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Induction of Neurotrophin Expression Via Human Adult Mesenchymal Stem Cells: Implication for Cell Therapy in Neurodegenerative Diseases

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In animal models of neurological disorders for cerebral ischemia, Parkinson's disease, and spinal cord lesions, transplantation of mesenchymal stem cells (MSCs) has been reported to improve functional outcome. Three mechanisms have been suggested for the effects of the MSCs: transdifferentiation of the grafted cells with replacement of degenerating neural cells, cell fusion, and neuroprotection of the dying cells. Here we demonstrate that a restricted number of cells with differentiated astroglial features can be obtained from human adult MSCs (hMSCs) both in vitro using different induction protocols and in vivo after transplantation into the developing mouse brain. We then examined the in vitro differentiation capacity of the hMSCs in coculture with slices of neonatal brain cortex. In this condition the hMSCs did not show any neuronal transdifferentiation but expressed neurotrophin low-affinity ($NGFR^{p75}$) and high-affinity (trkC) receptors and released nerve growth factor (NGF) and neurotrophin-3 (NT-3). The same neurotrophin's expression was demonstrated 45 days after the intracerebral transplantation of hMSCs into nude mice with surviving astroglial cells. These data further confirm the limited capability of adult hMSC to differentiate into neurons whereas they differentiated in astroglial cells. Moreover, the secretion of neurotrophic factors combined with activation of the specific receptors of transplanted hMSCs demonstrated an alternative mechanism for neuroprotection of degenerating neurons. hMSCs are further defined in their transplantation potential for treating neurological disorders.

Key words: Mesenchymal stem cells; Transplantation; Neurotrophin; Astroglial cells

INTRODUCTION

Brain repair has been a particular focus of attention in stem cell (SC) therapy. The benefits of neural SC transplantation as a means of restoring central nervous system (CNS) diseases or spinal cord injury are quite recognized (1,14,47,56). The use of human embryonic neural SCs raises ethical issues as well as practical problems due to transdifferentiation in the adult environment and the need for immunosuppression after grafting. One advantage of using adult SCs, therefore, is that they can be derived from and be transplanted into the same patient, thereby avoiding potential tissue rejection. The identification of a source of readily accessible neural progenitors that can be obtained from the individual requiring transplantation therapy without permanent damage could provide a great benefit.

An important biological finding from several studies is that SCs have a certain degree of flexibility in their differentiating capacity, resulting in lineage interconversion between precursor cells originating from the same and different germ layer (1,15,16,20). Such plasticity is supported by experiments in which bone marrow-derived SCs can yield not only blood cells but also mus-

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cle cells (mesodermal derivatives) and cells bearing neuronal markers (6,8,34,44,53,57). There is also evidence that mesenchymal stem cells (MSCs) can give rise to Purkinje neurons in the mouse brain through cell fusion (5,59).

The underlying mechanism of the neurological functional efficacy obtained after MSC transplantation remains unclear. One possible mechanism may be related to the production of neurotrophic factors from transplanted MSCs. MSCs secrete trophic factors active in neuroprotection, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), hepatocyte growth factor, and vascular endothelial growth factor (VEGF) (12). It is also reported that MSCs stimulate glial cells to produce neurotrophic factors such as BDNF and NGF (30). In the cerebral cortex, neurons in each layer express different neurotrophins and high-affinity (trk) receptors (26,32,49). More interesting is that the developing cortex uses neurotrophic signals to direct distinct aspects of neuronal growth and differentiation (7,25,35,41). Because of the established importance of neurotrophins in neuronal survival during development and their potential to promote recovery of neurons after injury in the adult nervous system (20,26,57), there has been a great deal of interest in characterizing the intracellular mechanisms by which neurotrophins may suppress neuronal degeneration due to apoptosis (23).

In this study we examined the hMSC potential to exhibit neurogenic and/or neuroprotective properties. Human MSCc were characterized in vitro and in vivo for the expression of neural markers, neurotropin low-affinity ($NGFR^{p75}$), high-affinity (trk) receptors, and neurotrophin production. After plating hMSCs in organotypic cocultures of neonatal brain sections we found the expression of $NGFR^{p75}$, trk receptors, and the release of human neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and NGF, whereas primary cultures of hMSCs showed only the expression of the growth cone associated protein 43 (GAP43). These results were also confirmed after the transplantation of hMSCs into the cortex of neonatal mice, suggesting that hMSCs respond to the neural tissue environment both in vitro and in vivo with neurotrophin expression with both protein release and receptor expression. Furthermore, human MSCs may provide a source of neuroprotective astrocytes for autologous cell transplantation therapies in different diseases, including neurodegenerative, ischemic, and traumatic CNS injuries.

MATERIALS AND METHODS

Isolation and Characterization of hMSC in Culture

Human MSC cultures were established as previously described (39) from the bone marrow of healthy volunteer subjects (30–55 years of age) after informed consent according to the guidelines of the Fondazione IRCCS Ospedale Maggiore of Milan Committee on the Use of Human Subjects in Research. Briefly, bone marrow samples were layered on a Histopaque-1077 gradient (specific gravity 1.077 g/ml; Sigma-Aldrich, St. Louis, MO, USA) and mononuclear cells plated in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose (Biowhittaker, Verviers, Belgium) supplemented with 10% FCS (Sigma-Aldrich), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Sigma-Aldrich) in 25-cm^2 tissue culture flasks (Costar, Cambridge, UK). After 72 h, the nonadherent cells were removed and fresh medium was added. The cultures were reefed weekly, grown until confluence, and expanded for 4–5 passages. Adherent cells were then analyzed by fluorescence-activated cell sorting (FACS) (FACS SCAN flow cytometer, Becton Dickinson). Cells were incubated for 30 min at 4°C with the FITC-conjugated mouse monoclonal antibodies against rat CD34 (clone ICO-115, Santa Cruz), CD45 (clone OX-1), and CD90 (clone OX-7) (Becton Dickinson). FITC-conjugated hamster anti-rat CD29 monoclonal antibody (clone Ha2/5, Becton Dickinson) and rabbit anti-rat c-Kit polyclonal antibody (clone C-19, Santa Cruz) were used. Isotype-identical antibodies served as controls. In experiments of neural differentiation, the hMSCs were harvested and plated on laminin-coated dishes in a B27 Neurobasal medium (Life Technologies) supplemented with 3–10% FBS (Gibco-BRL) and cultured for 3 weeks. For myoendothelial differentiation the hMSCs were cultured in the presence of 10 ng of VEGF (Sigma-Aldrich).

Organotypic Culture

Organotypic brain cultures were prepared from brains of 4-day-old mouse pups (P4) as previously described (43). Brains were collected under sterile conditions and transferred to Gey's balanced salt solution (Gibco, Grand Island, NY, USA) containing glucose (6.4 mg/ml). They were transversely sectioned into 400-μm slices with a Leica Tissue Chopper (Leica). Sections were carefully placed on Millipore Millicell-CM (Millipore Corp., Bedford, MA, USA) porous membranes (0.4 μm) in 35-mm culture wells (Nalgene, Naperville, IL, USA) containing 1 ml of incubation media (DMEM supplemented with 10% FBS, 25 mg/ml D-glucose, and 2 mM glutamine at a final pH of 7.2). Four microliters (20,000 cells) of hMSCs were placed within each organotypic culture brain sections after 24 h of tissue culture. Under these conditions hMSCs remained randomly distributed within the P4 brain sections. Cultures were incubated at 37°C in a 5% CO_2/95% air humidified environment.

Transplantation of hMSCs Into the Neonatal Mouse Brain

Balb/C and nude mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and were used

in two different sets of experiments according to institutional guidelines that are in compliance with national (D.I. No. 116, G.U. suppl. 40, Feb. 18, 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international law and policies (EEC Council Directive 86/609, OJ L358, 1 Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). The mice were kept in specific pathogen-free conditions, and maintained on acidified water and autoclaved food. Trimethoprim (60 mg/ml water) and sulfamethoxazole (300 mg/ml water) (Hoffmann-La Roche Inc., Nutley, NJ, USA) were given twice a week. The injection coordinates were determined in pilot studies by injecting hMSCs into Balb/C and nude neonatal mouse brains and locating the injection position in sectioned brains. The hMSCs were labeled with fluorescent dyes with a PKH26 Red Fluorescent Cell Linker Kit (Sigma Chemical Co., St. Louis, MO, USA) before implantation, as previously reported (33). By using bregma as a landmark, 3 μl of 50,000 hMSCs were slowly injected over a period of 5 min into the lateral right cortex of cryoanesthetized 4-day-old mice by using a stereotactic device. The needle of a 15-μl Hamilton was left in position for approximately 3 min and gradually withdrawn. Fifty Balb/C mice were transplanted with hMSCs and sacrificed at day 30.

