deficits). Two weeks after transplantation, neurological deficits were significantly ameliorated in rats with hMSC transplants when compared with the rats in the saline control group (**$P < 0.01$) and with the rats in CsA control group (**$P < 0.01$). Six weeks after grafting, hMSC-treated animals still exhibited functional improvement compared to saline controls (++$P < 0.01$) and CsA controls (++$P < 0.01$). Before transplantation, the rats that would receive hMSC transplants exhibited poor limb placement performance (mean score, 4.5), which did not significantly differ from saline controls and CsA controls ($P > 0.05$), while 2 weeks after grafting hMSC transplanted animals showed a significant functional improvement (mean score, 13, **$P < 0.01$) compared with their pregraft performance. The improved function by hMSC transplantation was seen at 6 weeks postgrafting (†$P = 0.03$, before grafting vs after grafting). Groups: sham ($n = 4$), sham ischemic rats with sham transplants; Saline ($n = 14$), ischemic rats with intracerebral PBS injections and ip saline injections; CsA ($n = 8$), ischemic rats with intracerebral PBS injections and ip cyclosporin A injections; MSCs ($n = 15$), ischemic rats with intracerebral hMSCs grafts and ip cyclosporin A injections. Nonparametric technique was used for statistical analysis. Data were presented as mean ± SE.

sented as a percentage of the contralateral cortex and a percentage of the contralateral hemisphere.

Statistical Analysis

The neurological evaluation data were statistically analyzed using a Kruskal–Wallis nonparametric test. When the results were significant ($P < 0.05$), a Mann–Whitney nonparametric analysis was used for determining the difference between two groups. The Wilcoxon signed-rank nonparametric test was used for examining the difference between any two time points. For difference in infarct size, one-way ANOVA with Scheffe's post hoc procedure was used at a 95% significant level. Data were presented as mean ± SE.

RESULTS

Functional Outcomes and Infarct Volume

Ischemia-induced neurological deficits were significantly ameliorated in rats that received MSC transplants when compared with other ischemic animals in the limb placement test (Fig. 2). One week prior to brain ischemia the SHRs did not display any neurological deficits (data not shown), the neurological scores were the same as sham animals. One week after brain ischemia, neurological deficits were observed in lesioned rats and there was no statistical difference among the three ischemic groups ($P > 0.05$). Two weeks after transplantation, animals with hMSC grafts achieved significantly higher limb placement scores when compared with the rats in the saline-control group (12.73 ± 0.53 vs 2.67 ± 0.58; MSCs vs saline; $P < 0.01$), and with rats in the CsA-control group (12.73 ± 0.53 vs 4.75 ± 0.84; MSCs vs CsA; $P < 0.01$). The greater functional performance was still significant 6 weeks postgrafting (11.80 ± 1.14 vs 3.33 ± 0.60; MSCs vs saline, $P < 0.01$; 11.80 ± 1.14 vs 5.38 ± 0.94; MSCs vs CsA, $P < 0.01$). The functional improvement observed at 2 weeks after MSC transplantation was also significant compared to the test performed before grafting (12.73 ± 0.52 vs 4.47 ± 0.68; 2 weeks after vs before grafting; $P < 0.01$). When comparing the test results 6 weeks after transplantation with the results before transplantation, this improvement also persisted (11.80 ± 1.14 vs 4.47 ± 0.68; 6 weeks after vs before; $P = 0.03$). MSCs induced functional recovery, however, did not reach levels comparable to intact sham animals (Sham vs MSC $P < 0.01$, 2 weeks and 6 weeks postgrafting). Nevertheless, animals with MSC transplants exhibited significantly improved neurological scores compared with their performance prior to implantation and with the two control groups.

