Background and Purpose—Hypoxic–ischemic (HI) brain injury in newborn infants represents a major cause of cerebral palsy, cognitive impairment, learning disability, and epilepsy. Stem cell-based therapy has the potential to rescue and replace the ischemic tissue caused by HI and to restore function. However, the mechanisms by which stem cell transplants induce functional recovery are yet to be elucidated. In the present study, we sought to investigate the efficacy of human neural stem cells derived from human embryonic stem cells in a rat model of neonatal HI and the mechanisms enhancing brain repair.

Methods—The human neural stem cells were genetically engineered for in vivo molecular imaging and for postmortem histological tracking. Twenty-four hours after the induction of HI, animals were grafted with human neural stem cells into the forebrain. Motor behavioral tests were performed the fourth week after transplantation. We used immunocytochemistry and neuroanatomical tracing to analyze neural differentiation, axonal sprouting, and microglia response. Treatment-induced changes in gene expression were investigated by microarray and quantitative polymerase chain reaction.

Results—Bioluminescence imaging permitted real time longitudinal tracking of grafted human neural stem cells. HI transplanted animals significantly improved in their use of the contralateral impeded forelimb and in the Rotorod test. The grafts showed good survival, dispersion, and differentiation. We observed an increase of uniformly distributed microglia cells in the grafted side. Anterograde neuroanatomical tracing demonstrated significant contralesional sprouting. Microarray analysis revealed upregulation of genes involved in neurogenesis, gliogenesis, and neurotrophic support.

Conclusions—These results suggest that human neural stem cell transplants enhance endogenous brain repair through multiple modalities in response to HI. (Stroke. 2010;41:516-523.)

Key Words: axonal sprouting ■ cell therapy ■ hypoxia ischemia ■ microglia ■ neural stem cells

Hypoxic–ischemic (HI) brain injury causes brain damage in the fetus and newborn infants and represents a major cause of cerebral palsy, cognitive impairment, learning disability, and epilepsy.1 Although mild body hypothermia has been shown to improve the outcome after neonatal HI encephalopathy when initiated within 6 hours of birth,2,3 there are no other effective interventions to improve the chronic sequelae of perinatal asphyxia. Neural stem cell-based therapy offers the prospect to rescue damaged tissue, to replace lost cells, and to restore neurological function after cerebral HI.

Early imaging studies in patients with stroke4–7 and microstimulation in experimental models of stroke8–10 reported that in response to ischemic injury, the brain undergoes limited compensatory changes in an effort to recover from structural and functional loss.11 These changes or neuroplasticity are most prominent in early weeks and include axonal, dendritic, and synaptic changes; inflammatory and immune adaptation; neurogenesis; gliogenesis; and angiogenesis.12 Axonal sprouting and reorganization manifest by changes in dendritic arborization, spine remodeling, branching into the denervated areas, and de novo formation of novel projections.13–17 Although limited, this spontaneous plasticity-mediated recovery is a promising target for drug and cell therapeutic interventions.17–21

In parallel to endogenous central nervous system plasticity, inflammation and immune components are markedly activated de novo in the neonatal brain and peripheral organs after HI.22,23 Noteworthy, this inflammatory response to ischemic injury may exert neuroprotective and regenerative effects on the central nervous system.24,25

In the present study, we evaluated functional recovery after transplantation of multipotent human embryonic stem cell-
derived human neural stem cells (hNSCs) into the rat model of neonatal HI. We also investigated modalities by which grafted hNSCs provide therapeutic benefits to the HI-damaged brain.

Materials and Methods

Derivation of Multipotent hNSCs

Human neural stem cells were derived from human embryonic stem cells and perpetuated as previously described.26,27

Induction of HI and Cell Transplantation

All animal experimentations were conducted according to the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee. Seven-day-old Sprague-Dawley rats (Charles River Laboratories; Wilmington, Mass) were subjected to permanent ligation of the left carotid artery followed by 90 minutes in a hypoxic chamber (8% O2 and 92% N2, at 37°C).28 The newborns were divided into HI vehicle (total n=12) and HI transplant (total n=12) groups. Twenty-four hours after the induction of HI, animals were placed in a stereotaxic apparatus with neonatal rat adaptor (Kopf Instruments). The skull bregma was determined and a single cell suspension (50 000 cell/μL) of hNSCs (passages P9 to P15) was transplanted, using a Hamilton syringe, into 3 sites (2 μL/site) in the left ischemic hemisphere at the following stereotaxic coordinates in millimeters: anteroposterior: +0.0, mediolateral: +3.0, dorsoventral: −4.0; anteroposterior: +0.5, mediolateral: +2.5, dorsoventral: −3.5; anteroposterior: −1.0, mediolateral: +2.0, dorsoventral: −4.0. The injection rate was 1 μL/min, and the cannula was left in place for 5 minutes before retraction.

