

# Early graft of neural precursors in spinal cord compression reduces glial cyst and improves function

## Laboratory investigation

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**Object.** Spinal cord injury (SCI) often results in irreversible and permanent neurological deficits below the injury site and is considered a pathological state of functional damage to local neurons and axon fibers. There are several experimental treatments to minimize tissue damage, and recently cell transplantation has emerged as a promising approach in spinal cord repair. The authors undertook this study to evaluate grafting of neural tube precursors as a possible therapeutic strategy in a model of spinal cord compression in the mouse.

**Methods.** Compression SCI was induced at the T-13 level in adult male mice. Immediately after injury, neural precursor cells (NPs) were transplanted into the SCI lesion cavity in 18 mice; the remaining 19 mice received saline injections into the lesion cavity and were used as controls. Spinal cords were examined 12, 19, and 26 days postinjury to investigate the survival of the NPs and their effects on the cellular environment, glial scar and glial cyst formation, astrogliosis, and microglial activation.

**Results.** Grafted NPs survived well and integrated into the host spinal cord tissue. Some NPs had differentiated into cells expressing glial and neuronal markers at all 3 end points. Analysis of glial cyst volume showed a lesion volume reduction of 63.2% in the NP-treated mice compared with volume in the injured but untreated mice. There appeared to be no difference in astrogliosis and microglial activation between untreated mice and treated ones. Sensory and motor tests demonstrated that transplantation of NPs promoted improvement in injured and treated animals compared with controls.

**Conclusions.** These results support the therapeutic potential of NPs, demonstrating that they can survive for a long time, differentiate, integrate into the injured spinal cord, and promote functional recovery after SCI.

(DOI: 10.3171/2011.1.SPINE10607)

**KEY WORDS** • spinal cord injury • cell therapy • CNS repair • glial cyst

**S**PIINAL cord injury often results in permanent neurological impairment due to glial activation, oxidative stress, inflammation, cell death, and axon fiber disruption.<sup>9,28</sup> Severed axons are unable to regenerate for many reasons. First of all, regeneration is made difficult by progressive tissue cavitation (formation of a glial cyst, in which macrophage and microglial infiltration occurs). In addition, astrocytes become hypertrophic through increased production of intermediate filaments, giving rise to reactive astrogliosis, and finally surrounding the lesion site and producing a glial scar.<sup>29,45</sup> The role of the glial scar is still unclear, but it represents a physical and biological barrier for axonal regeneration.<sup>13,45</sup> However, recent evidence indicates that it might play several beneficial roles in protecting tissue and preserving function

*Abbreviations used in this paper:* CNTF = ciliary neurotrophic factor; EGFP = enhanced green fluorescent protein; FGF = fibroblast growth factor; GFAP = glial fibrillary acidic protein; MAP-2 = microtubule associated protein-2; NeuN = neuronal nuclei; NGF = nerve growth factor; NP = neural precursor cell; PBS = phosphate-buffered saline; SCI = spinal cord injury; TBST = Tris-buffered saline with 0.1% Tween 20.

after neurotrauma; in fact, astrocytes can support axonal regrowth, limit neuronal degeneration and inflammation, and repair the blood-brain barrier through production of antiinflammatory cytokines and neurotrophic factors such as LIF (leukemia inhibitory factor), IGF (insulin-like growth factor), EGF (epidermal growth factor), NGF, FGF, and CNTF.<sup>12,33,41,42</sup>

One of the most promising strategies for spinal cord repair is transplantation of stem cells into the lesion site.<sup>2,14,44</sup> Different types of stem cells have been used,<sup>7,14,43,52</sup> but to date none has been found to result in a full repair. The greatest regenerative capacity and potential to repair the spinal cord were obtained with cells collected from the immature CNS. In particular, the mouse neural tube at embryonic Day 9 contains multipotent neuroepithelial stem cells that at embryonic Day 12 give rise to lineage-restricted self-renewing NPs, including neuronal- and glial-restricted precursor cells.<sup>31</sup> Neural precursor cells injected into injured rats showed great survival,

This article contains some figures that are displayed in color online but in black and white in the print edition.