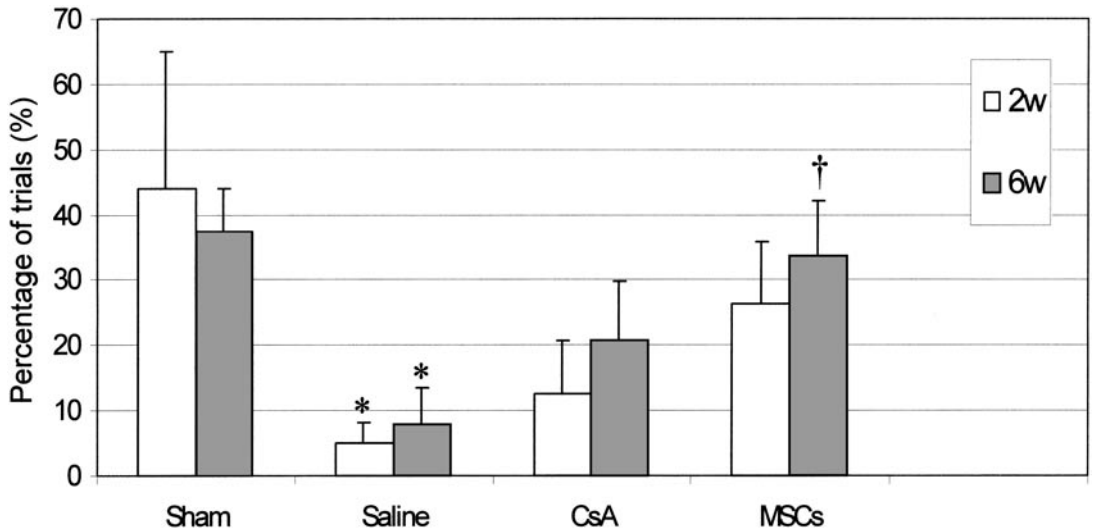

FIG. 3. Evaluation of somatosensory asymmetries. This graph illustrates the percentage of trials that the tape on the affected or left forepaw was first removed. The rats were examined by a tactile stimulation test applying adhesive tapes as stimuli to the forepaws. An intact rat typically shows an equal chance of removing the stimuli form either forepaw (i.e., left vs right = 50% vs 50%). Right MCA ligation causes somatosensory deficits in the left limbs, leading the rats to preferentially remove the tape from intact (right) forelimb first rather than the left one. As shown in this bar graph, somatosensorimotor response was significantly reduced in the effected forelimb (left) in saline-control rats when compared with sham rats (*$P < 0.05$, 2 weeks and 6 weeks). In comparison with saline controls, MSC-grafted animals showed a significant recovery of somatosensory (†$P < 0.05$) 6 weeks after grafting. Data were presented as mean ± SE and analyzed with nonparametric procedures.

The data from the tactile stimulation test (Fig. 3) showed that forelimb somatosensory asymmetries induced by unilateral brain ischemia were significantly increased in the saline control rats when compared with sham-operated rats (2 weeks: 5 ± 3.3% vs 43.8 ± 21.3% saline vs sham, 6 weeks: 7.8 ± 5.7% vs 37.5 ± 6.3%; $P < 0.05$), and the somatosensory asymmetries were significantly ameliorated in the rats received MSCs transplantation at 6 weeks after grafting in comparison with saline controls (33.5 ± 8.7% vs 7.8 ± 5.7%, MSCs vs saline, $P < 0.05$). MSCs-grafted animals also showed a relatively better recovery from ischemia caused somatosensory bias when compared to the rats in two ischemic control groups although it did not achieve significant levels. The SHRs did not display neurological asymmetries 1 week before brain ischemia (data not shown), the results were similar to sham operated animals.

Infarct volume did not show significant differences among MSC transplanted rats, saline-control rats and the rats in CsA-control group (Table 1), suggesting that the grafts did not protect the host brain from secondary tissue loss.

TABLE 1

Tissue Loss in the Rats of Ischemic Controls and MSC Graft

	Cortical tissue loss (% of contralateral cortex)	Total tissue loss (% of contralateral hemisphere)
Saline ($n = 14$)	24.60 ± 2.10	11.76 ± 1.62
CsA ($n = 8$)	27.57 ± 2.51	10.93 ± 1.35
MSCs ($n = 15$)	22.51 ± 1.63	10.32 ± 1.29

Note. Data were presented as mean ± SE.

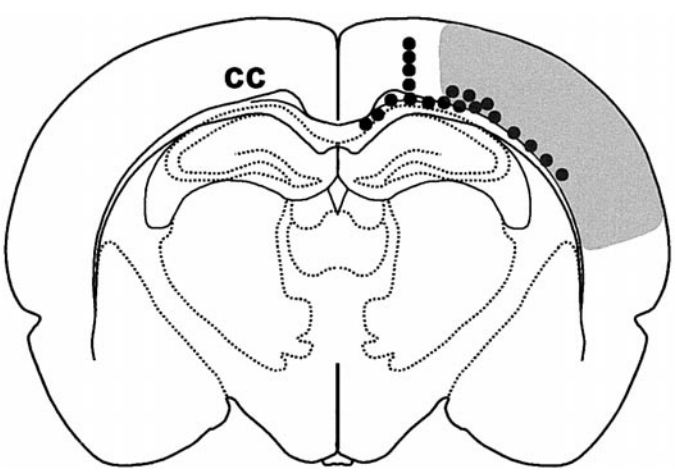

FIG. 4. The migration pattern of implanted hMSCs after cortical brain ischemia. Black dots represent the location of transplanted hMSCs. Shadow area, infracted cavity. CC, corpus callosum.