Of the nude mice, 30 were harvested either after 7 and 45 days after hMSC transplants. Brain tissues were removed and grossly dissected into four equal sized coronal sections. Specimens were then frozen in liquid nitrogen-cooled isopentane and cut on a cryostat into 7-μm serial sections. Grafted cells were detected by immunofluorescence assays for specific human antigens and the cell nuclei were stained for 15 min at 37°C with 4′,6-diamidino-2-phenylindole (DAPI).

Laser Microdissection Analysis of Human Transplanted hMSCs

The Leica AS LMD (Leica Microsystems, Germany) has been used to perform the isolation of the single cells from brain frozen sections. Sections of 8-μm thickness were prepared from frozen tissue and mounted on glass slides. These sections were stained without fixation by immunohistochemistry for identification of human lamin A/C nuclei. The slides were mounted on the Leica system with the sections downwards (10). Individual cells of the same brain area were pulled from sections by directing the laser through the sections over the selected cell. The RT-PCR and ELISA analyses were performed from 500 microdissected cells expressing the human lamin A/C+ nuclei. The isolation of such cell numbers by laser microdissection was selected after several preliminary experiments of genome or expression profile performed from a range of 20–1500 cells. The instrument works with a laser used to isolate the target after adjusting intensity, aperture, and cutting velocity. The laser was directed along the borders of the positive cells. Using a 20× objective setting was as follows: aperture 17, intensity 47, speed 10, offset −1. The cut area was transferred into a microcentrifuge tube cap placed under the section and collected by centrifugation and resuspended in RLT Buffer (RNeasy Mini Kit, Quiagen, Hilden, Germany) (9). Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions and RT-PCR was performed as reported above.

Immunohistochemistry and Immunofluorescence

Neuronal differentiation was tested using the following specific anti-human antibodies: neurofilament-M protein (1:100; Chemicon), neuronal-specific enolase (NSE) (1:2000; Polysciences), glial fibrillary acidic protein (GFAP) (1:50; Boehringer Mannheim, Indianapolis, IN, USA), β-tubulin type III (TuJ1) (1:100; Sigma), microtubule associated protein (MAP-2ab isoform) (1:200; Sigma), nestin (1:500; Pharmingen), galactocerebroside (Gal-C) (Promega, South San Francisco, CA, USA), trkA, trkC, and $NGFR^{p75}$ (1:500; Promega). For quantitative analysis, after immunostaining, coverslips were counterstained with DAPI and anti-human lamin A/C (Novocastra, 1:200). Immunostaining of MSCs was also performed using monoclonal antibodies against CD31 (1:100; Santa Cruz), VE-Cad (1:100; Santa Cruz), CD34 (1:50; Becton Dickinson), and STRO-1 (1:150; Sigma). Coverslips tested for neuronal markers were fixed in 4% paraformaldehyde (in PBS, pH 7.2) for 30 min, followed by three washes (10 min each) in PBS, pH 7.2. Nonneuronal antibodies were tested on coverslips fixed in ethanol 70% in PBS for 1 min and permeabilized for 5 min with 0.5% Triton X-100 in PBS. After washing with PBS, cells were incubated with appropriate secondary FITC/TRITC-conjugated IgG antibodies (1:100) for 1 h at room temperature and examined by a Leica TCS 4D confocal microscope.

Fluorescent In Situ Hybridization (FISH) on Transplanted Mice

FISH analysis was performed on frozen neonatal mouse brain tissue sections. The slides were first treated for 30 min with Histochoice Tissue Fixative (Sigma-Aldrich) and sections were dehydrated in 70%, 80%, and 95% alcohol. The denaturation was performed at 70°C for 2 min in 70% deionized formamide in 2× SSC and slides dehydrated again at −20°C. The hybridization was performed overnight at 37°C. A Cy3-labeled human Pan Centromeric probe and a FITC-labeled Pan Centromeric probe were used to identify human and mouse cells, respectively. Nuclei were counterstained with DAPI. Positive and negative controls were nontrans-

planted human and mouse brain sections. Slides were observed using a Leica TCS 4D confocal microscope.

GEArray Gene Expression and RT-PCR Analysis of hMSCs

Total RNA was isolated from Balb/C and nude brain sections injected with hMSCs using Trizol reagent (Invitrogen) as described by the manufacturer. Aliquots of total RNA (3 μg) were used to analyze the human neurotrophin and receptor gene expression profile by GEArray technology (Cat. No. HS-018N-2, SuperArray Inc., Bethesda, MD, USA). The biotin deoxyuridine triphosphate (dUTP)-labeled cDNA probes were generated by GEArray Ampolabeling-LPR kit. Total RNAs were annealed with random primers at 70°C for 3 min, then reverse transcribed to cDNA at 37°C for 25 min. These cDNAs were amplified by PCR using gene-specific primers and biotin 16-UTP (Roche). The PCR was performed as described by the following protocol: the first step at 85°C for 5 min, then 30 cycles (85°C 1 min, 50°C 1 min, 72°C 1 min), and the last step at 72°C for 5 min. After prehybridization membranes were incubated with biotin-labeled cDNAs overnight at 60°C. Membranes were then washed and blocked with GEAblocking solution. The chemiluminescent detection was performed by alkaline phosphatase-conjugated streptavidin and CDP-star substrate. Chemiluminescence was visualized by exposure to electrochemilumiscence film. Data were acquired with a scanner (Epson) and the digital image was converted to raw data file using ScanAlyze Software. The data acquired were analyzed with GEArray Analyzer Software (SuperArray). The expression level of each gene was compared with the signals derived from the housekeeping gene human glyceraldehyde 3-phophate dehydrogenase (GAPDH) (three spots in each array), whereas negative values were transformed to zero. Expression of neural markers in hMSCs was also investigated by means of RT-PCR technique and the primers used were: Hu-NSE_for ATGTGAT CACGCCTGGCTAATA and Hu-NSE_rev GAACC TAGGGTTGGGGAGAGAT (product size: 325 bps); Hu-GAP43_for TGATGCTGCCACAGAGCAGG and Hu-GAP43_rev TGGGAAAGGACAGACTCACAGAC GTG (product size: 498 bps); Hu-MAP2_for TGAG GATGTCACTGCTGCACTC and Hu-MAP2_rev TGT TGCAAATGGAAATCAGGTC (product size: 237 bps); Hu-GFAP_for GTGACTCATCCTCTTGAAGA TGC and Hu-GFAP_rev ACAGATCCCACCAGTCT GCTCAC (product size: 442 bps); Hu-β-tubulinIII_for AGATGTACGAAGACGACGAGGAG and Hu-β-tubulinIII_rev GTATCCCCGAAAATATAAACACAAA (product size: 315 bps); Hu-GAPDH_for TCCCCACTGC CAACGTGTCAGTG and Hu-GAPDH_rev ACCCTG TTGCTGTAGCCAAATTCG (product size: 268 bps).

Only samples expressing the human GAPDH gene were considered and we designed specific primers for several neural markers in unique regions on the basis of deposited human sequences. Briefly, total RNA was extracted with Trizol from frozen brains and tissue slices after positive immunohistochemical identification of transplanted cells. The contaminating genomic DNA was eliminated by DNAse I digestion at 37°C for 30 min (Roche, Hoffmann-La Roche Ltd), and samples (3 μg total RNA) were retro-transcribed in a final volume of 40 μl by SuperScript II RT (GIBCO; 50 min at 42°C) according to the manufacturer's protocol in the presence of oligo(dT) primers (500 pmol) and RNAsin Ribonuclease Inhibitor (Promega). cDNA was amplified for 40 cycles (35 for GAPDH) at annealing temperature optimized for each set of primers (50 pmol each), in the presence of 0.5 U of AmpliTaq Gold (Applied Biosystems). RT-PCR for several human neurotrophins and their receptors' expression was also performed under the following conditions: 94°C 5 min, then 35 cycles at 94°C 40 s, 68°C 40 s, 72°C 1 min. Human or murine brain cDNAs were amplified as positive controls, while in negative controls reverse transcriptase was omitted.