consistent migration, and ability to differentiate into mature CNS phenotypes, including neurons.<sup>17,22</sup>

We have previously demonstrated, in a mouse spinal cord hemisection model, that NPs transplanted after the acute phase of SCI are able to integrate into the circuits of the injured spinal cord and promote sprouting of serotonergic fibers and functional recovery.<sup>4</sup> In the present work, we transplanted NPs into another murine model of SCI, the compression injury, which better reproduces a typical lesion due, for example, to an accident. In particular, in this study, we aimed to understand the effects of NPs on the glial scar. Therefore, we studied NP survival and the effect of these cells on the cellular environment, glial cyst and glial scar formation, astrogliosis, microglial activation, and functional recovery compared with injured untreated mice. Another fundamental issue is the question of the best therapeutic time window for transplantation: in the present study, we performed acute NP transplantation.

## Methods

### *Experimental Animals*

We produced SCI (as described below) in adult C57BL/6 J male mice (weight range 22–32 g) purchased from Harlan Laboratories. The animals had free access to food and water. All experimental procedures on live animals were performed according to the European Communities Council Directive of November 24, 1986, and the University of Turin's institutional guidelines on animal welfare. Every effort was made to minimize the number of animals used and their suffering. The NPs for transplantation were collected from BCF1 mice that express EGFP under the influence of a  $\beta$ -actin promoter, kindly provided by Dr. M. Okabe (Osaka University, Suita, Japan)<sup>35</sup> and bred in our animal facility. Pregnant mice were killed at embryonic Day 12 by cervical dislocation, and NPs for transplantation were obtained from the embryonic neural tube.

### *Isolation of NPs*

The embryonic Day 12 EGFP<sup>+</sup> embryos were removed and placed in a petri dish containing chilled saline solution. Caudal neural tubes, corresponding to the 10 lower somites, were mechanically dissected from the surrounding connective tissue and gently triturated with a Pasteur pipette to dissociate the cells. Cells were collected by centrifugation and observed under the microscope (Leitz Diavert); counts were made on a Bürker chamber, after incubation in 0.4% trypan blue in PBS. Cells were resuspended in saline at a final concentration of  $50 \times 10^3$  cells/ $\mu$ l.

### *Surgery and Cell Injection Procedures*

Mice were divided into 2 groups as follows: animals in the control group (19 mice) were subjected to SCI and received saline solution instead of NPs; animals in the NP group (18 mice) were subjected to SCI and were then injected with NPs.

Spinal cord compression was induced according to the model described by Farooque.<sup>11</sup> Briefly, adult C57BL/6 J mice were deeply anesthetized with 3% isoflurane vaporized in O<sub>2</sub>/N<sub>2</sub>O 50:50. A laminectomy was performed at the level of the T-13 vertebra, corresponding to the L-2 neuromer. Compression was applied by means of a round plate (diameter 1.5 mm, weight 10 g), which was gently placed in a longitudinal orientation over the dorsal aspect of the spinal cord and held in place for 5 minutes to produce moderate injury. The plate was then removed. Immediately after injury, the suspension of NPs (2  $\mu$ l containing  $10^5$  cells in saline) was injected slowly (over about 60 seconds) into the spinal cord at the same level as above into the SCI lesion cavity via a glass micropipette (outer-tip diameter 50  $\mu$ m). The control animals received the vehicle (saline solution) alone.

### *Behavioral Testing*

The mice underwent a battery of behavioral tests before surgery to establish baseline for comparison with postsurgical/transplantation values, which were obtained at 4, 7, 12, 19, and 26 days postinjury: posture assessment, foot-fault test, hindlimb flexion, sensory test, and grip test. All behavioral tests were performed by 2 researchers, who were “blinded” to group assignment (control vs NP-treated).