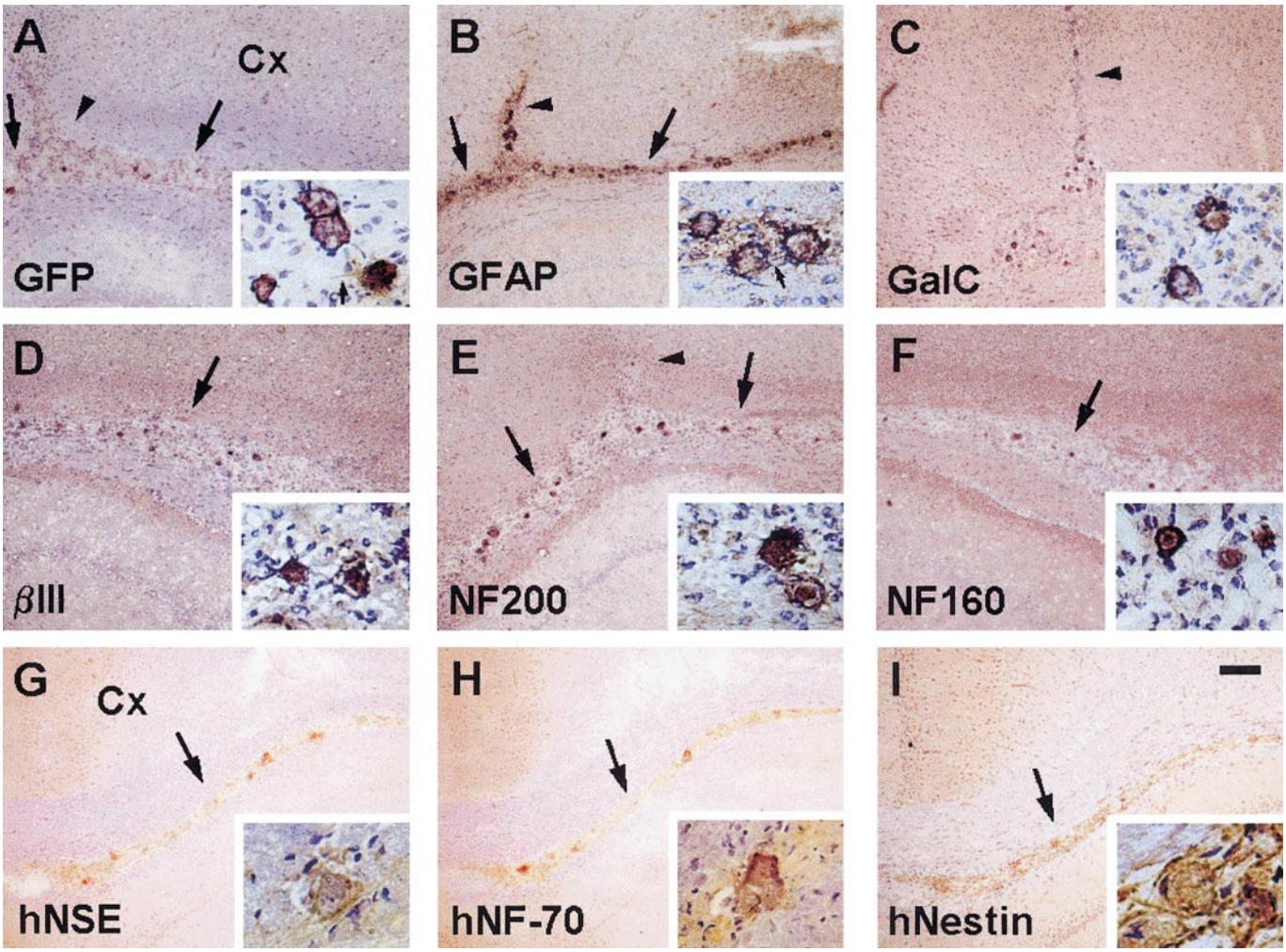

FIG. 5. Expression of neural markers by transplanted human mesenchymal stem cells. Human MSCs were grafted into the cortex surrounding the infarct area one week following cortical ischemia, and coronal cryostat sections were processed for immunohistochemistry 2 and 6 weeks after transplantation. (A–F) Representative sections form the rats sacrificed 2 weeks after grafting. (G–I) Representative sections form the rats sacrificed 6 weeks after grafting. (A) eGFP-labeled hMSCs were identified by anti-GFP antibody. GFP-positive cells were found at the site of grafting (arrowhead), and along the two migratory pathways (arrows). (B) Transplanted hMSCs were immunopositive for the astrocyte marker GFAP. The top right corner of the panel is the infarct area. $GFAP^+$ hMSCs were detected at the site of the graft and along the migratory paths. The right path extended to the region of infarction. (C) Grafted hMSCs expressed the oligodendrocyte marker GalC. Some of the transplanted hMSCs were positively stained with neuronal cell markers β III tubulin (D), NF-200 (E), and NF-160 (F). G–I show human-specific neural markers expressed by transplanted human mesenchymal stem cells. Human neuronal-specific markers, hNSE and hNF-70 were identified within the grafts (G and H, respectively). Human nestin positive cells were also found within the grafts (I). Insets in A–I are the higher magnification of A–I. Inset A: the arrow shows a process of the GFP^+ cell. Inset B: the arrow shows a process of a $GFAP^+$ cell. Scale bar (shown in I): A–I, 200 μm. Cx, cortex.