ELISA Assay

Supernatants of the organotypic brain tissue cultures and transplanted brain specimens were collected for neurotrophins measurement. Human NT-3, neurotrophin 4/5 (NT-4/5), brain-derived growth factor (BDGF), and NGF were measured using commercially available kits (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. The supernatant of the organotypic brain sections was collected after 7 days of coculture with the hMSCs. Transplanted hMSCs expressing the human lamin A/C antigen were isolated from different brain areas by laser dissection and homogenized. To test the species specificity of the ELISA kit, the specific expression level of each human neurotrophin was compared with the signals derived from the uninjected mouse brain areas (five specimens for each area per single neurotrophin). No detection of human neurotrophins was observed in all controls uninjected mouse brain areas.

In Vitro Neurotrophin Stimulation of hMSCs and Angiogenesis Assay

In order to verify whether neurotrophin stimulation can mimic the results obtained with the ELISA assay from the organotypic brain tissue cultures and transplanted brain specimens, 60–70% confluent cultures of adult hMSCs were incubated with NGF (50 ng/ml) and NT-3 (10 ng/ml) in combination or alone, in DMEM (Biowhittaker) supplemented with 10% FCS (Sigma-Aldrich), 100 U/ml penicillin, and 0.1 mg/ml streptomy-

cin (Sigma-Aldrich). Human MSCs were analyzed for their release of angiogenic factors on Angiokit (TCS Cell Works Ltd, Buckingham, UK) according to the manufacturer's protocols. Briefly, the 3-day-old medium from human MSCs was added at different dilutions (1:1; 1:2; 1:3) to the seeded human endothelial cells (HUVEC) in a 24-well plate. Control wells that received no conditioned medium were cultured in the presence of VEGF (2 μg/ml) and suramin (2 mM). Cultures were monitored daily for the tubules formation and fixed for immunohistochemistry analysis after 12 days of culture. Staining procedure for the expression of CD31 was performed as previously described (18). After the immunostaining, the degree of angiogenesis was assessed using the TCS "AngioSys" software package (TCS CellWorks, Cat No. ZHA-1800), which reduces in width to a single pixel each stained vessel. We analyzed the pixels' total number in six independent microscopic fields (40×). All data are expressed as percentage of the control wells cultured in the presence of VEGF and suramin (referred as 100%) and expressed as the mean ± SD values.

RESULTS

Characterization of Cultured hMSCs

Typically, hMSCs are isolated from bone marrow by their adherence to plastic (40). Most of cultured adherent cells expressed CD29 and CD90 (data not shown). In contrast, the majority of adherent cells were negative for CD34 and CD45. A small fraction of the adherent cells expressed c-Kit. Thus, we confirmed that the adherent cells were MSCs. To verify the neuropotential phenotype of the hMSCs, we subjected these cells in vitro to neural differentiation using B27 neurobasal medium supplemented with 3–10% FBS. In these conditions several hMSC were positive for the expression of vimentin antigen (Fig. 1A) and coexpressed β-tubulin III and GFAP (Fig. 1B) (11 ± 4.3% of the cells) displaying bipolar morphologies, whereas there was no expression of other neural markers, $NGFR^{p75}$, or trk receptors. However, in the presence of 10 ng of VEGF, the hMSCs (7 ± 2.9% of the cells) coexpressed the α-smooth muscle actin (α-SMA) (Fig. 1C) and desmin (Fig. 1D) myoendothelial markers. The RT-PCR analysis of these cells showed only the expression of GAP43. Furthermore, the hMSCs did not secrete detectable neurotrophins in these in vitro basal experimental conditions.

Transplantation of hMSCs Into Neonatal P4 Mouse Brain

To determine whether hMSCs can engraft into neonatal P4 brain, we initially injected 50,000 PKH-26-labeled cells into the right parietal cortex of neonatal Balb/C and nude mice. The use of PKH-26, a fluorescent membrane binding dye, provided a quick and convenient method to track the fate of the grafted cells in the brain. Thirty days after transplantation only 12 neonatal Balb/C mice survived, and the examination of cryostat sections by fluorescent microscopy revealed that 7 from these Balb/C mice contained labeled hMSCs (Fig. 1E, F). RT-PCR analysis of the counterstained sections showed the expression of human GAPDH (Fig. 1M). Thirty days after MSC transplantation neurons were shown to express the human neuronal antigens MAP-2ab isoform and human neurofilament 160 kDa (NF-160kD) (Fig. 1G, H) in the brain of one mouse of seven transplanted animals expressing the human GAPDH in brain specimens (Fig. 1M). No hMSC cells expressing the human GalC, a marker for oligodendroglia, were found in the transplanted mice brains (Fig. 1K). The hMSCs-derived neurons were positive for the human centromer nuclei by FISH as well (Fig. 1I) and exhibited characteristic polar morphologies with segregation of neurites into dendrites and axons with prominent fiber bundles running alongside, indicating a probable state of mature neurons. These cells were detected in layer 4 of the cortex of 10 serial brain sections and their number was 15 ± 6 (SD) cells/section. Some of them were found adjacent to host GFAP+ astrocytes (Fig. 1L). No human nuclei were detected in control mice after FISH labeling (Fig. 1J). Few hMSCs-derived cells expressing human GFAP with bipolar morphology and not the typical branching pattern were found. Moreover, fluorescent cells were observed clustered at the injection site, suggesting that hMSCs did not migrate throughout the brain of Balb/C mice. In this series of experiments of intracortical injections, 38 mice failed to survive. We attributed the poor cell survival to the local inflammation and immunological rejection related to the xenotransplant. We therefore repeated the injections into nude mice. In these experiments most of the transplanted mice survived after cell transplantation (Table 1).

At 7 days postinjection, the overall distribution of hMSCs throughout the brain was essentially identical in all nude animals examined. The transplanted cells were detectable by virtue of their human nuclei by FISH analysis and PKH expression. In the cortex, a large number of donor cells were found ipsilateral to the injection site throughout the cortical layers, spanning from the anterior commissure to the cingulate cortex (Fig. 2A). Many hMSCs, however, were also detected lining the ependyma throughout the ventricles. No human MSCs were localized into the cerebellum. All these data indicated that hMSCs can migrate in multiple directions after intracortical transplantation in nude mice. hMSCs expressing human centromer nuclei also were localized to areas undergoing active postnatal neurogenesis, including the Islands of Calleja (Fig. 3A–D) (54) in the ventral fore-

