

Neuroprotective effect of adult hematopoietic stem cells in a mouse model of motoneuron degeneration

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Degenerative spinal motor diseases, like amyotrophic lateral sclerosis, are produced by progressive degeneration of motoneurons. Their clinical manifestations include a progressive muscular weakness and atrophy, which lead to paralysis and premature death. Current pharmacological therapies fail to stop the progression of motor deficits or to restore motor function. The purpose of our study was to explore the possible beneficial effect of mouse adult hematopoietic stem cells (hSCs) transplanted into the spinal cord of a mouse model of motoneuron degeneration. Our results show that grafted hSCs survive in the spinal cord. In addition, the number of motoneurons in the transplanted spinal cord is larger than in non-transplanted mdm mice at the same spinal cord segments and importantly, motor function significantly improves. These effects can be explained by the increased levels of glial cell line derived neurotrophic factor (GDNF) around host motoneurons produced by the grafted cells. Thus, these experiments demonstrate the neuroprotective effect of adult hSCs in the model employed and indicate that this cell type may contribute to ameliorating motor function in degenerative spinal motor diseases.

Keywords: Motoneuron disease; Hematopoietic stem cell; ALS; Neurotropism; Motoneuron; Neurodegeneration; Cellular therapy; GDNF; Stem cell transplantation

Introduction

In degenerative spinal motor diseases such as motoneuron degeneration syndromes (MDS) and amyotrophic lateral sclerosis (ALS), the neurons of the motor system progressively die during a relatively short period of time. These diseases clinically manifest themselves as a progressive muscular weakness and atrophy, which lead to paralysis and premature death. A cascade of interdependent genetic and epigenetic factors may underlie the pathogenesis of most of these diseases. This would hinder the development of etiological therapies, so present therapies use a combination of pharmacological agents with neuroprotective activity to improve the quality and survival of the patient (Hugon, 1996; Eisen and Weber, 1999). However, these therapies currently fail to stop the progression of motor deficits or restore motor function. In the light of the extensive literature on the potential of stem cells in regenerative medicine, we decided to explore if stem cell transplantation into affected areas of the central nervous system has positive effects in animal models of motoneuron diseases and to examine if this could be an adequate strategy for treatment of neurodegenerative diseases in humans (Garbuzova-Davis et al., 2002, 2003; Park et al., 2002; Storch and Schwarz, 2002; Clement et al., 2003; Ourednik and Ourednik, 2004; Silani et al., 2004). The animal model used in this study, the muscle deficient (mdf) mouse, carries a currently unidentified spontaneous mutation in chromosome 19 (Poirier et al., 1998). This mutation results in progressive hindlimb paralysis with muscular atrophy, initially localized in the lumbosacral spinal cord, and pathological hallmarks of motoneuron degeneration with astrogliosis (Blot et al., 1995). This mouse has been described as a model for genetic diseases with motoneuron degeneration, such as MDS and ALS (Blot et al., 1995). In addition, since the mutant mice manifest clear motor deficits and long-term survival after disease initiation (12–15 months), it is an ideal model to assay stem cell based therapeutic approaches in symptomatic animals.

A number of studies have demonstrated that transplantation of human neuron-like cells, derived from a human teratocarcinoma cell line, delay the onset of motor deficits in the SOD1 mouse, a model of ALS (Garbuzova-Davis et al., 2001, 2002; Willing et al., 2001). Moreover, wild-type non-neural cells in the grey matter of the spinal cord extend survival of mutant SOD1 motoneurons in mouse chimeras (Clement et al., 2003). The regenerative potential of bone marrow stem cells in the demyelinated spinal cord has also been demonstrated (Sasaki et al., 2001; Akiyama et al., 2002; Inoue et al., 2003). Other studies explored the neural potential of bone marrow stem cells showing that grafts of these cells in mouse are capable of differentiating into neurons, astrocytes and microglia (Eglitis and Mezey, 1997; Azizi et al., 1998; Kopen et al., 1999; Mezey et al., 2000; Kogler et al., 2004).

In a previous study, we demonstrated the potential of bone marrow hematopoietic stem cells (hSCs) to differentiate into neural stem and mature neural cell lineages when grafted in neonatal mouse brain (Bonilla et al., 2002, 2005). The present study explores the possible regenerative and/or trophic effect of hSC transplantation into the spinal cord of mdm mice. Currently, there are no pre-symptomatic diagnostic tests for identifying pre-morbid stages for most of the motoneuron degenerative diseases in humans. Therefore, suitable therapeutic alternatives must be developed to treat patients who are already symptomatic. Here, we have tested the survival of hematopoietic progenitor cells from adult mouse bone marrow after transplantation into the spinal cord of symptomatic mdm mice. We analyzed their possible differentiation into neuronal and glial lineages, measured the expression of GDNF and studied the functional recovery of the transplanted mice.

Materials and methods

Detailed descriptions are available at: <http://www.salvadmartinezlab.com/materiales.php>.

Animals

Mice with the muscle deficient mutation (mdf) were obtained from the Pasteur Institute (Paris, France), as a gift from J.L. Guenet. As mentioned before, these mice suffer from a currently unidentified autosomal-recessive spontaneous mutation, which has been mapped to mouse chromosome 19. From 3 weeks of age, homozygous mutant mice develop a posterior waddle which is followed by additional clinical signs consisting of progressive hindlimb paralysis and forelimb weakness, leading to a serious disability between 4 and 6 weeks of age (Blot et al., 1995). A total of 86 symptomatic mdf animals were studied. Forty-three of them were transplanted with hSCs between the L4-S1 spinal cord segments, 19 were injected in the same spinal cord segments with serum-free medium (sham-operated), and 24 had no surgery at all. In addition, 14 wild-type mice were used for behavioral analysis and for immunohistochemistry. mdf mutants were selected for functional studies and transplants when motor symptoms were clearly detectable (about 3 weeks of age). After functional studies, performed during the next 3 weeks, the most functionally affected mice of each litter were selected for cell transplantation. The degree of affectation for this selection was determined by hindlimb atrophy and by the score of functional parameters. In these more severely affected mice, grafts were performed approximately at 6 weeks of age, while less affected animals were used as sham-operated and non-transplanted control mutants. Mice not showing motor symptoms were used as wildtype controls.

Isolation of bone marrow cells and CD117+ cell enrichment

We obtained hSCs from the bone marrow of adult green fluorescent protein (GFP) transgenic mice, which ubiquitously express GFP under the control of the chicken beta-actin promoter (Okabe et al., 1997). CD117+ bone marrow cells were enriched as previously described (Bonilla et al., 2002).

Surgery and transplantation

All experiments were carried out according to the guidelines of the European Council Directive for the Care of the Laboratory Animals. Adult mdf mice (6 weeks old, n=43) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), by intraperitoneal injection, and laminectomized at the L4–L5 vertebral level. Using a surgical microscope, a suspension of 300,000 hSCs in 0.5 µl of culture medium (RPMI 1640) was injected unilaterally into the right spinal cord through the posterior funiculus, at 0.1 mm from the dorsal medullar sulcus. After laminectomy, the meninges were cut using a tungsten needle and the cells were slowly injected into the spinal cord parenchyma (1.5–2 mm from the surface) with a glass micropipette connected to a silicon tube, under controlled air pressure. After the injection, the incision was sutured, and mice closely observed during recovery from anesthesia. In parallel, a group of mdf mice (sham-operated; n=19) were subjected to a similar laminectomy procedure and to an intraspinal injection of 0.5 µl of the suspension medium (RPMI 1640).