Migration of Transplanted MSCs

Our data showed that transplanted MSCs migrated along the corpus callosum. This migratory process is in agreement with the findings of Azizi *et al.* (3) that demonstrated the migration of grafted human MSCs was similar to implanted astrocytes migrating along the corpus callosum. From the graft sites two migratory pathways were noted: one extending toward the midline of the brain and another extending toward or into the infarct area (Fig. 4). MSCs in the infarct region were mostly located at the border between intact brain tissue and the area of infarction (adhering on the corpus callosum), and in other sections within the infarct cavity. The migratory pattern did not differ between the 2-week and 6-week postgraft groups.

The Phenotypes of Transplanted MSCs

Human MSCs gave rise to neuronal cells expressing neuronal, astrocyte, and oligodendrocyte markers after transplantation into the brain. Figure 5 shows that GFP-positive hMSC (Fig. 5A) were observed at the site of transplantation and along the two streams of migra-

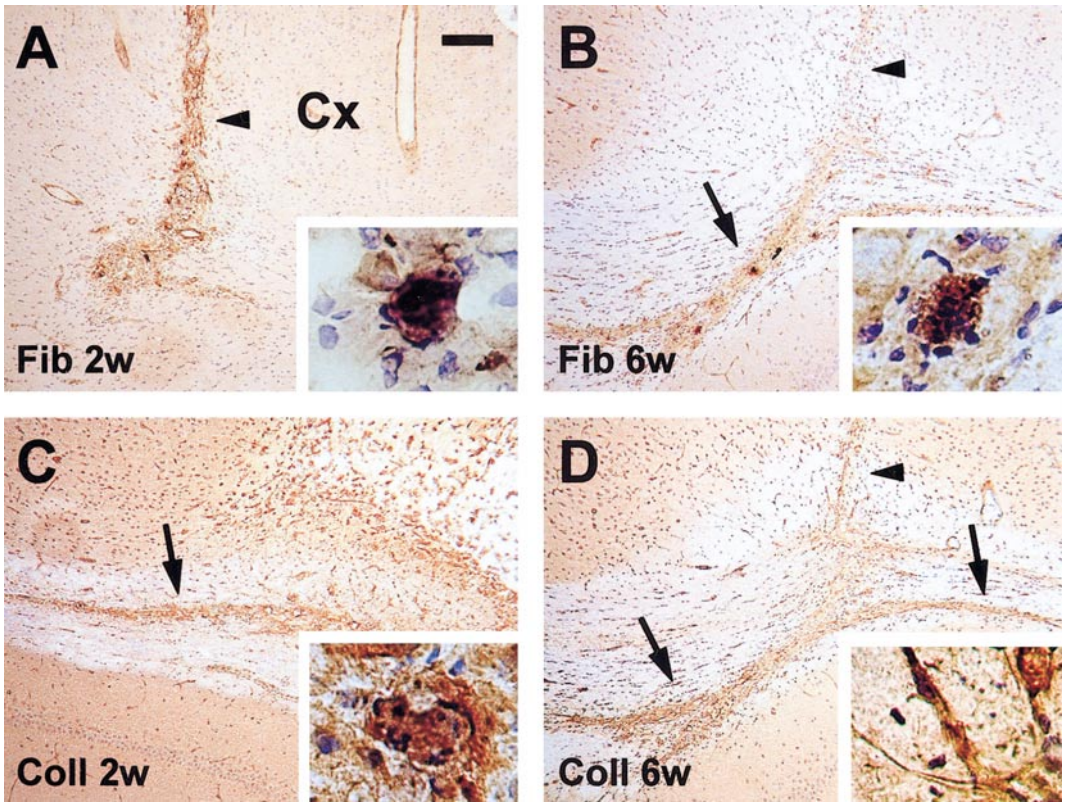

FIG. 6. Transplanted human mesenchymal stem cells express antigens for fibronectin and collagen type I. Human MSCs were implanted into the cortex surrounding the infarction 1 week after cortical ischemia. Within the grafts hMSCs were positively stained with fibronectin (A, B) and collage I (C, D) at 2 weeks (A, C) and 6 weeks (B, D) after transplantation. The top right corner of C is the infarct area. Insets in A–D are the higher magnifications of panels A–D. Arrows indicate migratory pathways of grafted MSCs and the arrowhead shows the site of the grafting. Scale bar (show in A): A–D, 200 μm. Cx, cortex.

tion 2 weeks postgrafting. Transplanted MSCs were immunopositive for the astrocyte marker GFAP (Fig. 5B), the oligodendrocyte marker GalC (Fig. 5C), and the neuronal markers tubulin β III (Fig. 5D), neurofilament −200 (Fig. 5E), and neurofilament-160 (Fig. 5F). These cells were found either at the site of grafting or along the migration pathways. Similar immunohistological features were also observed 6 weeks after transplantation (data not shown). Although transplanted MSCs exhibited the phenotypes of neuronal cells, the morphology of these cells at 2 and 6 weeks postgrafting was immature in nature, and rounded in shape with few fiber processes.