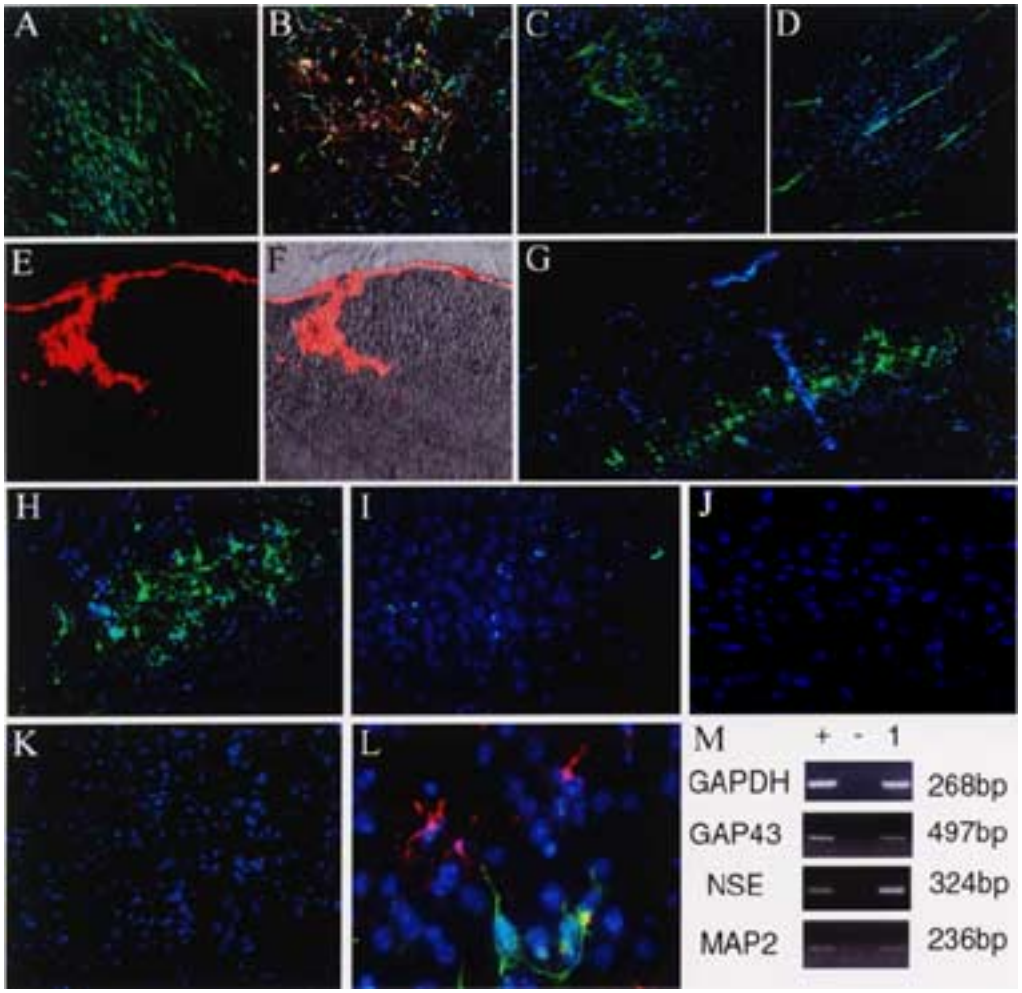


Figure 1. In vitro and in vivo differentiation of hMSCs. hMSCs, positive for the vimentin antigen (A), coexpressed β-tubulin III (green in B) and GFAP (red in B) displaying a typical bipolar phenotype (B). Some of them were able to express myoendothelial markers α-SMA (C) and desmin (D). To determine the neural potential of hMSCs we injected these cells directly into the cortical layer of neonatal P4 Balb/C mice. Before injection hMSCs were labeled with PHK26 red dye, and fluorescent cells were observed clustered near the injection site (E, original magnification 40×). The optic PKH26 counterstaining is shown in (F). After 30 days of transplantation several hMSCs were positive for human MAP-2ab isoform and neurofilament 160 kDa (NF-160kD) (G, H) and for human centromer in the same cortical layer of the serial section (I), showing the neuronal differentiation of injected hMSCs into cortical layer 4. No FISH+ cells were found in the same layer in control uninjected mice (J). No hMSC cells expressing GalC antigen were found in the transplanted brains (K). Few hMSC-derived human MAP-2ab neurons (green in L) were found adjacent to host GFAP-positive astrocytes (red in L). All nuclei are stained in blue by DAPI. The neuronal differentiation from injected hMSCs was also confirmed by RT-PCR analysis for the expression of specific human GAP43, NSE, and MAP-2ab products (M). The RT-PCR analysis was performed on mRNA extracted from injected hMSC mouse brains (lane 1), not injected mouse brains (lane −), and a biopsy of human brain (lane +). GAPDH was amplified in the same reaction tube to confirm the expression of human markers. The internal RT− controls were performed in the absence of reverse transcriptase.

brain and the subependyma of both the frontal cortex and olfactory bulb (4,5). The RT-PCR analysis from dissected human lamin A/C+ cells demonstrated the expression of the human marker GAPDH (Fig. 3H). Moreover, the RT-PCR with oligonucleotides specific for human NT3, NT4, NGF, trkA, trkB, and trkC revealed appearance of transcripts for several of these human genes both 7 and 45 days after transplantation (Figs. 2 and 3). The ELISA analysis of the laser dissected human lamin A/C+ hMSC-derived cells showed a production of human NT3 and NT4 after 7 days of transplantation from the cortical graft (hNT3 19.42 ± 6.88 pg/ml; hNT4 16.215 ± 1.97 pg/ml), ventral forebrain (hNT3 23.625 ± 17.677 pg/ml; hNT4 1.45 ± 0.10 pg/ml), ventricles (hNT3 17.975 ± 5.21 pg/ml; hNT4 14.57 ± 1.76 pg/ml), and olfactory bulb (hNT3 25.985 ± 7.44 pg/ml; hNT4 8.682 ± 5.403 pg/ml) (mean ± SEM; $n = 6$) (Table 2).

At 45 days postinjection, transplanted hMSCs were

Table 1. Summary of the Animal Survival After hMSC Transplantation

	Transplanted Animals	Sacrifice	Survived Animals	% Survival	Survived Animals With PHK26+ hMSC	Efficiency of Transplantation
P4 Balb/C	50	30 days	12	24%	7	14%
Nude mice	30	7 days	28	93.30%	24	80%
Nude mice	30	45 days	26	86.70%	18	60%

hMSCs (50,000) were injected into the lateral right cortex of cryoanesthetized 4-day-old mice by using a stereotactic device. Before transplantation, the hMSCs were labeled with a PKH26 fluorescent vital dye. In the first experiments, from the 50 Balb/C transplanted newborns, only 12 mice survived and were sacrificed after 30 days. Seven from these surviving animals showed clusters of fluorescent PKH26+ cells into their transplanted brains. We repeated the same experiments into nude mouse brains, and the animals were sacrificed 7 and 45 days after ($n = 30$ for each group). From the 28 animals that survived after 7 days of transplantation, 26 animals were positive for the PKH26 staining while only 18 from the 26 animals that survived after 45 days of transplantation showed PKH26+ cells into the injection site and distributed in different brain areas.

able to form clusters of 12–20 human GAP43+ cells, round or oval in shape, close to host striatal neurons (Fig. 3F). Moreover, the human GAP43+ cells did not exhibit characteristic polar morphologies with segregation of neurites into dendrites and axons with prominent fiber bundles running alongside, indicating a probable state of immature neurons. No donor-derived mature neurons and oligodendrocytes were found in the transplanted mouse brains by immunocytochemical analysis for human MAP-2ab, NF160, and GalC (data not shown). Colocalization of human nuclei and the astrocyte marker GFAP was unequivocally demonstrated at the graft–host border (i.e., within the area of mouse GFAP-positive reactive astrocytes surrounding the graft core) (Fig. 3G). Several transplanted hMSCs were also found close to blood vessels coexpressing the human centromer nuclei and Ve-cadherin after 45 days of transplantation, whereas these cells were negative for human KDR (marker for endothelial and hematopoietic stem cells) and CD45 (a marker of all mature nucleated hematopoietic cells) (Fig. 4A–D). The ELISA analysis of the laser dissected hMSC-derived cells after 45 days of transplantation confirmed the production of human NT3 and NT4 from the cortical graft (hNT3 16.39 ± 0.37 pg/ml; hNT4 18.5 ± 1.25 pg/ml), ventral forebrain (hNT3 14.29 ± 0.36 pg/ml; hNT4 8.85 ± 0.75 pg/ml), ventricles (hNT3 22.687 ± 5.41 pg/ml; hNT4 3.391 ± 2.012 pg/ml), and olfactory bulb (hNT3 25.812 ± 4.93 pg/ml; hNT4 4.143 ± 2.30 pg/ml) (mean ± SEM, $n = 6$) (Table 2).

hMSC Application Into Organotypic Cultures

hMSCs were labeled with PKH26 and cocultured for 7 days with brain sections of Balb/C and nude P4 mice. The application of hMSCs to the brain explants did not result in the expression of neuronal, astrocytes, and oligodendroglial markers in hMSCs. PKH26+ hMSCs only expressed the early neuronal marker βIII-tubulin (data not shown). We did not observe the expression of any further neuronal markers such as MAP-2, NeuN, and neurofilament. Moreover, the PKH26+ hMSCs did not label for the astrocytic marker GFAP or the oligodendrocytic GalC. We then examined the expression of the neurotrophin receptors. Double staining of PKH26+ hMSCs with anti-human nuclear lamin A/C (Fig. 5E) and trkC (Fig. 5F, J) showed that $55 \pm 3.7\%$ of lamin A/C+ cells expressed trkC. A few of these cells were binucleated, suggesting a cell fusion process (Fig. 5E–H). $NGFR^{p75}$+ cells were also observed ($35 \pm 0.6\%$ of the lamin A/C+ cells). Immunostaining for trkA and trkB receptors was negative in all coculture wells. Most of the applied hMSCs remained aggregated in clusters within the explants. Moreover, several lamin A/C+ hMSCs were also found around the explants after 7 days of coculture (Fig. 5A). Most of these cells coexpressed the PKH26 dye (Fig. 5B) and the human trkC ($85 \pm 5.9\%$) (Fig. 5C), while few of them coexpressed $NGFR^{p75}$ ($4 \pm 0.7\%$). To elucidate the possible production of human soluble factors by hMSCs in the organotypic brain cocultures, analysis of the supernatants using specific human ELISA for human neurotrophic factors (NGF, BDNF, NT3, NT4/5) was performed. This revealed that after 7 days of cocultures, the hMSCs secrete NT3 (140 ± 23.4 pg/ml), NT4 (121 ± 16 pg/ml), and NGF (89 ± 17.7 pg/ml), but no traces of BDNF were found in these conditions (mean ± SEM; $n = 5$). In order to improve these observations, we analyzed the human neurotrophin and receptor gene expression profile by RT-PCR analysis (Fig. 5M) and GEArray technology (Fig. 6) of the organotypic cocultured hMSCs (12,29). The expression patterns of human NT3, NT4, and NGF mRNA were consistent with the results of ELISA.