Behavioral testing

We analyzed motor function by several behavioral tests including open field, footprint, and rotarod performance. To evaluate locomotion evolution, these tests were performed weekly 3 weeks before and then 3 weeks after transplantation. The open field test was a modification of the method described by Broadhurst (1960). In each session the animal was tested 3 times and the mean value was used for further analysis. For footprint analysis, we used a modification of the method described by De Medinacelli et al. (1982). For quantification we computed the number of complete prints versus the number of total prints. We considered a complete print as four visible toes and the plantar print. In each testing session, the animal was explored 3 times, with at least a 60-min interval between each test. For rotarod analysis, mice were first given 2 days to become acquainted with the rotarod apparatus (Letica, LE8500, Panlab, Spain). After that, the rotarod test was performed weekly. Mice were placed on the rotating rod at speeds of 5, 10, 15 and 20 rpm, and the time each mouse remained on the rod was registered automatically. The onset of disease was defined as the time when the mouse could not remain on the rotarod for 7 min at a speed of 20 rpm, as described previously (De Medinacelli et al., 1982).

Electromyography

Compound muscle action potentials (CMAPs) were recorded as previously described (Ruiz et al., 2005). We analyzed a group of 18 mice (5 wild-type, 5 sham-operated and 8 transplanted symptomatic mice), who had been anesthetized with ketamine and xylazine as reported above. Briefly, we used sciatic stimulation needle electrodes, the anode being inserted at the sciatic notch and the cathode 1 cm more distal. The active needle (recording electrode) was inserted into the dorsal foot muscles and the reference electrode into the base of the 5th phalanx. A ground electrode was placed at the base of the tail. Square-wave stimuli of 5 mA current with a duration of 0.05 ms were delivered at a frequency of 10–100 Hz by means of an isolated pulse stimulator (A-M Systems, Model 2100). Recorded outputs were differentially amplified (Brownlee Precision, Model 210A), digitally acquired at 20,000–40,000 samples/s (ADInstruments, PowerLab/4SP) and stored in a computer for later analysis.

Immunohistochemistry

At 1-, 3-, 5- and 10-weeks post-grafting, animals were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused through the heart (left ventricle), first with 30 ml PBS and then with 100 ml fixative solution containing 4% paraformaldehyde in phosphate buffer, pH 7.4. The spinal cord was carefully excised and kept in the same fixative overnight. After fixation, spinal cords were cryopreserved in 20% sucrose in PBS

and embedded in Tissue-Tek. Transverse sections of 20 μm were cut and mounted on slides. The following antibodies were used to analyze the possible differentiation of hSCs *in vivo*: anti-O4 IgM monoclonal antibody (Chemicon, Temecula, CA; 1/1000); anti-GFAP rat monoclonal antibody (IgG isotope; Calbiochem, San Diego, CA; 1/200); anti-TUJ-1 polyclonal antibody (IgG isotope; Eurogenec, Belgium; 1/300); anti-GDNF polyclonal antibody (IgG isotope; Santa Cruz Biotechnology, INC; 1/500) and anti-nestin monoclonal antibody (IgG isotope; Chemicon; 1/100). For analysis of microglia, we used biotinylated tomato lectin (*Lycopersicon esculentum* lectin-LEA-Sigma-Aldrich, Madrid; 1/300). Histological samples were observed using a fluorescence microscope (Leica DMR, Leica Microsystems) and photographs were taken with the same microscope or with a confocal microscope (Leica DMR).

In situ hybridization

Digoxigenin-UTP-labeled (Dig-UTP) single-strand antisense RNA probes were prepared according to instructions of the manufacturer (Boehringer-Mannheim) from GDNF plasmid provided by Dr. Arenas from the Karolinska Institute. *In situ* hybridization was performed following the protocol described previously by Schaeren-Wiemers and Gerfin-Moser (1993).

Motoneuron counting after sciatic nerve axotomy

Cell counting was performed using three wild-type, five symptomatic hSC-grafted, and nine sham-operated symptomatic mice injected with cell culture media. One week before being sacrificed, the right sciatic nerve was exposed by dissection, cut at the level of the knee, and then placed over a small piece of parafilm. A drop of distilled water was applied to the proximal end of the nerve, followed by the application of a few crystals of tetramethylrhodamine-dextran-lysine (Rho+) (Molecular probes). After a survival period of 7 days, which is the time required for complete retrograde motoneuron labeling after sciatic nerve axotomy, the animals were over-anesthetized and processed for histological techniques. To compare the number of motoneurons in the lumbosacral enlargement of the spinal cord in hSC-grafted and non-grafted mice, we counted only neurons located in the ventral horn that were Rho+ and contained a distinct nucleus.

ELISA measurements

A group of 27 animals was used to study the possible neuroprotective effects of glial cell line derived neurotrophic factor (GDNF) levels in the grafted spinal cord area. Seven days after surgery, mice were anesthetized with an overdose of pentobarbital (100 mg/kg) and decapitated. Different groups of animals were used: wild-type mice (n=3), non-operated mdm clinically affected animals (n=6), hSC transplanted mdm affected animals (n=11) and symptomatic sham-operated mice (n=7). The lumbosacral (L4-S1 segments) enlargement of the spinal cords was removed within 10–15 min after decapitation. Tissue was homogenized in 150 μl of ice-cold extraction buffer and mixed completely by vortexing. This mix was then centrifuged, and the collected supernatant was stored at $-70\text{ }^{\circ}\text{C}$. All samples were used within 1 month after freezing. The tissue levels of GDNF were measured with an ELISA kit (GDNF Emax ImmunoAssay System; Promega, Madison, WI), according to the protocol of the supplier.

Statistical analysis

Data are presented as means \pm SEM. Statistical comparisons were made using the Student's t-test for means when the distribution was normal and the Mann-Whitney Rank Sum Test (MWR) when the distribution was not normal. The Z-test was used for comparison of proportions (Sigmastat 2.0; Jandel Scientific Software, Erkrath, Germany).

Results

hSCs survive in the spinal cord

Histological analysis of spinal cords from transplanted mdm mice revealed the presence of GFP+ hSCs in the experimental spinal cord segments up to 10 weeks after transplantation. The injection tract, crossing the dorsal funiculus, and the mass of grafted cells in the dorsal horn were partially encapsulated by an astroglial reaction at 1 week after transplant (Figs. 1A–C). Abundant engrafted GFP+ hSCs were also detected outside the reactive glial capsule in the dorsal and ventral spinal regions, both in white and grey matter (n=12; Figs. 1D–I). Grafted hSCs appeared with a polygonal or spheroid small-shaped morphology with short radially distributed dendrites and exhibited no evidence of morphological differentiation (Figs. 1G–I). Ten weeks after transplantation, the grafted hSCs still remained in the injected spinal cord segments, with a similar cell morphology and dispersion pattern (n=5; Figs. 1J, M, P, S). Between 1 and 3 weeks after transplant, the number of grafted cells was increased in the anterior horn of the grafted spinal cord segments, around the motoneurons (Figs. 5A–C, G–L). Although the distribution of the grafted cells in the host spinal cord was similar between analyzed cases at each different period of time, the number of donor cells showed some variability. We counted a mean of 95 ± 25 GFP-positive cells per section in 10 consecutive sections of the central grafting area at 1 week after transplant (representing a spinal cord segment of 200–300 μm ; n=3) and 212 ± 50 GFP-positive cells under similar conditions at 3 weeks after transplant (n=3). Dispersed cells were observed along 500–800 μm rostral and caudal to this central area, in a symmetrically decreasing distribution. This shows that in the grafted spinal segments, approximately 10000 donor cells survived at 1 week and donor cell number increased to 24000 cells during the 3 weeks after

transplants, suggesting an important process of cell loss immediately after the grafting procedure, followed by an increase in cell number in the donor cell population because of cell proliferation. These data are in agreement with our previous results showing important cell loss and donor cell proliferation with a similar cell population after intracerebral grafts in mouse (Bonilla et al., 2002, 2005). Cell aggregation masses, epithelial pseudo-rosettes or perivascular cell clusters indicative of a tumor at or near the injection site, were not observed in any of the transplanted animals.