*Posture Assessment.* The mouse was placed on a smooth flat surface and its general posture was observed from behind. The following scores were applied: 0, not different from normal; 1, balance shifted to the lesioned side.<sup>48</sup>

*Foot-Fault Test.* The mice were placed on elevated grids. They put their paws on the wire while moving along the grid. With each weight-bearing step, if the paw fell or slipped between the wires, a foot fault was recorded. The observers counted the total number of foot faults in 30 steps.<sup>39,54</sup>

*Hindlimb Flexion.* The animal's head was covered with a hand and the hindlimbs were gently pulled toward the tail so that they extended backward with the plantar surfaces of the paws directed upward. The results were scored as follows: 0, no difference in retractive power between hindlimbs; 1, weaker retraction on the lesioned side; 2, abnormal extension of hindlimb on the lesioned side with retraction of the limb when the plantar surface of paw is touched with a finger; and 3, abnormal extension of hindlimb and absence of retraction in response to touch.<sup>48</sup>

*Sensory Test.* The mouse was touched with a blunt stick on each side of the body and the reaction to the stimulus was observed. The responses were scored as follows: 3, mouse reacted by turning head and was equally startled by the stimulus on both sides; 2, mouse reacted slowly to stimulus on the impaired side; and 1, mouse did not respond to the stimulus.<sup>15</sup>

*Grip Test.* The mouse was placed on the wire lid of a conventional housing cage. The lid was gently shaken to prompt the mouse to hold on to the grid before the lid was

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swiftly turned upside down. Grip score was measured as the length of time (expressed in seconds) that the mouse hung on to the grid (up to a maximum of 90 seconds).<sup>53</sup>

### *Histological Examination*

At 12, 19, and 26 days after compression and transplantation or saline injection, some animals (10, 7, and 9 mice at 12, 19, and 26 days, respectively) from the 2 groups were deeply anesthetized by intraperitoneal injection of chloral hydrate and subjected to intracardiac perfusion with 4% buffered paraformaldehyde, pH 7.4. To determine the spread of EGFP<sup>+</sup> cells induced by the injection pressure, another 2 animals were subjected to the compression injury and injection of NPs and were killed immediately after surgery.

The spinal cord was removed, cut between the T-8 and L-2 vertebral segments, and postfixed in paraformaldehyde for 2 hours at 4°C.

Samples were transferred into 30% sucrose in 0.1 M phosphate buffer and maintained overnight at 4°C for cryoprotection, embedded in cryostat medium (Kilik; Bio-Optica), cut on the cryostat (Microm HM 550; Thermo-Scientific) in serial transverse 50- $\mu$ m-thick sections, and kept in PBS at 4°C or mounted onto gelatin-coated slides to be processed for immunostaining. Before any further reactions, all sections were mounted in PBS, coverslipped, and examined with a Nikon Eclipse E800 epifluorescence microscope under an FITC filter set to count surviving transplanted EGFP<sup>+</sup> cells as a percentage of those injected: to avoid double counting in consecutive sections, only cells contained entirely within the section were counted (partial cells, cut at the edges of the section, were not included in the counts). All morphological analyses were performed by examiners blinded to the group assignment of the animal from which the specimen was obtained.

### *Immunofluorescence and Immunohistochemistry*

For immunofluorescence, serial sections (1 section every 600  $\mu$ m) were immunolabeled with the following antibodies. After nonspecific binding sites were blocked for 30 minutes at room temperature with 0.3% Triton X-100 and 10% normal donkey serum (Sigma-Aldrich) in PBS pH 7.4, the sections were incubated with primary antibodies in the same solution at 4°C overnight: 1:500 dilution polyclonal rabbit anti-GFAP (DakoCytomation), 1:200 monoclonal mouse anti-MAP-2 (Chemicon), 1:200 monoclonal mouse anti-*nestin* (Chemicon), 1:200 polyclonal rabbit anti-NG2 chondroitin sulfate proteoglycan (Chemicon), 1:200 polyclonal anti-myelin basic protein (Immunological Sciences), 1:500 monoclonal anti-*NeuN* (made in mouse; Chemicon), 1:200 monoclonal mouse anti-vimentin (DakoCytomation), 1:400 polyclonal rabbit anti-Ki 67 (Novocastra Laboratories, Ltd.), and 1:150 monoclonal mouse antisynaptophysin (Immunological Sciences). Sections were washed in PBS and incubated in 1:200 cyanine 3-conjugated secondary antibodies, either anti-rabbit or anti-mouse (Jackson ImmunoResearch Laboratories).