Bioluminescence Imaging of Grafted hNSCs

In Vivo

Bioluminescence imaging was performed using the Xenogen in vivo imaging system. Due to space limitation, we refer the reader to our recent publication27 where we described in detail the bioluminescence imaging technique.

Behavioral Tests

The animals were evaluated 4 weeks after transplantation for their sensorimotor skills in the cylinder and in the Rotorod tests, as we previously reported.26,29

Transcriptome Analysis

Total RNA was isolated from the ipsilateral hemisphere of normal, HI, and HI transplant groups (n=3) using the RNAeasy kit (Qiagen) according to the manufacturer’s instructions. The Oligo GEArray neurogenesis and neural stem cell microarrays were purchased from SuperArray (SA Biosciences Corporation). The protocol for the microarray analyses followed the manufacturer’s recommendations. Array spot density and differential probe expression were calculated using SuperArray’s GEArray Expression Analysis Suite software. Spot density was normalized to select positive and negative controls spotted onto each array.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from tissue dissected from the ipsilateral hemisphere of vehicle and hNSC-treated animals (n=3). Aliquots (1 μg) of total RNA from the cells were reverse transcribed as previously described.30 Real-time polymerase chain reaction was performed using Maxima SYBR Green qPCR Master Mix (Fermentas) in the Stratagene thermocycler MX3000P (Stratagene) according to the supplier’s instructions. Data were expressed as mean±SEM. The ΔCt for each candidate was calculated as ∆Ct of [Ct (gene of interest)−Ct (18S)] and the ∆∆Ct was the difference between the Ct of the treated sample (transplanted animals) and the Ct of the control vehicle sample. The relative expression was calculated as the 2^ΔΔCt according to the methods31 and plotted as relative levels of gene expression. The rat primers were designed using the Primer3 software and are available on request.

Figure 1. Imaging of human embryonic stem cell-derived hNSCs in HI neonates. A, Neural stem cells were isolated through a multistep process previously described.26,27 Flow cytometry analysis demonstrated the purity of the eGFP-expressing hNSCs. B, In vitro imaging analysis of genetically engineered hNSCs show increasing fLuc activity with cell density and a linear correlation (R2=0.98). C, Data are representative of 3 independent experiments performed in triplicate. Representative bioluminescence imaging of a neonate rat transplanted with the hNSCs and monitored for 4 weeks. D, Color scale bar is in photon/s/cm2/sr.
Biotinylated Dextran Amine Injections
The last week before euthanasia of the animals, 3 randomly selected animals from both animal groups were anesthetized and placed in the stereotaxic apparatus. After craniotomy, 0.5 mL of biotinylated dextran amine (BDA; 10 000 molecular weight; Molecular Probes; 10% wt/vol solution in sterile phosphate-buffered saline) was injected stereotaxically into the sensorimotor cortex opposite to the HI lesion site at the stereotaxic coordinates: anteroposterior: +0.5 mm, mediolateral: 2.5 mm, and DV: −1.5 mm. The scalp was then closed and the animal returned to its cage.

Histopathology, Immunocytochemistry, and Microscopical Analysis
Immunocytochemistry was performed as we previously reported.26 The following primary antibodies were used: antihuman Nuclei (hNuc), anti-NeuN, anti-GAD65/67, antiglial fibrillary acidic protein, antigalactocerebroside, anti-Nestin (Chemicon), anti-TuJ1 (Covance), anti-CNPyse (Aves Labs), and antidualleocortin (SantaCruz Biotechnology). Secondary antibodies raised in the appropriate hosts and conjugated to fluorescein isothiocyanate, RITC, AMCA, CY3, or CY5 chromogenes (Jackson ImmunoResearch) were used. Cells and sections were counterstained with the nuclear marker 4',6-diamidine-2'-phenylindole dihydrochloride. Fluorescence was detected, analyzed, and photographed with a Zeiss LSM550 laser scanning confocal photomicroscope (Carl Zeiss).

For the rat, anti-Iba-1 (ionized calcium binding adapter molecule; Wako Bioproducts) and BDA immunohistochemistry was carried out, as we previously described.29 For each animal, quantitative estimates of the number of Iba-1+ cells were stereologically determined using the optical fractionator procedure,23 as we previously described in detail.29 The quantitative analysis of BDA-labeled fibers was performed, as we previously reported.29 The infarct

Figure 2. Characterization of hNSC grafts. A, Example of triple labeling of transplanted animal with the human nuclear specific marker hNuc (purple) and the neuronal markers doublecortin (red) and TuJ1 (green). B, Higher magnification shows the dispersion of the grafted hNuc+ (purple) hNSCs in the striatum. Photomicrographs show coexpression of hNuc (purple) and the neuronal markers: TuJ1 (green; C), doublecortin (green; D), and NeuN (green; E). F, Colocalization of hNuc and the astrogial marker glial fibrillary acidic protein, and (G) example of grafted cell expressing the GABAergic neurotransmitter marker GAD. Bars: (A–B) 100 μm; (B, far right, E) 20 μm; (C, D, F, G) 10 μm.
volume was estimated by summing up the infarcted areas in all animals of each group. To eliminate the effects of edema, infarct size was calculated as the contralateral hemisphere–ipsilateral nonischemic hemisphere/contralateral hemisphere and expressed as percentage (% of infarcted hemisphere.