We also analyzed microglial activation in response to grafting using biotinylated tomato lectin (L. esculentum lectin-LEA) immunohistochemistry. Three weeks after transplantation, abundant microglial cells were found to be localized in the areas of the spinal cord where the engrafted hSCs were detected (n=7; Figs. 2A–G). Whereas most of the grafted hSCs (GFP-positive) were not LEA labeled (red), colocalization of GFP and LEA was observed in about 5–10% of cells in the grafted area (we observed 8–23 GFP+/LEA+ cells in each section of the central transplanted area; Figs. 2E, F). This result suggests that some grafted hSCs differentiated into microglial cells or, alternatively, that microglial reactive cells have phagocytosed the donor cells and therefore show GFP in the cytoplasm. We cannot exclude one of these alternatives at the present time. In sham-operated mice, microglial reaction was not evident in the operated spinal cord segments (Fig. 2H).

hSCs do not differentiate into neural cells

In contrast to our previous results with adult hSC intracerebral grafts in neonatal mice (Bonilla et al., 2002, 2005), in the adult spinal cord, hSCs do not differentiate into any type of CNS derived cell. In all analyzed cases (n=32), hSCs did not express, at the 3rd and 10th post-grafting weeks, specific markers for neurons (TUJ-1) (Figs. 1E, F, N, O and 5A–C), astrocytes (GFAP) (Figs. 1B, C, Q, R) or oligodendrocytes (O4) (Figs. 1T, U). Conversely, analysis of microglial cells (LEA) suggests that some hSCs may be able to differentiate into microglia when engrafted in the spinal cord (Fig. 2). The common mesodermal origin of hSCs and microglia during embryogenesis supports the possible existence of microglial histogenic potential in donor hSCs (Chugani et al., 1991). To study the possibility that transplanted hSCs differentiated into a progenitor neural state, we used an anti-nestin antibody. GFP was not found to be colocalized with nestin either (Figs. 1J–L) (n=19 mice).

Behavioral test

We compared the motor function of non-grafted sham operated and hSC-grafted symptomatic mdf mice at different times, using the open field test, the footprint test and rotarod performance. In the open field test, the locomotion frequency ratio post-surgery/pre-surgery was calculated for each animal. The pre-surgery values were obtained 1 week before surgery, and the post-surgery values 1 and 2 weeks after surgery. At both times after surgery, the ratios in hSC-transplanted mdf mice were >1 (n=6) (Table 1). In contrast, in non-grafted mice the ratios were <1 (n=5) (Table 1).

The footprint test was used as a semi-quantitative test to compare locomotion patterns in wild-type and symptomatic mdf mice, and also to evaluate qualitative changes in mdf mice before and after the hSCs graft. In wild-type mice (n=8), prints were complete and the walking pattern was very regular (Fig. 3A). However, in the non-transplanted symptomatic mice (n=8), the pattern was irregular with fewer complete plantar footprints and, sometimes, the mice stopped before the end of the walking pathway (Fig. 3B). The absence of backward or exploratory movements at the stopping point in the test trajectory suggested muscular weakness as the cause of these premature stops. In the transplanted symptomatic mice, the walking trajectory was always completed and there was a significant increase in the number of complete plantar footprints 3 weeks after the transplant (compare panels B and C in Fig. 3). Complete plantar footprints before and after the transplant were 8.1 ± 3.1 and 16.7 ± 6.3 , respectively (n=8, $p<0.05$ paired t-test). In contrast, in the sham-operated symptomatic mice, we never observed an increase in the number of complete plantar footprints (0/8).

In the rotarod test, hSC-grafted mice showed a tendency to improve their motor performance (63% improvement at 5 rpm and 50% at 10 rpm, see Table 2) 1–2 weeks after the transplant (n=8) in comparison with symptomatic mice without surgery (n=8) and sham-operated mice (n=4). Nevertheless, this improvement in the rotarod performance of hSC-grafted mice was temporary, being present from 1 to 8 weeks post-surgery, since performance subsequently decreased progressively after 8 weeks. Nevertheless, motor performance after the post-transplant recovery period seems to be clearly better than the motor function predicted by the observed progressive deterioration in the symptomatic control groups.

hSCs improve motor function in mdf mice

To further characterize motor function in mdf mice and to evaluate the possible recovery of physiological activity in the hSC-grafted animals, we recorded the evoked activity of the extensor muscles from the dorsal side of the right hindlimb foot 1 week after transplantation or equivalent protocol (see below). In addition, animals were tested for the presence of spontaneous electrical muscular activity. In symptomatic mice, spontaneous electrical activity in paraspinal muscles was found in non-transplanted and transplanted mice, while it was not detected in wild-type mice. Fig. 4A shows an example of such activity recorded in a symptomatic animal, indicating a certain degree of denervation.

For evoked responses, we first examined the responses to a single stimulus. The shapes and peak-to-peak amplitudes of the CMAPs (compound muscle action potentials) (Fig. 4B) were similar in wild-type (12.98 ± 6.21 mV; mean \pm SD, n=5) and nontransplanted symptomatic mutant mice (control and sham-operated) (12.28 ± 7.35 mV; n=5). However, the amplitudes of the CMAPs recorded in transplanted symptomatic mutant mice were much more variable (21.36 ± 19.96 mV; n=8), although the mean was not statistically different to those recorded in the wild-type and non-transplanted symptomatic mutant mice. With repetitive nerve stimulation, CMAPs gradually declined in amplitude until reaching a plateau of maximal depression after several pulses. The amount of depression is directly proportional to the stimulation frequency. We compared this physiological response in wild-type and in operated mice (transplanted with hSCs and sham-operated mice) (Fig. 4C). The decline in amplitude of the three first CMAPs was much larger in non-transplanted mice (dashed lines) than in wild-type and transplanted mice. The normalized amplitude of the CMAPs at the quasi steady-state (see Fig. 4D) was much less in the non-transplanted symptomatic mice (0.58 ± 0.04 , n=5) than in the wild-type (0.75 ± 0.04 , n=5) and in the transplanted symptomatic mice (0.7 ± 0.03 , n=8), indicating a tendency to normality of the short-term neuromuscular depression in transplanted symptomatic mice.