In addition to identifying the grafted cells with an antibody for GFP, antibodies that specifically label human cells were utilized. Some of the transplanted hMSCs were immunoreactive for human neuronal specific enolase (hNSE, Fig. 5G) and human neurofilament-70 (hNF-70, Fig. 5H). Positive staining for human nestin, a human neural stem cell marker, was also observed in the transplants (Fig. 5I). Fibronectin- and collagen type-I-positive hMSCs were also found in the grafts at 2 weeks (Figs. 6A and 6C) and 6 weeks (Figs. 6B and 6D) after transplantation, suggesting that a subset of the hMSCs maintained a primitive stem cell phenotype (42, 44).

DISCUSSIONS

The main findings of the present study are that transplanted human MSCs survived and expressed markers of neuroectodermal cells in the rodent brain, and that ischemic rats showed improved neurological function after transplantation. By 2 weeks after grafting, human MSCs expressed markers for neurons, astrocytes, and oligodendroglia. Previous *in vivo* studies have examined the differentiation of murine MSC. Transplantation of murine MSCs gave rise to neurons (8, 29, 35) and astrocytes (29). Two recent reports have shown that human MSCs could differentiate *in vitro* into neurons (49, 57) and astrocytes (49). The present study confirms these *in vitro* findings and extends them *in vivo* with the phenotypic expression of human MSCs into cells with neuroectodermal characteristics when implanted into the rodent brain.

The morphology of the grafted hMSCs in this study after 2 weeks appeared quite primitive in that they were spherical in shape with few processes extending

from the soma. These morphological features were maintained 6 weeks after transplantation. In contrast, a study that purified murine MSCs labeled with BrdU and then transplanted into lateral ventricle of neonatal mice (29) showed donor-derived mature astrocytes in host brain 12 days postgrafting. The differences between the morphological features in the grafted cells of the present study and those of Kopen *et al.* (29) may be due to differences in the donor species, since it has been demonstrated that differentiation of human fetal brain cells is significantly slower after transplantation in comparison to fetal rodent brain cells (9, 45). Another difference is that the transplant recipients used in the present study were adult rats while those used by Kopen *et al.* (29) were neonatal mice. As a consequence, environmental differences between the adult and neonatal brain may also contribute to differences in the speed of differentiation of the grafted cells.

The prolonged maturation of the transplanted hMSCs noted in this study may also be due to the adoption of an ectodermal fate. Turning bone marrow into neural cells requires transcending the barrier between mesoderm and ectoderm. Thus fate determination, differentiation, and maturation may involve more complex processes and additional steps than the differentiation of MSCs into mesodermal cells. In transplanting *human* neural progenitor cells into the brain of adult rats, Fricker *et al.* (15) and Ostenfeld *et al.* (40) have demonstrated that graft-derived mature neurons are observed 6 weeks after implantation. Whereas our data showed that *human* MSC-derived neural progeny still maintained an immature morphology at the same period (6 weeks) after grafting. A study conducted by Bjornson *et al.* (6) showed that when both neural stem cells (NSCs) and bone marrow (BM) cells were injected into adult irradiated mice, the time required for NSCs to turn into blood cells was much longer than that required by bone marrow. The authors postulated that "NSCs undergo additional steps of fate determination, differentiation, and maturation with respect to BM cells to produce hematopoietic progeny." Accordingly, the induction of hMSCs to neural cells may also require extended maturation latency when compared with neural progenitor cells.

The fact that functional improvement in the rats that received hMSC transplants was not dependent on establishing new neuronal circuits between grafts and host or dependent on reducing the infarction size, suggests that functional restoration was probably a result of proteins released by grafted hMSCs which promoted brain plasticity in the host brain. Collagen I and fibronectin may be involved in hMSC-induced functional improvement. At 6 weeks postgrafting, the latest time point of the present study, the grafted MSCs still maintained their primitive properties producing collagen I and fibronectin (41, 44). Numerous studies have shown that extracellular matrix (ECM) proteins influence cell proliferation, migration, survival, and differentiation (20, 21, 46, 58). Moreover, cell–ECM attachment is crucial for nerve fiber growth (10, 31). Recently, Yoshii and Oka (59) reported that collagen type-I-derived filaments guided peripheral nerve regeneration. Fibronectin, another important component of the ECM, integrates with the receptors of the integrin family and modulates many basic cellular functions, including cell adhesion, migration, cell growth, differentiation, and programmed cell death (1, 19, 20, 22, 24, 60). Fibronectin stimulates nerve fiber growth in culture (10). Recently fibronectin has been demonstrated to exert a neural protective effect after ischemic injury. Fibronectin knockout mice exhibited a larger infarction size and an increased number of apoptotic cells in the infarct area following brain ischemia (48). In our study, hMSCs continued to express collagen type I and fibronectin 6 weeks after grafting. Therefore, it is possible that both of collagen I and fibronectin may participate in hMSCs survival and differentiation, and may also involve the functional restoration by activating intergrin signal transduction, and reestablishing new neuronal circuits in host brain tissue. Neurotrophic factors could also participate in hMSC-mediated functional improvement. Numerous studies suggest that neurotrophic factors play important roles in brain plasticity. Neurotrophic factors can enhance neuronal sprouting (11), synaptogenesis (18), long-term potentiation (2, 30, 41), and neurotransmission (25) and increase neurotransmitter release (26, 27, 34). Humpel *et al.* reported that human fetal cortical grafts expressed nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 mRNA after transplantation to cortical cavities in rats (23). We speculated that grafted hMSCs, their immature progenies, and/or the host brain cells stimulated by the grafts might produce neurotrophic factors that participate in functional reorganization in the host brain.