In Vitro Neurotrophin Stimulation of hMSCs and Angiogenesis Assay

We further examined whether the in vivo release of neurotrophins may correspond to an autocrine pathway

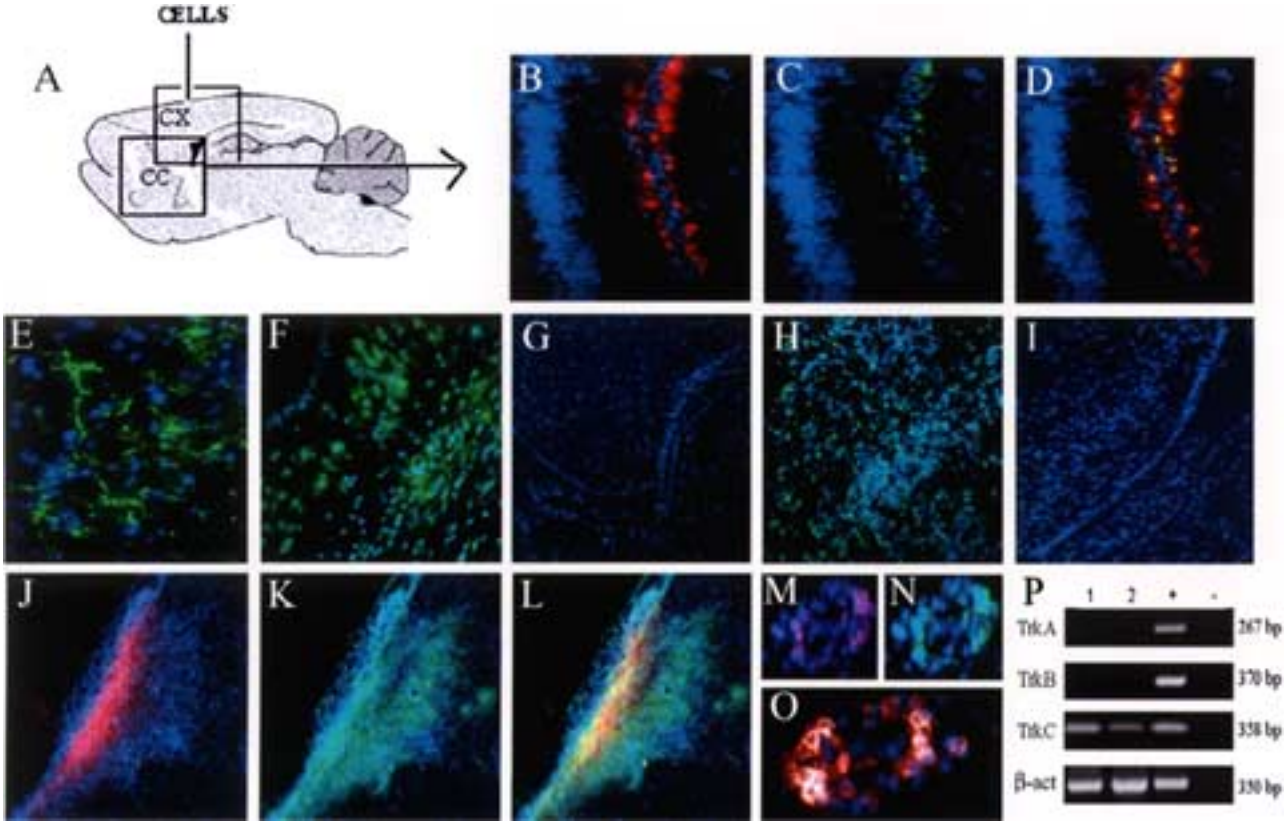


Figure 2. Schematic representation showing the longitudinal view of the mouse brain indicating the maps of the injection fronto-parietal cortex area (cx) and the area of distribution of anti-human nuclei-positive injected hMSC cells (cc) (A). PKH26-labeled cells (B) coexpressing the human lamin A/C (C) were localized in the fronto-parietal cortex and ventricular zone. (D) Merged (B) and (C) pictures (yellow, where bright red and green overlap). Control reactions for GalC, $NGFR^{p75}$, and trkC performed from human (E, F, and H, respectively) and mouse (G and I) brain sections confirmed the specificity of these antibodies. After 7 and 45 days of transplantation most of PKH26+ cells (J, L, and O) coexpressed trkC (green in K and L), the human lamin A/C (magenta in M and O), and the human $NGFR^{p75}$ (green in N). (L) Merged (J) and (K). The neurotrophin receptors' expression from transplanted areas after 7 (lane 1) and 45 (lane 2) days of transplantation was also confirmed by RT-PCR analysis (P). The internal RT− control (lane −) was performed in the absence of reverse transcriptase. The human cDNA library (lane +) was used as control. cx, cortex; cc, corpus callosum.

required to generate neuroglial cells from hMSCs. For this purpose we decided to treat hMCSs with the same neurotrophins expressed in the organotypic brain coculture experiments, namely NGF and NT3. Human MSCs were cultured for 15 days in the presence of these neurotrophins (50 ng/ml of NGF; 10 ng/ml of NT3), followed by immunostaining with anti-MAP-2ab, GAP43, GFAP, and GalC antibodies. NGF, NT3, and combination of the two did not induce any neural cell differentiation. As for the neural cell markers, the immunostaining for $NGFR^{p75}$, trkA, trkB, and trkC was negative in all the tested conditions. To further test the hypothesis that neurotrophins and their receptor induction may be related to soluble factors, hMSCs were cultured with different dilutions (1:1, 1:2, 1:3) of the condition medium obtained from the organotypic brain sections. In these conditions, ELISA, immunostaining and GEArray analysis did not recognized the expression of neurotrophins and their receptors in hMSCs.

These results may suggest that the release of neurotrophins in hMSCs is not sufficient for their neural differentiation and seems to be dependent on cell–cell contact in the developing brain. A tube formation assay (Angiokit, TCS CellWorks Ltd, Buckingham, UK) was also used to determine the capability of conditioned hMSCs media to regulate the angiogenesis (Fig. 4E–G). Increasing conditioned media of hMSCs (1:1, 1:2, 1:3) added to the Angiokit wells seemed to promote the formation of capillary-like structure (Fig. 4F) in a dose-dependent manner after 10 days of culture. Counts of total CD31+ vessels were performed by using an inverted microscope, and the results found in VEGF-stimulated cultures (Fig. 4E) were expressed as 100%. The count of CD31+ vessels confirmed the high angiogenetic