Neuroprotective effect of hSCs on spinal cord motoneurons

To assess the neuroprotective effect of the hSCs on motoneurons, we counted neurons that retained intact their peripheral projections through the sciatic nerve. We compared the number of Rho+ spinal motoneurons in three different mice groups (wildtype, mdf hSC-grafted and sham-operated symptomatic), 1 week after rhodamine-dextran-lysine application (5 weeks after grafting). Sham-operated and non-transplanted symptomatic mice showed severe loss of Rho+ motoneurons compared to wild-type (860 ± 8 in wild-type, n=3, versus 300 ± 53 in both sham-operated and symptomatic mice without surgery, n=5, mean \pm SE; t-test, $p < 0.001$) (Fig. 5F). In contrast, in the hSC-grafted mice, a significantly larger number of motoneurons remained in the lumbosacral segment (441 ± 27 , n=9, $p < 0.05$, t-test) in comparison with symptomatic mice without surgery (Figs. 5E, F), suggesting a markedly protective effect of the hSCs on affected motoneurons.

An increased concentration of GDNF generates the cellular neuroprotective effect

Previous work has suggested that GDNF exerts a neuroprotective effect on motoneuron loss (Wang et al., 2002; Saito et al., 2003; Aszmann et al., 2004). To test if the positive effects that we observed in mdf-grafted mice were related to an increase in the levels of this neurotrophic factor in the spinal cord, we measured the concentration of GDNF in the lumbosacral region of the spinal cord by enzyme-linked immunosorbent assay (ELISA) in four groups of mice. In the wild-type mouse group, the mean basal level of GDNF (133 ± 6.5 pg/ml, n=3) was significantly lower than in the symptomatic non-operated mice group (205 ± 37.2 pg/ml, n=6) (t-test, $p < 0.05$). GDNF levels in the sham-operated mouse group (209 ± 42 pg/ml, n=7) were not different to those of the nonoperated mouse group (t-test, $p = 0.849$), suggesting that the increase in GDNF level was not evoked by surgery itself. GDNF levels in the symptomatic grafted mouse (244 ± 33.5 pg/ml, n=11) was significantly higher than in the sham-operated symptomatic mouse (t-test, $p < 0.05$) (Fig. 6A). These results suggest that hSCs engrafted into the spinal cord were able to increase the concentration of GDNF in the neural parenchyma.

We also investigated the expression and cellular distribution of GDNF in the graft area by in situ hybridization and immunohistochemistry. GDNF mRNA transcripts were detected in the grafted area, where the majority of grafted cells were found to express GDNF (n=3). Moreover, some small host cells around the graft also expressed GDNF transcripts (Figs. 6B–C). In the sham-operated symptomatic mice no GDNF transcripts were found (data not shown). Immunohistochemistry using anti-GDNF antibody (n=3) revealed intense GDNF immunoreactivity in grafted cells, as well as in some host cells (n=3; Figs. 6D–G). In sham-operated symptomatic mice, no GDNF immunoreactivity was found (data not shown).

Discussion

This study demonstrates that hSCs obtained from the adult mouse survive for at least 10 weeks after being transplanted into the spinal cord of mdf mice, a model of motoneuron degeneration. We also found that in the spinal cord, the transplanted hSCs did not differentiate into any primary neural cell, in contrast to our previous results with this bone marrow population of cells which differentiates into neurons and glial cells when grafted into neonatal brains (Bonilla et al., 2002, 2005). The restricted differentiation of stem cells in the spinal cord has also been previously reported using neural stem or fetal spinal cord cells (Onifer et al., 1997; Liu et al., 1999; Cao et al., 2001; McDonald et al., 2004; but see Koshizuka et al., 2004). Conversely, grafts of hSCs or neural stem cells are able to undergo neural differentiation in the brain (Eglitis and Mezey, 1997; Azizi et al., 1998; Kopen et al., 1999; Mezey et al., 2000, 2003; Kogler et al., 2004). These distinct fates may be due to the fact that the brain is more permissive and has more inductive capability to develop a neural phenotype from undifferentiated stem cells than the spinal cord (Teng et al., 2003). The possible microglial differentiation of the grafted cells suggests that some adult hSCs may retain the potential to develop brain microglia under appropriate environmental conditions. The common origin of hSCs and microglia in bone marrow supports this hypothesis, but the existence of GFP-positive vesicles in brain reactive microglia after hSC phagocytosis as an alternative explanation cannot be excluded.

In the mutant grafted mice, a significant functional recovery of motor performance was found, as assessed by electromyography, and also by rotarod performance, the open field test and the footprint test. These results

corroborate those obtained by other authors using human neuron-like cells (hNT cells) or non-neural cells in an animal model of familiar ALS (Clement et al., 2003; Garbuzova-Davis et al., 2002; Willing et al., 2001). Moreover, McDonald et al. (2004) demonstrated that transplantation of embryonic stem cells promotes functional recovery after spinal cord injury. A recent report using bone marrow stromal cells in a clinical trial of cellular therapy in ALS, has shown preliminary positive results in motor performance of transplanted patients (Silani et al., 2004; Mazzini et al., 2003, 2004). In five additional mdm mice, we injected hSCs into the spinal subdural space without lesion to the spinal cord. We detected GFP positive cells engrafted in the dorsal root ganglia, nerve roots and pial surface of the spinal cord, but never in the neural parenchyma 3 weeks after cell injection (data not shown). Functional recovery was never observed in these cases.

In the majority of cell therapy studies using motoneuron degenerative models, transplants are performed prior to the onset of behavioral symptoms (Garbuzova-Davis et al., 2002; Willing et al., 2001; Boillee et al., 2006). In contrast, in our experimental strategy, transplantation is carried out in already symptomatic mice. We believe that this is specially relevant because for the moment, in most human motoneuron diseases, and in particular in ALS, there is currently no marker for the pre-clinic diagnosis of patients before the onset of the symptomatic phase, when it is assumed that important cell loss has already occurred.

The functional recovery of transplanted mdm mice, observed by the rotarod test, lasted for up to 8 weeks post-transplantation, before declining. This loss of function may be due to reduced GDNF secretion by donor and host cells or to receptor desensitization. Because of the allogenic character of the cell grafts it is possible that after an initial beneficial effect, donor cells become detrimental in their neurotrophic activity either by attacking recipient elements (reaction of the graft versus the host due to the immunogenic character of hSCs) or by inducing some kind of immunological reaction in the donor tissue. Experiments in progress are exploring the cellular and molecular mechanisms which underlies this reduced neurotrophic effect and the progression of motor deficits after this post-surgery time.

Although it has been demonstrated that hSCs in neonatal brains can differentiate into neural cells (Bonilla et al., 2002, 2005), in agreement with Hudson et al. (2004), we observed that grafted hSCs did not express any neuronal or macroglial markers, thus corroborating reports suggesting a lack of neural inducing properties of the spinal cord (Hammang et al., 1997; Onifer et al., 1997; Zompa et al., 1997; Cao et al., 2001). Therefore, motoneuron regeneration does not appear to be the cause of the functional motor improvement in the transplanted mice. An alternative explanation is that the increased motor activity in the mdm mice after the transplant could be attributable to a delivery of trophic factors produced directly or indirectly by the hSCs. Beneficial effects due to neurotrophic factors were first described by Nieto-Sampedro et al. (1984), studying the survival of brain transplants in the cortex of the neonatal rat.