It has been reported that transplantation of other cell sources such as rodent embryonic hippocampal formation cells (37, 39), MHP36, a conditionally immortal neuroepithelial stem cell line derived from embryonic mouse (53), and human neuroteratocarcinoma-derived neurons (7, 50, 28) ameliorated neurological deficits induced by brain ischemia. Here we demonstrated that *human* bone marrow mesenchymal stem cells could engraft and reduce neurological deficits in a rat model of stroke. Compared with other cell sources, hMSCs have the advantages that they can be readily harvested and expanded *ex vivo.* These cells can thus provide an unlimited source of autologous cells for transplantation in conditions of stroke, head injury, and neurodegeneration without ethical concerts and immune rejection considerations. However, the biological mechanisms involved in the reparative process mediating functional improvement as a result of stem

cell transplantation should be clarified prior to the initiation of clinical studies (5).

In summary, hMSCs adopted ectodermal cell fates expressing neural cell phenotype antigens when implanted to the brain. Transplanted hMSCs ameliorated neurological deficits after experimental stroke in rats. The mechanisms underlying the functional recovery remain to be further investigated.

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REFERENCES

1. Almeida, E. A., D. Ilic, Q. Han, C. R. Hauck, F. Jin, H. Kawakatsu, D. D. Schlaepfer, and C. H. Damsky. 2000. Matrix survival signaling: From fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase. *J. Cell Biol.* **149:** 741–754.
2. Arvanov, V. L., B. S. Seebach, and L. M. Mendell. 2000. NT-3 evokes an LTP-like facilitation of AMPA/kainate receptor-mediated synaptic transmission in the neonatal rat spinal cord. *J. Neurophysiol.* **84:** 752–728.
3. Azizi, S. A., D. Stokes, B. J. Augelli, C. DiGirolamo, and D. J. Prockop. 1998. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats—Similarities to astrocyte grafts. *Proc. Natl. Acad. Sci. USA* **95:** 3908–3913.
4. Beresford, J. N., J. H. Bennett, C. Devlin, P. S. Leboy, and M. E. Owen. 1992. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. *J. Cell Sci.* **102:** 341–351.
5. Bjorklund, A., and O. Lindvall. 2000. Cell replacement therapies for central nervous system disorders. *Nat. Neurosci.* **6:** 537–544.
6. Bjornson, C. R., R. L. Rietze, B. A. Reynolds, M. C. Magli, and A. L. Vescovi. 1999. Turning brain into blood: A hematopoietic fate adopted by adult neural stem cells *in vivo. Science* **283:** 534–537.
7. Borlongan, C. V., Y. Tajima, J. Q. Trojanowski, V. M. Lee, and P. R. Sanberg. 1998. Transplantation of cryopreserved human embryonal carcinoma-derived neurons (NT2N cells) promotes functional recovery in ischemic rats. *Exp. Neurol.* **149:** 310–321.
8. Brazelton, T. R., F. M. Rossi, G. I. Keshet, and H. M. Blau. 2000. From marrow to brain: Expression of neuronal phenotypes in adult mice. *Science* **290:** 1775–1779.
9. Brundin, P., O. G. Nilsson, R. E. Strecker, O. Lindvall, B. Astedt, and A. Bjorklund. 1986. Behavioural effects of human fetal dopamine neurons grafted in a rat model of Parkinson's disease. *Exp. Brain Res.* **65:** 235–240.
10. Carbonetto, S. T., and M. M. Gruver. 1982. Turner DC nerve fiber growth on defined hydrogel substrates. *Science* **216:** 897–899.