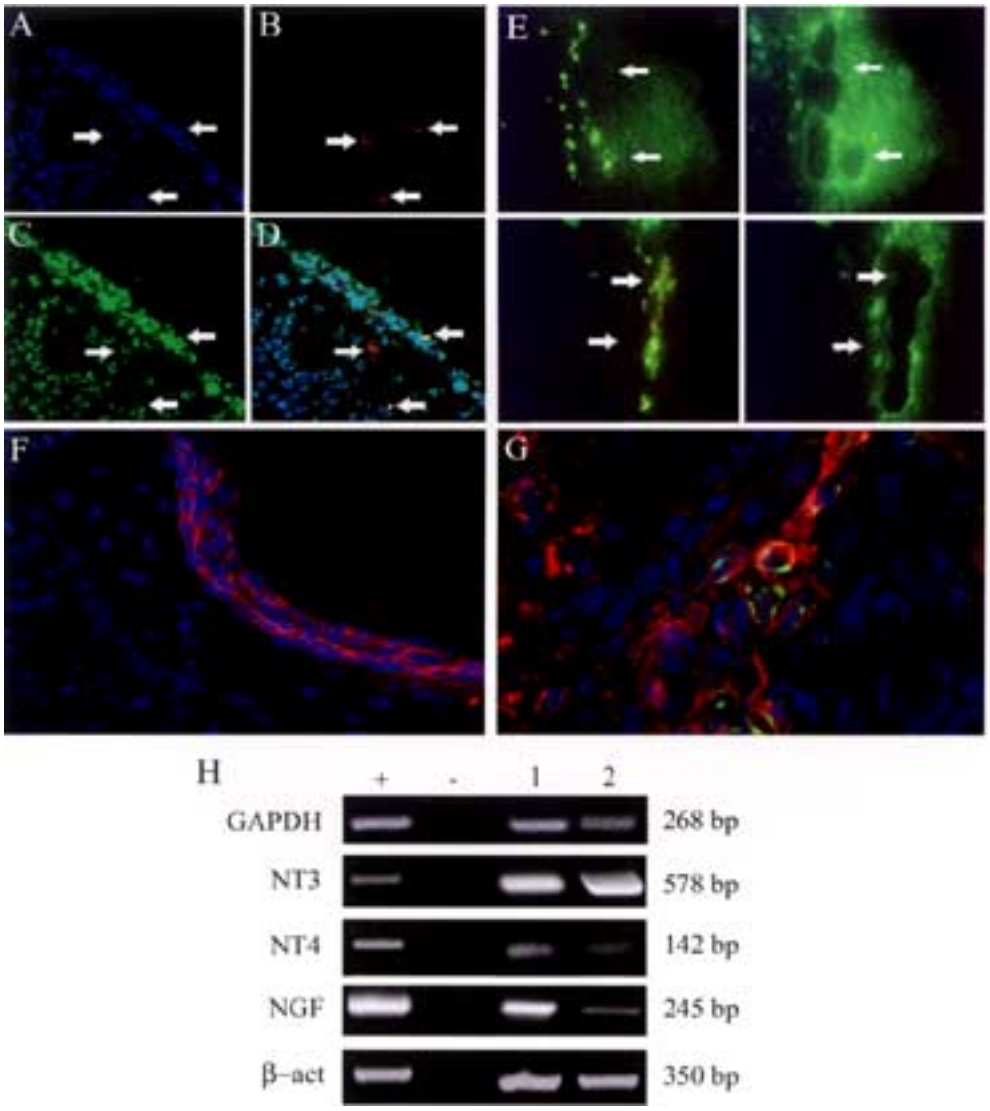


Figure 3. Distribution and neural differentiation of hMSCs after 45 days of transplantation in the brain of neonatal P4 nude mice. FISH analysis for the expression of mouse (green in C and D) and human (red in B and D) centromeric DNA into the same region of injected brains with hMSCs indicates the presence of cells with both human (arrowheads) and mouse-positive centromeric DNA into cells, demonstrating cell fusion process. Cell nuclei were counterstained with DAPI (blue in A). (E) Laser microdissection of human lamin A/C+ cells 45 days after intracortical transplantation of P4 nude mice from the cortical layer 4–6. Expression of the human GAP43 in cortex sites of injected animals is shown in (F). These cells did not extend neuronal process toward the cortex. Confocal images demonstrated rare astrocytes coexpressing GFAP (red in G) and human lamin A/C (green in G) generated by the transplanted hMSCs into the white matter regions such as under the corpus callosum. The expression of human GAPDH from laser captured cells demonstrated the presence of transplanted hMSCs (H). Moreover neurotrophin production from transplanted hMSCs after 7 (lane 1) and 45 (lane 2) days of transplantation was demonstrated by RT-PCR analysis for the expression of NT3, NT4, and NGF products.

potential of the hMSCs conditioned medium (58.19%) (Fig. 4G). No tube formation was observed when HUVEC from the Angiokit were cultured with conditioned media obtained from human fibroblasts (data not shown).

DISCUSSION

This study examined the capacity of adult hMSCs to differentiate into the neural lineage both in vitro and in vivo. The in vitro analysis using different cell induction protocols further confirmed the reduced capability of hMSCs to transdifferentiate, overcoming the barriers of germ layer commitment. These data further expand our previous observations in different subpopulations of human adult bone marrow cells (6). To further test the neural potential of the hMSCs, we injected these cells into the lateral parietal cortex of neonatal Balb/C and immu-

Table 2. ELISA Analysis of the Laser Dissected Areas of hMSC-Derived Cells After 7 and 45 Days of Transplantation

	hNT3 (pg/ml)	hNT4 (pg/ml)	hNGF (pg/ml)	hBDNF (pg/ml)
CTR+	218.13 ± 5.303301	131.45 ± 2.311127	99 ± 2.6728	100 ± 1.67923
CTR−	0 ± 0	0 ± 0	0 ± 0	0 ± 0
P4 A (7 days)	19.42 ± 6.88722	16.215 ± 1.972828	0 ± 0	0 ± 0
P4 B (7 days)	23.625 ± 17.67767	1.455 ± 0.106066	0 ± 0	0 ± 0
P4 C (7 days)	17.975 ± 5.211377	14.57 ± 1.767767	0 ± 0	0 ± 0
P4 D (7 days)	25.985 ± 7.441483	8.6825 ± 5.403428	0 ± 0	0 ± 0
P4 A (45 days)	16.935 ± 0.374767	18.5 ± 1.25865	0 ± 0	0 ± 0
P4 B (45 days)	14.29 ± 0.367696	8.855 ± 0.756604	0 ± 0	0 ± 0
P4 C (45 days)	22.6875 ± 5.418151	3.39175 ± 2.012758	0 ± 0	0 ± 0
P4 D (45 days)	25.8125 ± 0.934466	4.14325 ± 2.305945	0 ± 0	0 ± 0

The ELISA analysis of the laser dissected areas of hMSC-derived cells after 7 and 45 days of transplantation confirmed the production of human NT3 and NT4 from the cortical graft (P4 A), ventral forebrain (P4 B), ventricles (P4 C), and olfactory bulb (P4 D) regions (3H). The human specificity of ELISA analysis was demonstrated from human (CTR+) and mouse (CTR−) brain sections.