Trophic factors could either recover and stimulate the function of surviving neurons, or rescue neurons in reversible phases of cell death. GDNF is intensely expressed in motoneurons during mouse embryogenesis indicating its possible involvement in motoneuron differentiation and maturation (see Supplementary figure). Our data on sciatic nerve motoneuron counting and the increase in GDNF concentration in grafted mutant mice strongly support the trophic interpretation. It has been reported that neurotrophic factors such as insulin growth factor 1 (IGF-1) and GDNF delay the onset of neurological symptoms and extend survival in the SOD1 mouse (Ascadi et al., 2002; Wang et al., 2002; Kaspar et al., 2003; reviewed by Ekestern, 2004). Thus, we analyzed GDNF expression by mRNA in situ hybridization and anti-GDNF immunohistochemistry and evaluated GDNF protein levels by ELISA immunoassay. Our results show that hSCs express GDNF when grafted into the spinal cord and induce the host cell to produce it. The localization of host cells expressing GDNF in the anterior horn and around the motoneurons, together with their small size suggests their astroglial or microglial character. The intimate association between the GDNF producing cells and the motoneurons could permit the accumulation of this trophic factor locally, just in the microenvironment around motoneurons. This might explain how the small increase observed in the ELISA analysis could result in such a substantial functional improvement. Taylor et al. (2006) reported similar cell nonautonomous benefits in a mouse model of Krabbe leukodystrophy, using neural stem cells. Taken together these results demonstrate that wild-type non-neuronal cells, in our case adult bone marrow hematopoietic progenitors, can be activated to express GDNF and act as local "pumps" of these molecules, resulting in improved motor function when transplanted into the spinal cord of a mouse model of motoneuron degeneration. Then, the two main problems in the use of neurotrophic factors in therapeutic trials, the short lifetime of these molecules and the difficulties for continuous local administration (reviewed by Ekestern, 2004) could be resolved with the local production of such factors by transplanted hSCs. In addition to these partially identified cell non-autonomous trophic effects, cell-cell interactions between normal grafted and damaged host cells may also be a source of unknown factors with beneficial effects, such as those reported by Boillee et al. (2006) between normal microglia and motoneurons in the SOD1 mutant mouse.