For immunohistochemistry, sections were washed in PBS and incubated for 30 minutes in PBS containing 0.3% Triton X-100 at room temperature in a humidified chamber; endogenous peroxidase activity was neutralized by incubating the sections for 30 minutes in the presence of 0.3% H<sub>2</sub>O<sub>2</sub>. After the sections were rinsed in PBS, nonspecific binding sites were blocked with 0.3% Triton X-100 and 10% normal goat serum (NGS, Sigma-Aldrich) in PBS for 1 hour at room temperature. On the following day, sections were incubated overnight with polyclonal rat anti-mouse CD11b antibody (1:100; Serotec) in the same solution at 4°C, washed in PBS, and incubated with specific secondary goat anti-rat antibody (1:50; Serotec) in 2% NGS, followed by avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories) for 30 minutes. Immunoreactivity was visualized with Vector SG (Vector Laboratories). Finally, sections were dehydrated in ascending concentrations of alcohol and coverslipped with Eukitt mounting medium (Bio-Optica).

The sections were examined with a Nikon Eclipse E800 light and epifluorescence microscope and photographed with a Nikon Coolpix 995 digital camera. Photomicrographs were manipulated and mounted in plates with the Photoshop CS2 software, with autocontrast enhancement. To check for double staining and to make 3D reconstructions, some preparations were examined also with an Olympus FluoView 300 confocal laser scanning microscope.

To evaluate microglial and astroglial activation, respectively, GFAP and CD11b immunoreactivities were analyzed. The density of immunopositive profiles was quantified with Scion Image software (Scion Corporation) by 2 blind observers. For semiquantitative analysis, we considered 3 sections, one at the lesion site, one 600  $\mu$ m rostral to the lesion, and one 600  $\mu$ m caudal to the lesion. In particular, we quantified immunoreactivity in laminae I, II, III, IV, V (represented as the average of GFAP- or CD11b-immunoreactive percentages of the area in the whole dorsal horn for a thickness of 1200  $\mu$ m), and IX. These areas of the spinal cord were photographed using a Nikon Coolpix 995 digital camera at a magnification of 40. The percentage of the overall GFAP- or CD11b<sup>+</sup> area was quantified using the Scion Image software for Windows (freeware version of NIH image; Scion Corporation).

### *Analysis of Lesion Volumes*

Fifteen animals (9 controls and 6 NP-treated mice) were killed 26 days postinjury, and their spinal cords were frozen and sectioned on the cryostat as described above. One of every two 50- $\mu$ m-thick sections (total spinal cord segment 1700  $\mu$ m in length) was drawn at the computer using the NeuroLucida software program (MicroBrightField, Inc.), and the volume obtained was analyzed with NeuroLucida Explorer (MicroBrightField, Inc.). The lesion volume was then expressed as a percentage of the total volume of the segment analyzed.

### *Western Blot Analysis*

An additional 8 mice were subjected to the same pro-

cedure and injection of NPs (4 mice) or vehicle alone (4 mice) as described above in *Surgery and Cell Injection Procedures*. All 8 of these mice were killed at postinjury Day 26 and their spinal cords were dissected: the lesioned spinal cord segment was used for Western blot analysis. Twenty micrograms of total protein was separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After being blocked for 2 hours in TBST and 5% bovine serum albumin (BSA), membranes were incubated overnight at 4°C with 1:1000 monoclonal mouse anti-GFAP (Cell Signaling Technologies) in TBST containing 5% BSA. After 3 washing steps with TBST, membranes were incubated with 1:2000 horseradish peroxidase-conjugated secondary antibody anti-mouse (Pierce) for 1 hour. Western blots were developed with the Amersham ECL detection system (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare). The density of the bands on the membrane was scanned and analyzed with a GS-800 calibrated densitometer (Bio-Rad Laboratories, Inc.).

#### Statistical Analysis

Data are presented as mean values  $\pm$  SEM, except for the analysis of the lesion volume, where data are presented as mean values  $\pm$  SD. Intergroup differences were evaluated by means of a paired 2-tailed Student t-test. Differences were considered significant at  $p \leq 0.05$ .