11. Cohen-Cory, S., and S. E. Fraser. 1995. Effects of brain-derived neurotrophic factor on optic axon branching and remodelling *in vivo. Nature* **378:** 192–196.
12. Coyle, P. 1982. Middle cerebral artery occlusion in the young rat. *Stroke* **6:** 855–859.
13. Dennis, J. E., S. E. Haynesworth, R. G. Young, and A. I. Caplan. 1992. Osteogenesis in marrow-derived mesenchymal cell porous ceramic composites transplanted subcutaneously: Effect of fibronectin and laminin on cell retention and rate of osteogenic expression. *Cell Transplant* **1:** 23–32.
14. Eglitis, M. A., and E. Mezey. 1997. Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc. Natl. Acad. Sci. USA* **8:** 4080–4085.
15. Fricker, R. A., M. K. Carpenter, C. Winkler, C. Greco, M. A. Gates, and A. Bjorklund. 1999. Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. *J. Neurosci.* **14:** 5990–6005.
16. Gage, F. H. 2000. Mammalian neural stem cells. *Science* **287:** 1433–1438.
17. Galli, R., U. Borello, A. Gritti, M. G. Minasi, C. Bjornson, M. Coletta, M. Mora, M. G. De Angelis, R. Fiocco, G. Cossu, and A. L. Vescovi. 2000. Skeletal myogenic potential of human and mouse neural stem cells. *Nat. Neurosci.* **10:** 986–991.
18. Garofalo, L., A. Ribeiro-da-Silva, and A. C. Cuello. 1992. Nerve growth factor-induced synaptogenesis and hypertrophy of cortical cholinergic terminals. *Proc. Natl. Acad. Sci. USA* **89:** 2639–2643.
19. George, E. L., E. N. Georges-Labouesse, R. S. Patel-King, H. Rayburn, and R. O. Hynes. 1993. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* **4:** 1079–1091.
20. Giancotti, F. G., and E. Ruoslahti. 1999. Integrin signaling. *Science* **285:** 1028–1032.
21. Gospodarowicz, D., and I. Vlodavsky. 1982. The role of the extracellular matrix and growth factors in the control of proliferation of anchorage-dependent cells. *Prog. Cancer Res. Ther.* **23:** 73–104.
22. Hedin, U., B. A. Bottger, J. Luthman, S. Johansson, and J. Thyberg. 1989. A substrate of the cell-attachment sequence of fibronectin (Arg-Gly-Asp-Ser) is sufficient to promote transition of arterial smooth muscle cells from a contractile to a synthetic phenotype. *Dev. Biol.* **2:** 489–501.
23. Humpel, C., I. Stromberg, and L. Olson. 1995. Expression of nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 mRNAs in human cortical xenografts. *J. Neural Transplant Plast.* **5:** 257–264.
24. Hynes, R. O. 1992. Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **1:** 11–25.
25. Kang, H., and E. M. Schuman. 1995. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science* **267:** 1658–1662.
26. Knipper, M., M. da Penha Berzaghi, A. Blochl, H. Breer, H. Thoenen, and D. Lindholm. 1994a. Positive feedback between acetylcholine and the neurotrophins nerve growth factor and brain-derived neurotrophic factor in the rat hippocampus. *Eur. J. Neurosci.* **6:** 668–671.
27. Knipper, M., L. S. Leung, D. Zhao, and R. J. Rylett. 1994b. Short-term modulation of glutamatergic synapses in adult rat hippocampus by NGF. *Neuroreport* **5:** 2433–2436.
28. Kondziolka, D., L. Wechsler, S. Goldstein, C. Meltzer, K. R. Thulborn, J. Gebel, P. Jannetta, S. DeCesare, E. M. Elder, M. McGrogan, M. A. Reitman, and L. Bynum. 2000. Transplantation of cultured human neuronal cells for patients with stroke. *Neurology* **4:** 565–569.
29. Kopen, G. C., D. J. Prockop, and D. G. Phinney. 1999. Marrow stromal cells migrate throughout forebrain and cerebellum, and