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Figure 1

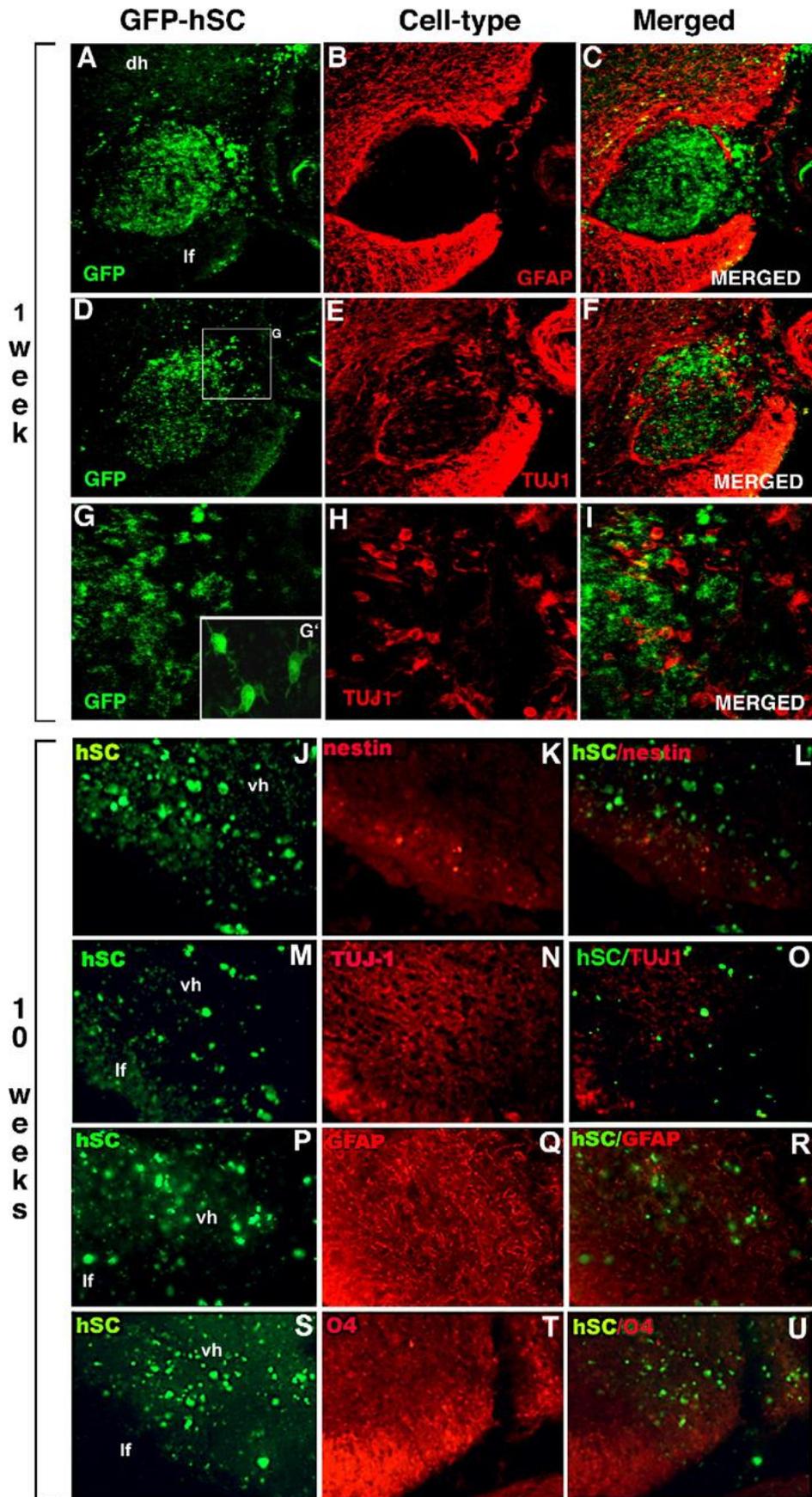


Figure. 2

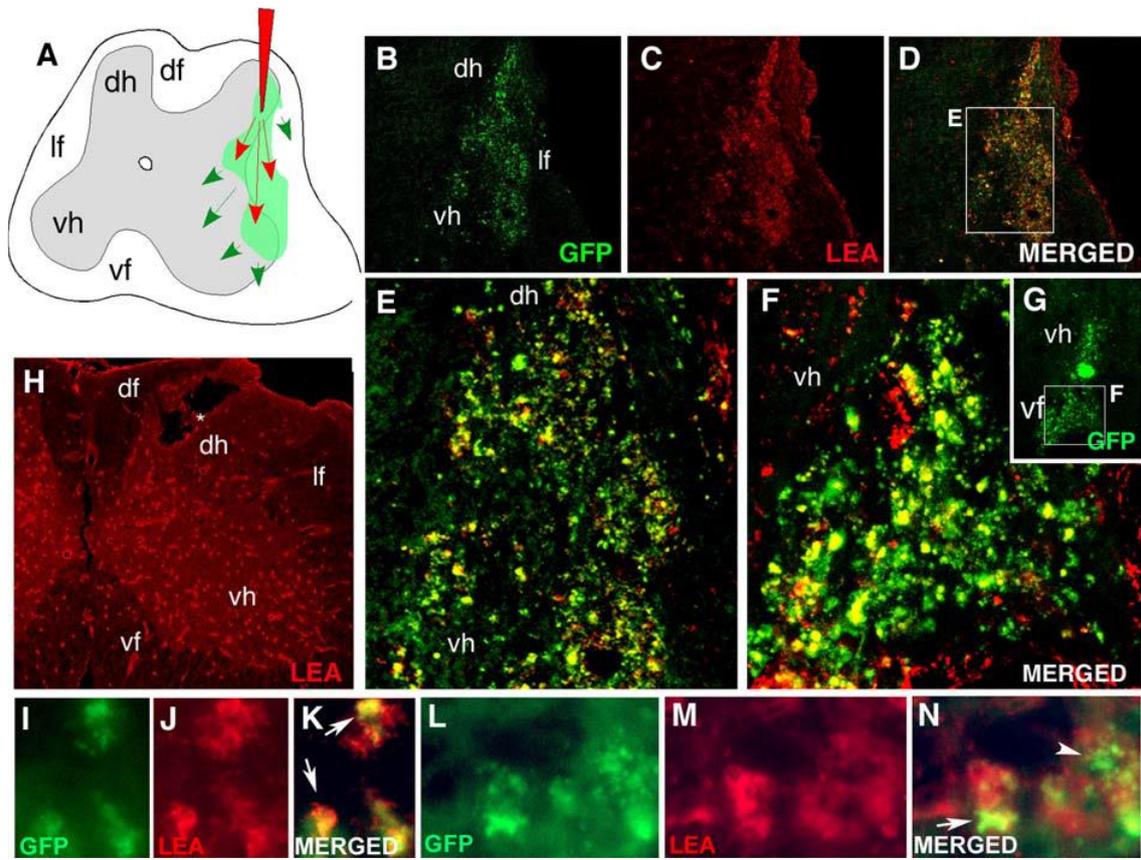


Figure 3

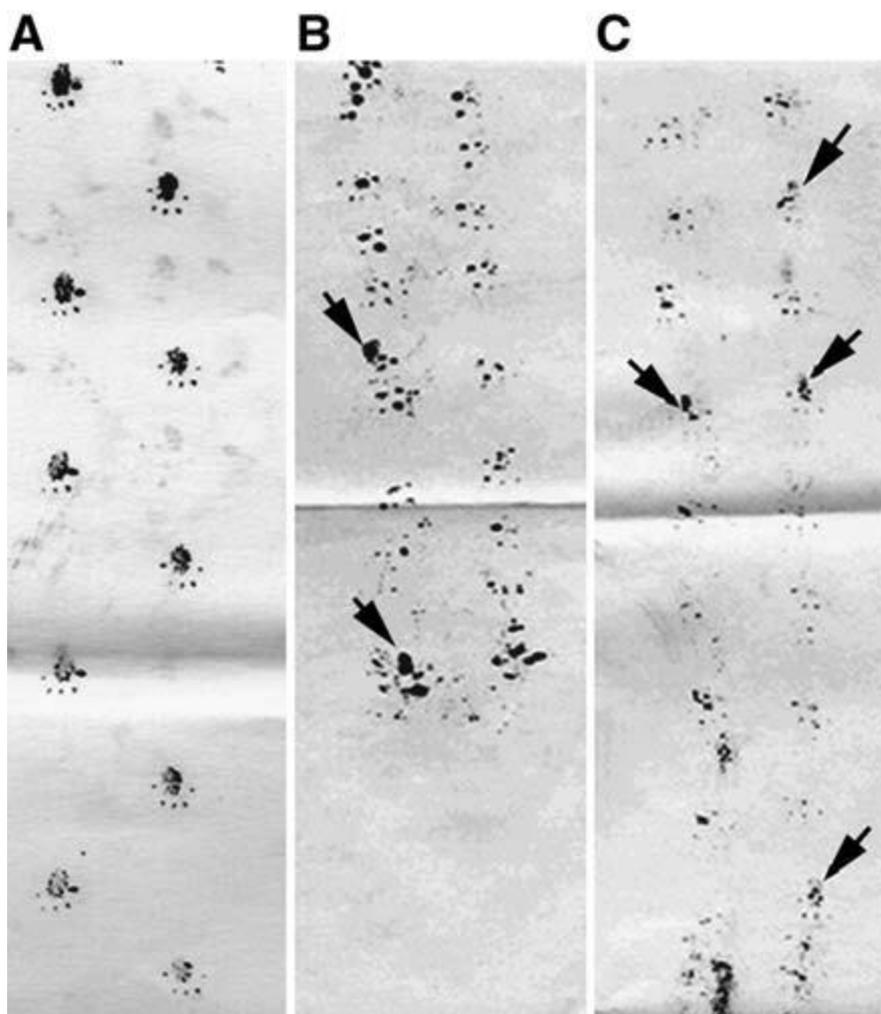


Figure 4

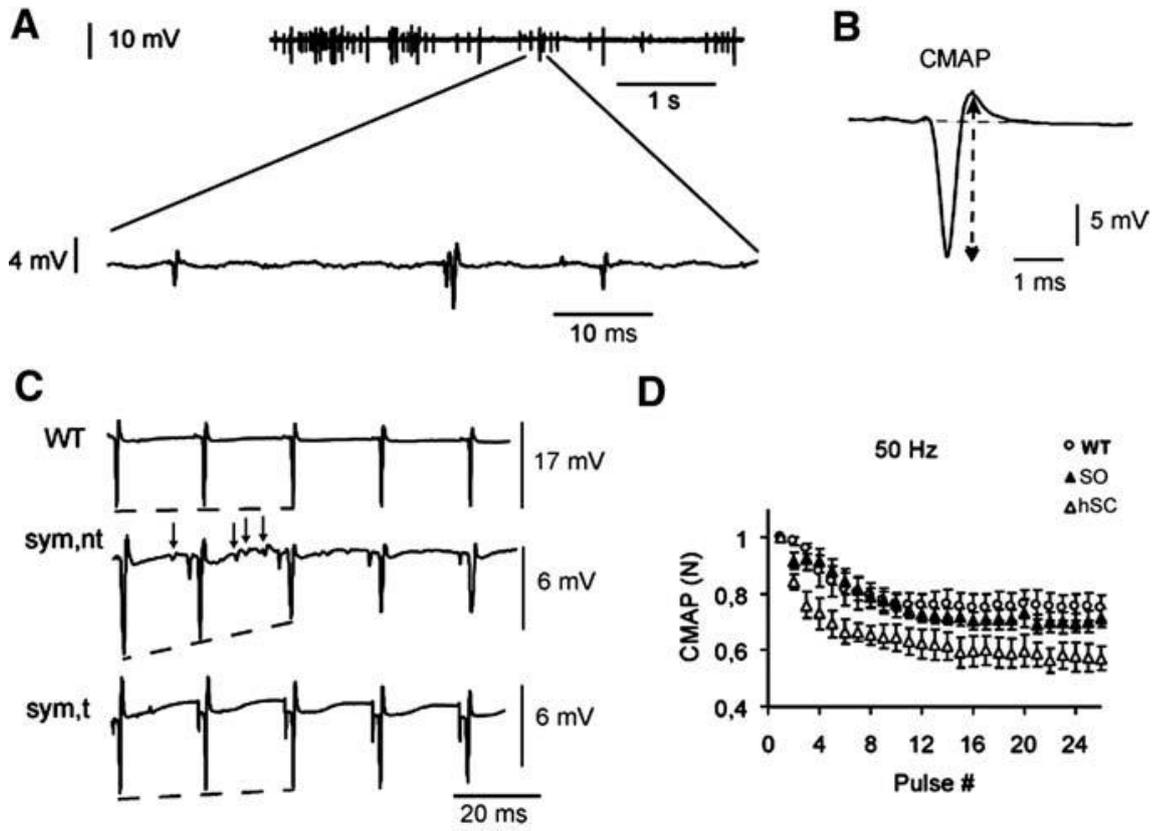


Figure 5

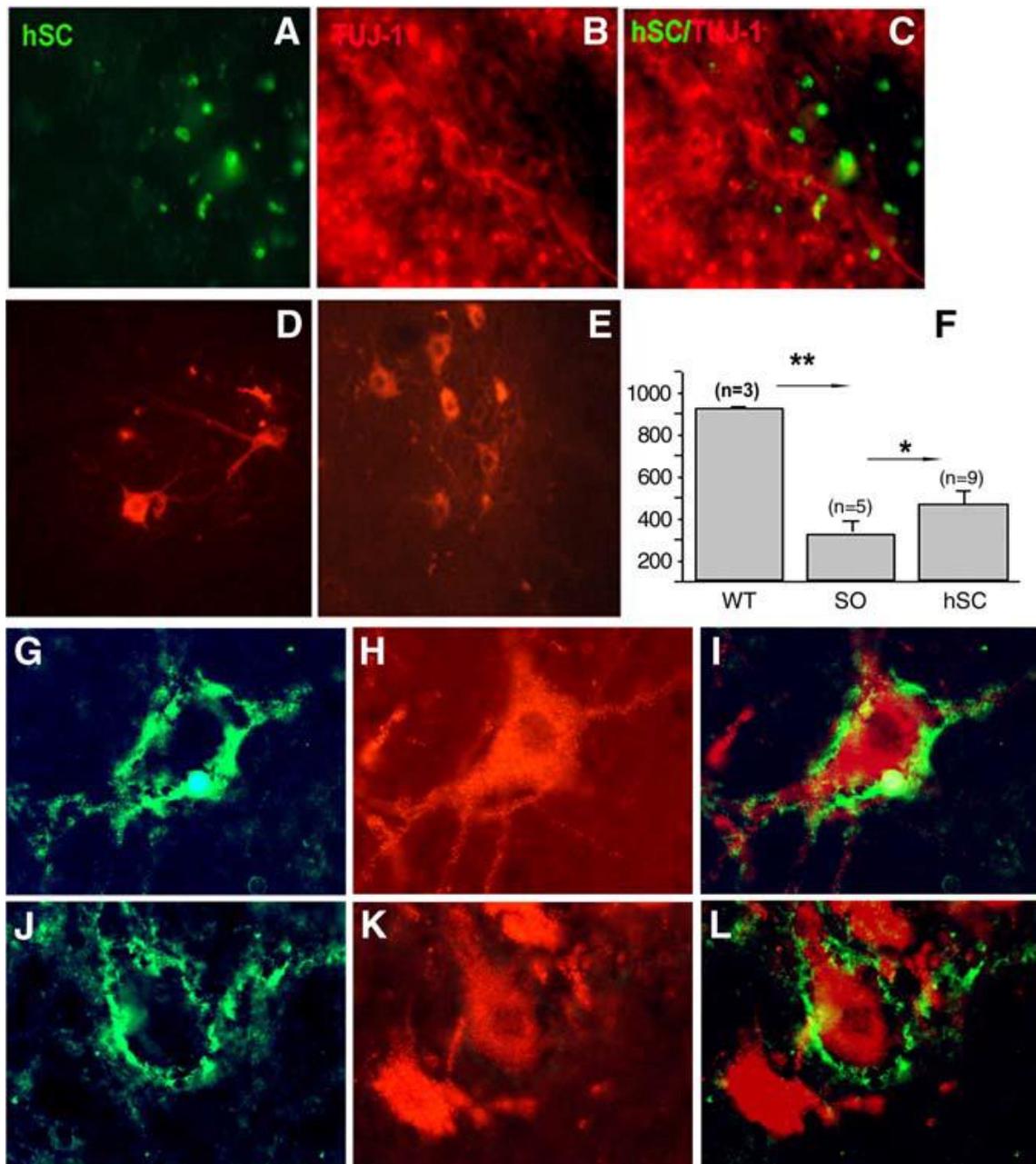


Figure 6

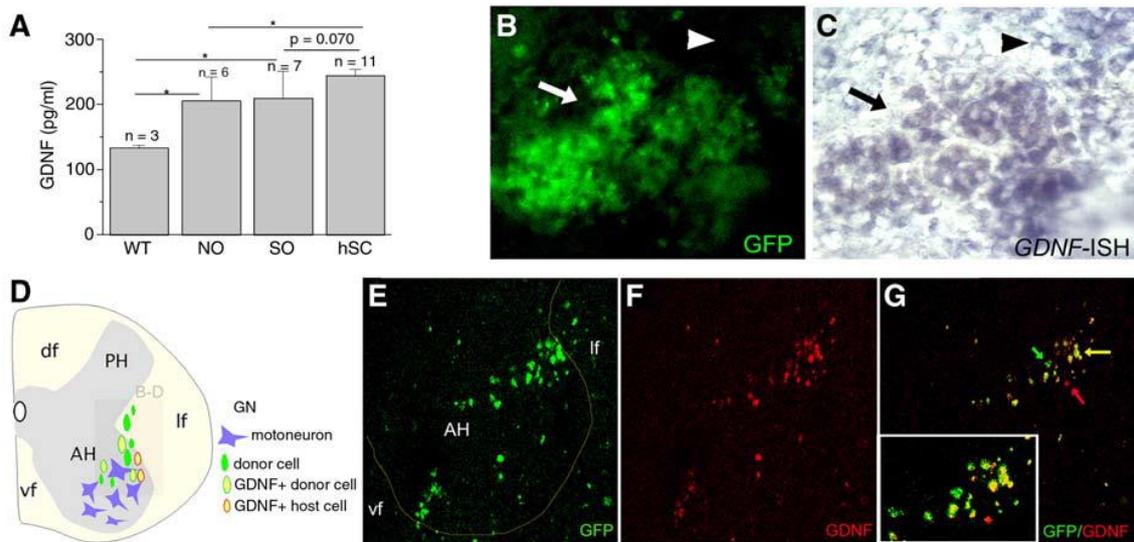


Figure legends

Fig. 1. Hematopoietic stem cells (hSCs) survive in the host spinal cord and do not differentiate into neural or macroglial cells. (A–I) Histological analysis of mice 1 week after transplantation revealed intraparenchymal engraftment of hSCs. Donor cells were detected by their direct GFP fluorescence in the spinal cord (A, D, G). Immunohistochemistry using an anti-GFAP antibody (B, C) revealed an astroglial reaction around the hSC graft. (J–U) Images obtained from the spinal cord of a mouse 10 weeks after hSC grafting, confirming that the hSCs have survived 10 weeks after transplantation into the host spinal cord. Immunohistochemistry was performed on spinal cord sections to determine if the transplanted hSCs express neural markers such as TUJ-1 (D–I at 1 week after hSC grafting and M–O at 10 weeks after hSC grafting), or glial markers such as GFAP (A–C and P–R at 1 and 10 weeks after hSC grafting, respectively) or O4 (S–U). Colocalization of TUJ-1, GFAP or O4 with the green fluorescence of the hSCs was not observed either 1 or 10 weeks post-transplantation. (J–L) The neural progenitor marker nestin (K) is not expressed by hSCs (J). (G) Insert in panel G shows a higher magnification of donor cells in a spinal cord section from a grafted mdm mouse. df: Dorsal funiculus, dh: dorsal horn; lf: lateral funiculus; vf: ventral funiculus; vh: ventral horn.