Statistics
Outcome measurement for each experiment was reported as mean±SEM. Data were analyzed using SPSS 11 for Mac OS X (SPSS Inc). Significance of intergroup differences was performed by applying Student t test where appropriate. One-way analysis of variance was used to compare groups. Differences between the groups were determined using Bonferroni post hoc test. A probability value of <0.05 was considered statistically significant.

Results
Real-Time Imaging of hNSC Transplants
The hNSCs grew as an adherent monolayer culture and all progeny expressed double fusion construct carrying fLuc and eGFP reporter genes (Figure 1A).
Image analysis (Figure 1B) demonstrated a linear correlation between plating density and the fLuc activity (Figure 1C). This stable and efficient expression of fLuc gene enabled us to noninvasively image, in real time, the survival of the grafted hNSCs at different time points (Figure 1D).

Characterization of the HI Infarct Size and the hNSC Graft
Microscopic examination of cresyl violet-stained sections showed significant loss of brain tissue in HI animals in the neocortex, the striatum, and extending caudally to the hippocampus. This atrophy was accompanied sometimes by the formation of a porencephalic cyst. The estimation of the infracted region revealed 17.8%±8.6% of the hemisphere (n=6) for the transplanted group and 20.66%±12.5% (n=6) for the vehicle group. Although hNSC-grafted animals showed smaller infarct volume, the difference from the vehicle group was not significant (n=6). Grafted hNSCs identified with hNuc demonstrated good survival dispersion and integration into the HI-damaged tissue (Figure 2A–B). Transplanted cells expressed the neuroepithelial stem cell marker nestin in 12.8%±3.4% and differentiated into neurons expressing NeuN, TuJ1+ (42.9%±3.5%), doublecortin (4.8%±1.1%; Figure 2C–E), and the GABAergic marker GAD (47.6%±4.3%; Figure 2G). Grafted cells also differentiated into glial fibrillary acidic protein-expressing astrocytes (Figure 2F).

hNSC Grafts Enhance Axonal Sprouting
To investigate whether the transplanted hNSCs influenced the rewiring of the HI-damaged tissue, we used BDA to anterograde label the sensorimotor cortical projection originating from the contralateral side. We measured the density of BDA+ crossing fibers in the corpus callosum and of BDA+ terminals in the ipsilateral sensorimotor cortex, the striatum, and thalamic nuclei. The quantitative analysis of BDA-labeled fibers and terminals, normalized to the total number of labeled somas at the injections site, revealed an increase in both the number of fibers crossing toward the ipsilateral hemisphere (Figure 3B) and in the number of terminals in the ipsilateral sensorimotor cortex (Figure 3A), the striatum (Figure 3C), and thalamic nuclei (Figure 3D).

Transcriptome Analysis
Gene microarray analysis revealed, in comparison to the vehicle group, a significant increase in central nervous system rat endogenous genes involved: (1) in neurogenesis and cell migration (doublecortin, chemokine [C-X-C motif] receptor 4 [CXCR4], oligodendrocyte lineage transcription factor 2, and FGF2); (2) in myelination (myelin basic protein); and (3) in genes involved in cell survival and neurite outgrowth (glial derived neurotrophic factor, Neurturin, and insulin growth factor-1; Figure 4A). Subsequently, we confirmed the microarray expression patterns of selected genes using real-time quantitative reverse transcribed–polymerase chain reaction (Figure 4B).

Grafts of hNSCs Modulate Microglial Presence in the HI-Damaged Area
Immunostaining with the pan-microglial marker Iba1 demonstrated that microglia were homogenously distributed throughout the brain in both vehicle and hNSC-grafted animals without adverse infiltration or reaction against the grafts (Figure 5A–B). Stereological quantitative analysis of the Iba1-positive cells demonstrated a significant increase in the transplanted striatum (Figure 5D).

Improvement of Motor Behavior in Rats That Received the hNSC Grafts
To determine the ability of hNSCs to functionally engraft, 1 month after transplantation, the sensorimotor skills of animals were evaluated using 2 neurobehavioral tests. Our results showed that during the fourth week, HI transplanted animals significantly improved in their use of the contralateral impeded forelimb (Figure 6A–B). The hNSC grafts significantly ameliorated the locomotor deficits in the Rotorod test (Figure 6C).