they differentiate into astrocytes after injection into neonatal mouse brains. *Proc. Natl. Acad. Sci. USA* **19:** 10,711–10,716.

30. Korte, M., P. Carroll, E. Wolf, G. Brem, H. Thoenen, and T. Bonhoeffer. 1995. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. USA* **92:** 8856–8860.
31. Letourneau, P. C., M. L. Condic, and D. M. Snow. 1994. Interactions of developing neurons with the extracellular matrix. *J. Neurosci.* **3:** 915–928.
32. Li, Y., M. Chopp, J. Chen, L. Wang, S. C. Gautam, Y. X. Xu, and Z. Zhang. 2000. Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after stroke in adult mice. *J. Cereb. Blood Flow Metab.* **9:** 1311–1319.
33. Liechty, K. W., T. C. MacKenzie, A. F. Shaaban, A. Radu, A. M. Moseley, R. Deans, D. R. Marshak, and A. W. Flake. 2000. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. *Nat. Med.* **6:** 1282–1286.
34. Lindholm, D. 1997. Neurotrophic factors and neuronal plasticity: Is there a link? *Brain Plast. Adv. Neurol.* **73:** 1–6.
35. Mezey, E., K. J. Chandross, G. Harta, R. A. Maki, and S. R. McKercher. 2000. Turning blood into brain: Cells bearing neuronal antigens generated *in vivo* from bone marrow. *Science* **290:** 1779–1782.
36. Morrison, S. J., N. Uchida, and I. L. Weissman. 1995. The biology of hematopoietic stem cells. *Annu. Rev. Cell Dev. Biol.* **11:** 35–71.
37. Netto, C. A., H. Hodges, J. D. Sinden, E. LePeillet, T. Kershaw, P. Sowinski, B. S. Meldrum, and J. A. Gray. 1993. Foetal grafts from hippocampal regio superior alleviate ischaemic-induced behavioural deficits. *Behav. Brain Res.* **58:** 107–112.
38. Ohlsson, A. L., and B. B. Johansson. 1995. Environment influences functional outcome of cerebral infarction in rats. *Stroke* **4:** 644–649.
39. Onifer, S. M., and W. C. Low. 1990. Spatial memory deficit resulting from ischemia-induced damage to the hippocampus is ameliorated by intra-hippocampal transplants of fetal hippocampal neurons. *Prog. Brain Res.* **82:** 359–366.
40. Ostenfeld, T., M. A. Caldwell, K. R. Prowse, M. H. Linskens, E. Jauniaux, and C. N. Svendsen. 2000. Human neural precursor cells express low levels of telomerase *in vitro* and show diminishing cell proliferation with extensive axonal outgrowth following transplantation. *Exp. Neurol.* **1:** 215–226.
41. Patterson, S. L., T. Abel, T. A. Deuel, K. C. Martin, J. C. Rose, and E. R. Kandel. 1996. Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* **16:** 1137–1145.
42. Pereira, R. F., K. W. Halford, M. D. O'Hara, D. B. Leeper, B. P. Sokolov, M. D. Pollard, O. Bagasra, and D. J. Prockop. 1995. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone,cartilage, and lung in irradiated mice. *Proc. Natl. Acad. Sci. USA* **11:** 4857–4861.
43. Poungvarin, N. 1998. Stroke in the developing world. *Lancet* **352:** 19–22.
44. Prockop, D. J. 1997. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276:** 71–74.
45. Pundt, L. L., T. Kondoh, J. A. Conrad, and W. C. Low. 1996. Transplantation of human fetal striatum into a rodent model of Huntington's disease ameliorates locomotor deficits. *Neurosci. Res.* **4:** 415–420.
46. Reddi, A. H. 1980. *Biochemistry of Collagen* (G. N. Ramachandran and A. H. Reddi, Eds.), pp. 449–478. Plenum Press, New York and London.
47. Rosenberg, C. H., and G. M. Popelka. 2000. Post-stroke rehabilitation. A review of the guidelines for patient management. *Geriatrics* **9:** 75–81.
48. Sakai, T., K. J. Johnson, M. Murozono, K. Sakai, M. A. Magnuson, T. Wieloch, T. Cronberg, A. Isshiki, H. P. Erickson, and R. Fassler. 2001. Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. *Nat. Med.* **3:** 324–330.
49. Sanchez-Ramos, J., S. Song, F. Cardozo-Pelaez, C. Hazzi, T. Stedeford, A. Willing, T. B. Freeman, S. Saporta, W. Janssen, N. Patel, D. R. Cooper, and P. R. Sanberg. 2000. Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Exp. Neurol.* **2:** 47–56.
50. Saporta, S., C. V. Borlongan, and P. R. Sanberg. 1999. Neural transplantation of human neuroteratocarcinoma (hNT) neurons into ischemic rats. A quantitative dose–response analysis of cell survival and behavioral recovery. *Neuroscience* **2:** 519–525.
51. Schallert, T., and I. Q. Whishaw. 1984. Bilateral cutaneous stimulation of the somatosensory system in hemidecorticate rats. *Behav. Neurosci.* **98:** 518–540.
52. Schallert, T., T. D. Hernandez, and T. M. Barth. 1986. Recovery of function after brain damage: Severe and chronic disruption by diazepam. *Brain Res.* **379:** 104–111.
53. Sinden, J. D., F. Rashid-Doubell, T. R. Kershaw, A. Nelson, A. Chadwick, P. S. Jat, M. D. Noble, H. Hodges, and J. A. Gray. 1997. Recovery of spatial learning by grafts of a conditionally immortalized hippocampal neuroepithelial cell line into the ischaemia-lesioned hippocampus. *Neuroscience* **3:** 599–608.
54. Van Gijn, J., and M. S. Dennis. 1998. Issues and answers in stroke care. *Lancet* **352:** 23–27.
55. Wakitani, S., T. Saito, and A. I. Caplan. 1995. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve* **12:** 1417–1426.
56. Weissman, I. L. 2000. Translating stem and progenitor cell biology to the clinic: Barriers and opportunities. *Science* **287:** 1442–1446.
57. Woodbury, D., E. J. Schwarz, D. J. Prockop, and I. B. Black. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. *J. Neurosci. Res.* **4:** 364–370.
58. Yamada, K. M., S. Aota, S. K. Akiyama, and S. E. LaFlamme. 1992. Mechanisms of fibronectin and integrin function during cell adhesion and migration. *Cold Spring Harbor Symp. Quant. Biol.* **57:** 203–212.
59. Yoshii, S., and M. Oka. 2001. Peripheral nerve regeneration along collagen filaments. *Brain Res.* **1:** 158–162.
60. Zhang, Z., K. Vuori, J. C. Reed, and E. Ruoslahti. 1995. The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc. Natl. Acad. Sci. USA* **13:** 6161–6165.