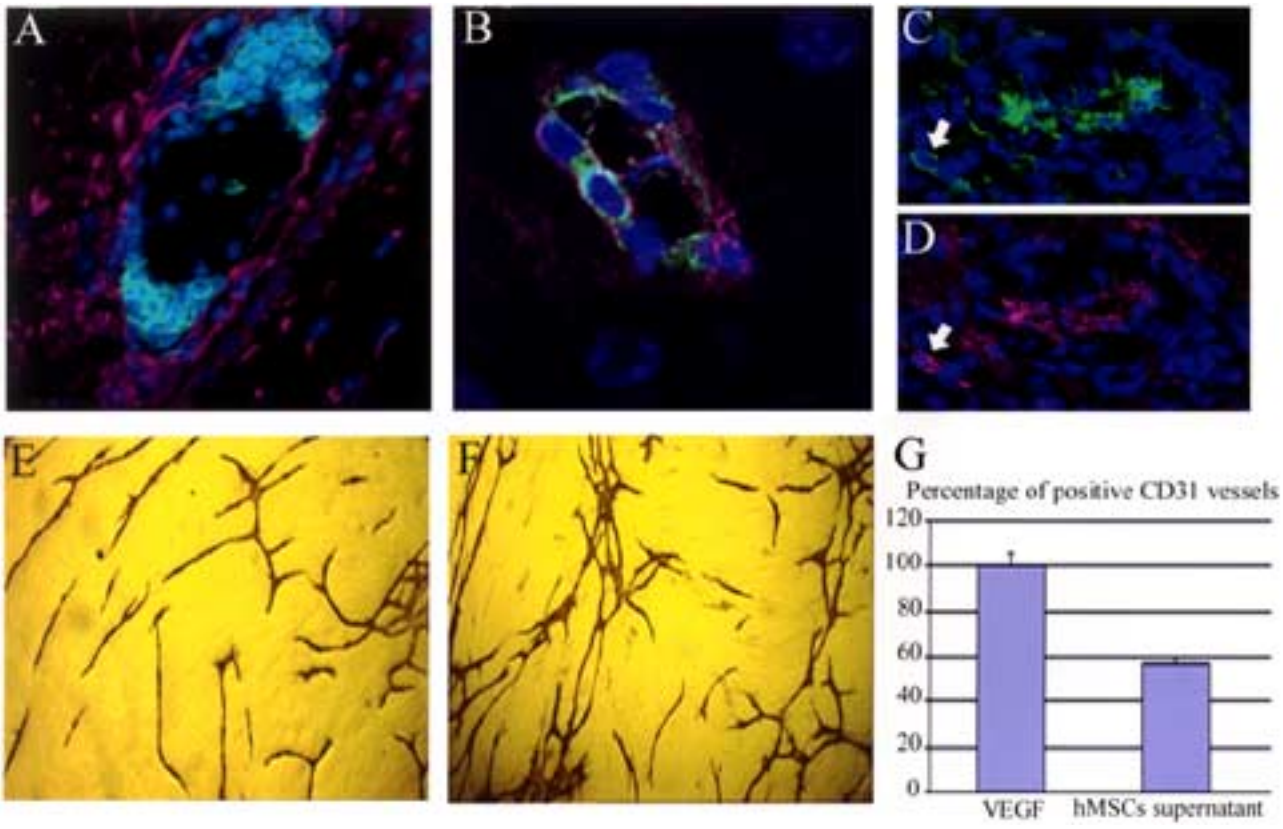


Figure 4. The hMSC-derived cells expressing human lamin A/C (green in A, B, and C) assumed perivascular locations (A–D), with one or several processes extending to the capillary wall of the cortical cortex (B–D). Host GFAP+ cells (magenta in A) were found near these vessels. Several small rounded human lamin A/C cells (green in C; arrowhead in C), similar to the hematopoietic/endothelial precursors, were found at the host vascular border and within the wall vessel. These cells did not express any of the neuronal and glial markers but were positive for human Ve-cadherin (magenta in D; arrowhead in D). A tube formation assay was also used to determine the capability of conditioned MSCs media to regulate the angiogenesis (E–G). The human endothelial cells (HUVEC) from the Angiokit differentiate in a network of CD31+ vessels in the presence of VEGF (E). Capillary sprouts, tubular structures, and vessel arborization in human MSC conditioned medium after 10 days of culture is shown in (F). (G) The CD31+ tubular structures obtained in the different conditions were quantified using the TCS "AngioSys" software package and expressed as a percentage of the number of the tubular structures obtained from the VEGF-treated HUVEC cells (referred as 100%).

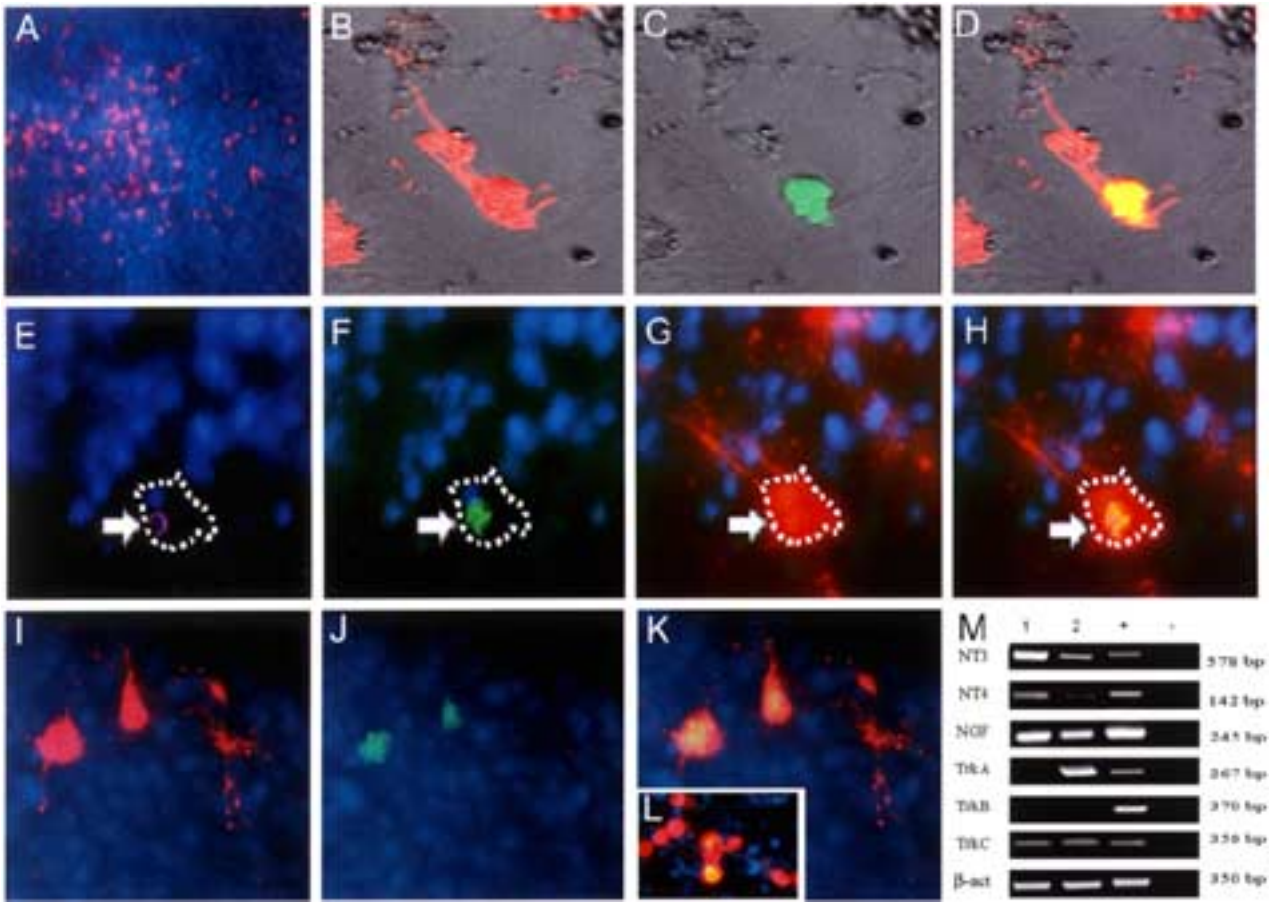
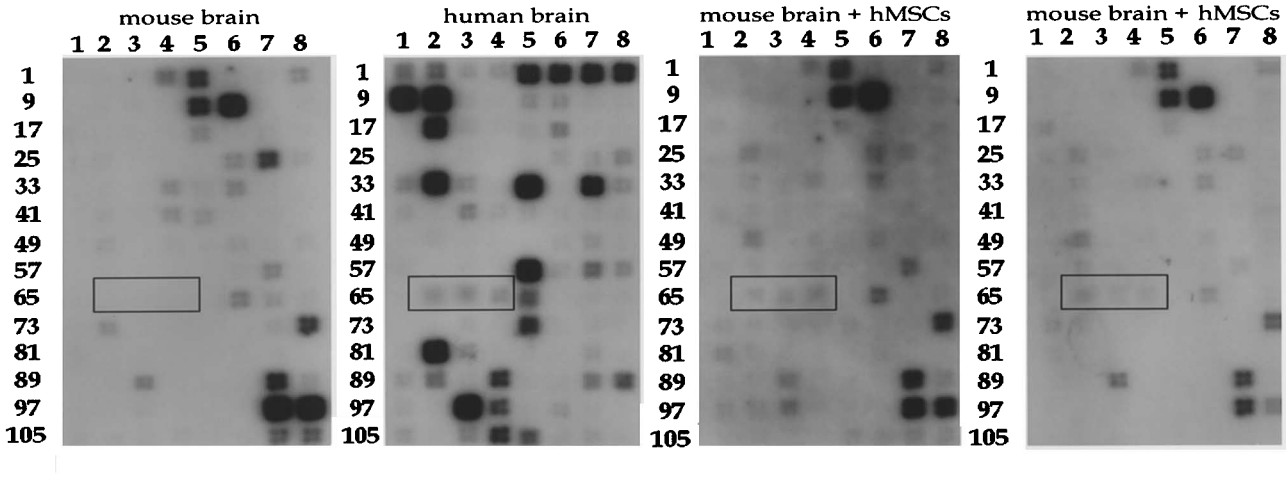


Figure 5. We examined the expression of neurotrophin receptors from hMSCs in organotypic cultures of nude P4 brains. Before the experiment, hMSCs were labeled with PKH26 dye. After 7 days of coculture, several lamin A/C+ cells were found aggregated in clusters within the explants (A). In these conditions, the labeled cells (red in B) coexpressed the human trkC (green in C and D; yellow where bright red and green overlap). Double staining of PKH26+ hMSCs (red in G–I) with anti-human trkC (green in F and J), and nuclear lamin A/C (magenta in E), showed that few hMSCs were binucleated, suggesting a cell fusion process (arrowheads in E–H; the plasma membrane of a cell with two nuclei is outlined in white). The RT-PCR analysis (M) showed the human neurotrophin and receptor gene expression. Lanes 1 and 2 in (M) represent the same experiments of hMSC organotypic coculture analyzed by GEArray technology. The internal RT– control (lane –) was performed in the absence of reverse transcriptase. The human cDNA library (lane +) was used as controls.

nodeficient nude mice and into slice culture tissues obtained from the same brain areas. One of the major problems of our study was the high mortality rate of transplanted P4 Balb/C mice and the low levels of engraftment in these animals, whereas the results obtained with nude mice demonstrated improvement of the engraftment with migration of hMSCs from the graft. These data suggest that the status of the immunoprivileged brain should be reconsidered in relation to the inflammatory response and immune system activation produced after brain xenotransplantation. The low percentage of hMSC-derived neural cells found in the brain after grafting confirms this statement.