Discussion
Genetically engineered hNSCs were efficiently and noninvasively imaged in real time after transplantation into the HI model of newborn rats and up to 5 weeks of age. We report that hNSCs engrafted into the ischemic brain enhanced axonal sprouting and the expression of genes involved in neurogenesis, gliogenesis, and neurotrophic support; modulated microglial response; and improved motor function of the animals.
It is generally believed that transplanted nonneural cells such as those derived from bone marrow or cord blood exert neurotrophic effects on ischemia-injured tissue and may not survive long term, whereas neural stem cells are thought to provide cell replacement and neurotrophic support. This neurotrophic support may be responsible for the significant axonal sprouting that we measured in the cortex, the striatum, and the thalamus. The number of BDA-labeled axon

Figure 5. Stereological analysis of Iba1-expressing microglia in vehicle and transplanted groups. Brain sections from vehicle (A) and hNSC-grafted (B) animals were processed for the pan-microglial marker Iba1 immunohistochemistry. Stereological quantification of the Iba1+ cells in the cortex (C) and striatum (D) of grafted animals. Tx indicates transplant. Bars: (A, B) 20 μm.

Figure 6. Transplantation of hNSCs improves sensorimotor function. The independent use of the impaired contralateral forelimb significantly increased in the transplant group (B; n=3) on Days 28 and 30 posttransplantation (*P<0.05 versus vehicle group. A, Bars represent percentages±SEM of steps taken by the ipsilateral, contralateral, and both forelimbs simultaneously. On Days 29 and 30, the transplant group (n=9) showed significant improvement (*P<0.05) on the Rotarod test.
terminals may vary with the size of the injection site and the number of BDA+ cell bodies. The BDA infusion led to small (3 to 5 mm²) and circumscribed injection sites in the sensorimotor cortex. There was no significant difference in the size of the injection site and the total number of labeled cells between vehicle and transplanted groups. In addition, the data were normalized to the total number of labeled cells. Thus, it is unlikely that the increase of axonal sprouting we observed is due to differences in the size or location of the injection site.

After ischemia, regions of the contralateral hemispheres become activated during the early phase of partial regain of function or spontaneous recovery in experimental models and in patients with stroke. In neonate HI rats, contralateral sprouting could give rise to alternative motor descending pathways from the motor cortex relaying in subcortical structures such as the red nucleus and the pontine formation or direct corticospinal projections may be formed to compensate for functional loss. The newly formed pathways could be generated by multiple mechanisms, including sprouting from the surviving neurons, unmasking of existing pathways that are functionally inactive, or the compensatory descending control channels through alternative functionally active but redundant pathways.

The intracerebral or intravenous delivery of multipotent adult progenitor cells provided protection to HI-damaged tissue in neonates and improved motor and neurological scores compared with the vehicle group. Using retrograde and anterograde neuroanatomical tracking, Park et al demonstrated that neural stem cells grafted into HI-lesioned neonates, along with a polyglycolic acid-based biodegradable polymer scaffold, re-established long-distance cross-callosal neuronal connections. Recent genomic analysis studies demonstrated that in addition to these growth factors, axonal sprouting is activity-dependent in constraint-induced movement therapy. Together, these data suggest that grafted hNSCs could exert neurotrophic and/or activity-mediated effects on a HI-damaged local network and enhance axonal sprouting. Based on these observations, it seems reasonable to propose that the newly innervated and recruited area of the contralateral hemisphere is becoming part of a reorganized network promoting motor recovery.

Our data are in line with previous studies that show an increase in the resident microglia/macrophage cells (CD11b) in ischemic animals that received neurosphere-derived cells. Capone et al suggest that microglia activation is required for neurosphere graft neuroprotective action through secretion of growth factors, including insulin growth factor-1, vascular endothelial growth factor-A, transforming growth factor-β1, and brain-derived neurotrophic factor. Indeed, brain-derived neurotrophic factor treatment of HI neonates improves spatial memory and selective ablation of microglia through the mutant thymidine kinase gene driven by myeloid-specific CD11b promoter exacerbated ischemic injury. Thus, these findings support the notion that microglia play a dual role, proinflammatory or anti-inflammatory/neurotrophic, depending on their state of activation and functional phenotype.

In conclusion, we provide evidence that growth factor-isolated and perpetuated hNSCs from human embryonic stem cells are amenable to genetic modification for real time in vivo imaging and potentially for other therapeutic genes. We showed that these hNSCs are able to modify the host microenvironment and enhance neuroanatomical plasticity after HI in neonates and improve sensorimotor skills.

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Disclosures
None.

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