Fig. 2. Microglial reaction and differentiation of donor hematopoietic stem cells. (A) Representative diagram of the graft localization and cellular dispersion in grafted spinal cords. (B–D) Low magnification images showing the grafted area and the microglial reaction (C) in the experimental side at 3 weeks after graft. (E, F) High magnifications of the grafted area in panels (B, G) respectively (the amplified area is labeled by a polygonal line). (I–N) High magnification images showing the co-expression of GFP and LEA in yellow cells (arrows), while some donor cells do not exhibit microglial labeling (arrowhead in N).

Fig. 3. Comparison of the normal footprint pattern in an adult wild-type mouse (A) and a symptomatic mouse before (B) and after (C) hematopoietic stem cell transplantation. The images show a clear improvement in the motor pattern of the symptomatic mouse grafted with hSCs. Note the increase in the number of complete plantar footprints (indicated by arrows) after the transplant. The footprint test was performed 1 week before (B) and 1 week after (C) the hSC graft.

Fig. 4. Physiological recovery of hematopoietic stem cell transplanted mice. Electromyographs were obtained from the different groups of mice 1 week after graft transplantation. (A) Spontaneous electrical activity in paraspinous muscles recorded in symptomatic mice. (B) Example of a compound muscle action potential (CMAP) recorded from dorsal foot muscles. (C) Representative evoked responses obtained from a wild-type (WT, upper trace), a sham-operated symptomatic mouse (SO, middle trace) and a transplanted symptomatic mouse (hSC, lower trace) upon applying a stimulation train of 0.5 s at 100 Hz (only the first five responses are shown). Note the presence of asynchronous spontaneous electrical activity in the recording of the non-transplanted mutant mouse (arrows). (D) Normalized amplitude of the CMAPs in wild-type (n=5), transplanted (n=8) and non-transplanted (n=5) mutant mice in response to 50 Hz train stimuli.

Fig. 5. Localization in the anterior horn and neuroprotective effect of hematopoietic stem cells. Micrographs (A–C) show the grafted hSCs localized in the ventral horn around symptomatic mice motoneurons, identified by their localization, morphology, and TUJ-1 immunoreactivity. Panels D–E show the rhodamine-positive cells at the level of L2 1 week after sciatic axotomy in sham-operated symptomatic mice (D) and in a symptomatic mouse grafted with hSCs (E) at the same medullary level. In panel F, the mean value for the different groups is plotted, showing motoneuron rescue by hSC graft (WT: wild-type; SO: sham-operated symptomatic mice; hSC: symptomatic mice grafted with hSCs). (G–L) High magnification images showing the localization of small GFP-positive cells intimately surrounding sciatic motoneurons retrogradely labeled by rhodamine.

Fig. 6. Glial cell line derived neurotrophic factor (GDNF) is expressed by host cells and by grafted hSCs. ELISA assays revealed that 1 week after the graft, GDNF levels in the symptomatic mice were higher than in wild-type mice, and the presence of the hSC graft increases this level further (A). Statistically significant differences between experimental and control mice are represented by asterisks (WT: wild-type; NO: non-operated symptomatic mice; SO: shamoperated mice and hSC: symptomatic mice grafted with hSCs). GDNF in situ hybridization shows that the GDNF is expressed by the grafted hSCs (arrow) and by some host cells (arrowhead) (B–C). Schematic representation of cell graft localization in the analyzed spinal cord sections (D). Immunohistochemistry using a GDNF antibody (E–G) revealed hSCs expressing GDNF (G, yellow arrow), or not expressing GDNF (G, green arrow), and some cells from the host tissue also expressing GDNF (G, red arrow). (E–G) Images from confocal microscopy of 1- μ m-thick sections. The type of labeling is indicated in the lower right corner.

Tables

Table 1

Open field	hSC (n=6)	Symptomatic (n=5)
Locomotion performance ratio		
Post/Pre-surgery 1 week after	1.3±0.09	0.9±0.08*
Post/Pre-surgery 2 weeks after	1.3±0.18	0.75±0.17*

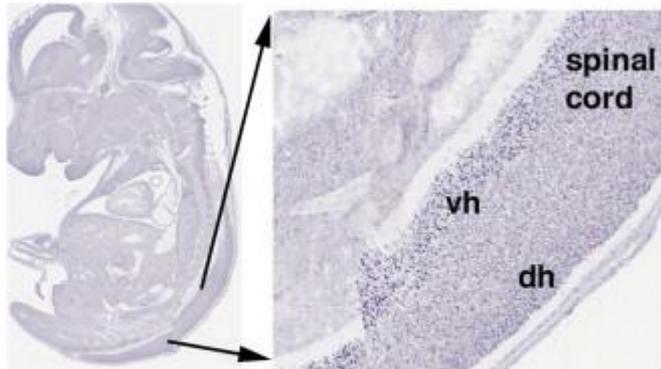
Comparison of the open field test post/pre-surgery ratio in symptomatic animals grafted with hSCs and non-grafted symptomatic animals. The ratio for each animal was obtained by measuring locomotor frequency (see Materials and methods) after and before surgery. A mean value was obtained for each individual animal (3 tests/session). Subsequently, the mean value for each group was subtracted. The t-test was applied; * indicates p<0.05.

Table 2

Rotarod test						
Motor Performance post/pre-surgery	5 rpm Symptomatic (n=8)	5 rpm hSC (n=8)	5 rpm Sham-operated (n=4)	10 rpm SYMT control (n=8)	10 rpm hSC (n=8)	10 rpm Sham-operated (n=4)
Better performance	0/8 0%	5/8 63%	0/4 0%	0/8 0%	4/8 50%	0/4 0%
No change	8/8 100%	1/8* 12%	3/4 75%	7/8 87%	2/8* 25%	2/4 50%
Worse performance	0/8 0%	2/8 25%	1/4 25%	1/8 13%	2/8 25%	2/4 50%

Values shown are the percentage of mice in each group showing improvement (ratio >1), no variation (ratio=1) or deterioration (ratio <1) in rotarod performance 1 week after versus 1 week before surgery, at speeds of 5 rpm and 10 rpm. The ratio is between the time remaining on the rotarod, 1 week after and 1 week before the transplant for individual mice in the different groups (symptomatic non-operated mice, symptomatic hSC-grafted mice and symptomatic sham-operated mice). The Z-test was applied. * Indicates p<0.05.

Supplementary data



E15.5 mouse embryo. Sagittal section. GDNF expression was detected by ISH in cells of the ventral horn (vh). Source: www.eurexpress.org