On the contrary, cells grafted into nude mice demonstrated better survival, migration, and expression of neurotrophin receptors and neurotrophin release within the grafted regions. This is probably the effect of the local neural microenvironment on the grafted hMSCs. To complement these data, hMSCs respond in vitro to a similar epigenetic signal(s) from organotypic brain tissues and demonstrate in vitro a broad capacity to express human neurotrophins. Additionally, the GEArray and RT-PCR analysis confirmed the expression of human neurotrophins in these conditions. This in vitro system could thus be considered as a developing microsystem, inducing neurotrophin production from hMSCs that is presumably the result of a partial reprogramming of the hMSC genome at different stages of maturation. These data would indicate that hMSCs may have neuroprotective capacity after direct cell–cell contact in developing



Position	Description	Gene name
2	Apoptotic protease activating factor	Apaf-1
3	Homo sapiens artemin	artemin (Artn)
4	cAMP responsive element binding protein 2	ATF-2 (creb-2)
10	Homo sapiens ciliary neurotrophic factor receptor mRNA	CNTFRa
11	Homo sapiens corticotropin releasing hormone	CRF
12	Corticotropin releasing hormone-binding protein	CRF-BP
18	Homo sapiens v-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1)	Ets1
19	human prothrombin (phii-3 clone)	Prothrombin kringle-1
20	Fibroblast growth factor 2 (basic)	FGF2
26	Homo sapiens GDNF family receptor alpha 1	GFRalpha1
27	Homo sapiens GDNF family receptor alpha 2	GFR-a2
28	Homo sapiens GDNF family receptor alpha 3	GFR-a3
34	Homo sapiens hypocretin (orexin) receptor 1	Orexin-R1
35	Homo sapiens hypocretin (orexin) receptor 2	Oresin-R2
36	Heat shock 27 KD protein	hsp27 (hsp b1)
42	Interleukin 6 (interferon, beta 2)	IL-6
43	Interleukin 6 receptor	IL-6 Ra
44	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	gp130
50	Metallothionein 3 (growth inhibitory factor (neurotrophic))	MT3
51	v-myc avian myelocytomatosis viral oncogene homolog	c-myc
52	Nuclear receptor coactivator 1	NCOA1
58	Homo sapiens neuropeptide Y	neuropeptide Y
59	neuropeptide Y receptor type 1	NPY1R
60	neuropeptide Y receptor type 2	NPY2R
66	Neurotrophin 3	NT-3
67	Homo sapiens neurotrophin 5 (neurotrophin 4/5)	neurotrophin
68	neurotrophic tyrosine kinase, receptor, type 1	Trk A
74	Homo sapiens persephin	persephin
75	PTGER2 prostaglandin E receptor 2 (subtype EP2), 53kD	PTGER2
76	Pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)	PTN (pleiotrophin)
82	Signal transducer and activator of transcription 2, 113kD	STAT 2/SGF-3
83	Signal transducer and activator of transcription 3 (acute-phase response factor)	Stat3
84	Signal transducer and activator of transcription 4	STAT 4
90	Transforming growth factor, beta 1	TGFb1
91	Tumor necrosis factor receptor superfamily, member 5	CD40 (NGFR)
92	Tumor necrosis factor receptor superfamily, member 6	Fas (Apo-1) (CD95)
111	Beta Actin	b-actin

Figure 6. GEArray technology of the organotypic cocultured human MSCs. After 7 days of coculture, we found the expression patterns of human trkA, trkC, NT3, NT4, and NGF mRNA.

brain tissues, becoming able to produce neurotrophic factors (12,30).

MSCs have great promise as a source for introducing new neurons and glial cells to the damaged CNS. Once transplanted into the brain, MSCs can eventually adapt to the region of engraftment by differentiating into the appropriate neuronal subpopulations (42,46,50). One of the most important questions is whether transdifferentiated neuronal cells from bone marrow become functional neurons. The neuronal cells are defined not only by polarity in cytology and specific protein expression in defined locations, but more importantly by their excitability and ability to communicate with other cells by releasing neurotransmitters at synapses (22). No study has shown so far generation of functional neurons from adult bone marrow stem cells using the above criteria (28). If the neuronal marker expression of the transplanted hMCSs in our experiments is poor, the expression of human GFAP by immunocytochemical analysis after grafting of hMSCs represents unequivocal data that further complement previous observations in a large series of cultured hMSCs, demonstrating limited expression of the astroglial marker GFAP after systematic evaluation of several neuronal induction protocols (6). In the prospective of a neuroprotection in which degenerating neurons may be saved, increasing the number of

normal surrounding astroglial cells (13), hMSCs acquire further potential as therapeutic agents.

Furthermore, our data demonstrate an undescribed neurogenic feature of transplanted hMSCs both in vitro and in vivo, namely the neurotrophin activation. In two key studies (8,34), numerous marrow-derived cells were reported to migrate into the brain and to express neuronal marker genes. The present data further confirm the capability of hMSCs to migrate into the host brain, at least in nude mice. The activation of neurotrophins provides a novel marker capable of identifying the grafted cells but, more importantly, may reveal a new neuroprotection mechanism for injured or degenerating neurons. Neurotrophins and their receptors, which are normally involved in neural cell maturation and proliferation during CNS development, have also been found to be reexpressed in brain lesions from patients with stroke, multiple sclerosis, and neurodegenerative disease as ALS (24,36,38,51). This reexpression is probably the result of a recapitulation of developmental programs and may represent an important mechanism of brain plasticity and repair (55).

Secretion of neurotrophic factors or neurotrophins has been advocated as an underlying mechanism of neuroprotection after transplantation, but the difficulty in discerning in clinical studies has been considered a major obstacle (17). The present experiments unequivocally indicate the capability of hMSCs to activate the neurotrophin metabolism after transplantation with release and receptor activation for NT3 and NT4. Although trk receptors account for most of the biological responses of neurons to neurotrophins, $NGFR^{p75}$ can facilitate trk ligand binding and neurotrophin responses and can initiate other pathways for intracellular signaling independent of trk receptors (11,19). The ability of the hMCS-derived astroglial cells to produce neurothophic factors amplifies the possible use of bone marrow-derived cell as chaperones that offer neuroprotection and mediate rescue of degenerating host populations (52).

In the developing brain neurogenesis correlates closely with angiogenesis, suggesting that an angiogenic microenvironment may be important for adult neurogenesis (31,37,58). In vitro, hMSCs have the potential to induce a neovascular response in an angiogenesis assay as previously described (2). In vivo, local MSC implantation induces therapeutic angiogenesis in a rat model of hindlimb ischemia (3). On the other hand, hMSCs directly injected into the neonatal brain of nude mice express human endothelial markers within host brain vessels. Endothelial cells stimulate proliferation and neurogenesis of neural stem cells (45), suggesting that hMSCs-derived endothelial cells may also produce neurogenic factors.

The availability of hMSCs from bone marrow and the possibility of autologous transplantation provide exciting perspectives for the treatment of CNS human diseases such as ALS (48). The described capability to express neurotrophin molecules and receptors give to the hMSCs the potential to positively interact with the recipient without need for integration in the brain circuitry or for replacing lost neurons. Moreover, transplantation of engineered SCs, providing a constant source of neurotrophic factors, may offer several advantages to conventional therapies in neurodegenerative diseases (55). Further studies are necessary to investigate whether or not engrafted hMSCs are really neuroprotective and if they are able to improve functional outcome in animal models of neurodegenerative diseases.

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