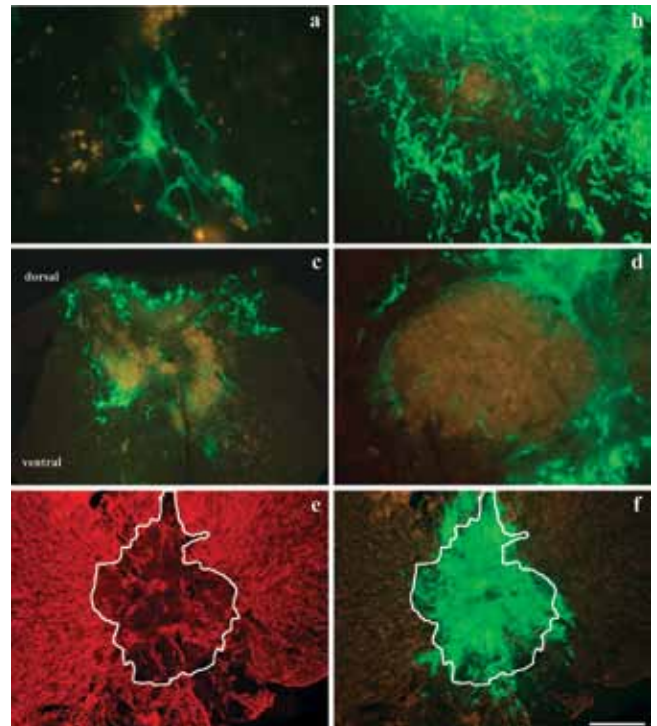
## Results

### Survival and Distribution of NPs

Neural precursor cells (Fig. 1a and b) were injected into the injured spinal cords of C57BL/6 J mice immediately after injury. At all survival points considered (12, 19, and 26 days postinjury), we detected clusters of surviving cells (Fig. 1b). At 26 days postinjury, EGFP<sup>+</sup> cells were found to be distributed especially in the dorsal spinal cord (Fig. 1c), including the dorsolateral funiculi (29.2%  $\pm$  10.7%), dorsal horns (27.4%  $\pm$  7.3%), and the overlying posterior meninges (1.5%  $\pm$  1.3%); additionally, we observed transplanted cells in the ventral funiculi (20.5%  $\pm$  11.6%), gray commissure (18.4%  $\pm$  8.8%), ventral horns (1.9%  $\pm$  1.1%), and anterior meninges (1.2%  $\pm$  0.5%). Even though the cells were grafted directly into the injury site, generally only a small portion of the lesion cavity was filled with EGFP<sup>+</sup> cells: in fact, they preferentially surrounded the damaged area (Fig. 1c and d). Sometimes, however, grafted cells were also detected in the lesion cavity (Fig. 1e and f).

Many transplanted NPs survived, even at 26 days postinjury, when we found 0.2%–2% of injected cells. We tend to exclude cell proliferation as a factor, since no Ki 67<sup>+</sup> cells were found at any age considered (data not shown). In addition, no cell masses infiltrating the surrounding tissue or along blood vessels were observed, thus excluding tumor formation and teratomas.

Grafted cells spread craniocaudally 1000  $\mu$ m from the injection site, thus indicating that they migrated out of the transplant/injury site into the host parenchyma. The finding of EGFP<sup>+</sup> cells far away from the injection site is not likely to be related to injection pressure: in fact,



**FIG. 1.** Photomicrographs showing survival and distribution of NPs. Neural precursor cells display extremely polymorphic morphological characteristics, ranging from a neuron-like phenotype characterized by numerous processes (a) to a fibroblast-like shape (b). After transplantation, NPs are preferentially distributed in the dorsal spinal cord (c): they typically surround the injury site (d), but occasionally enter the lesion cavity (e and f, area outlined in white). Due to the low magnification, cellular profiles are not clearly detectable. Bar = 50  $\mu$ m (a), 100  $\mu$ m (b and d), and 200  $\mu$ m (c, e, and f).

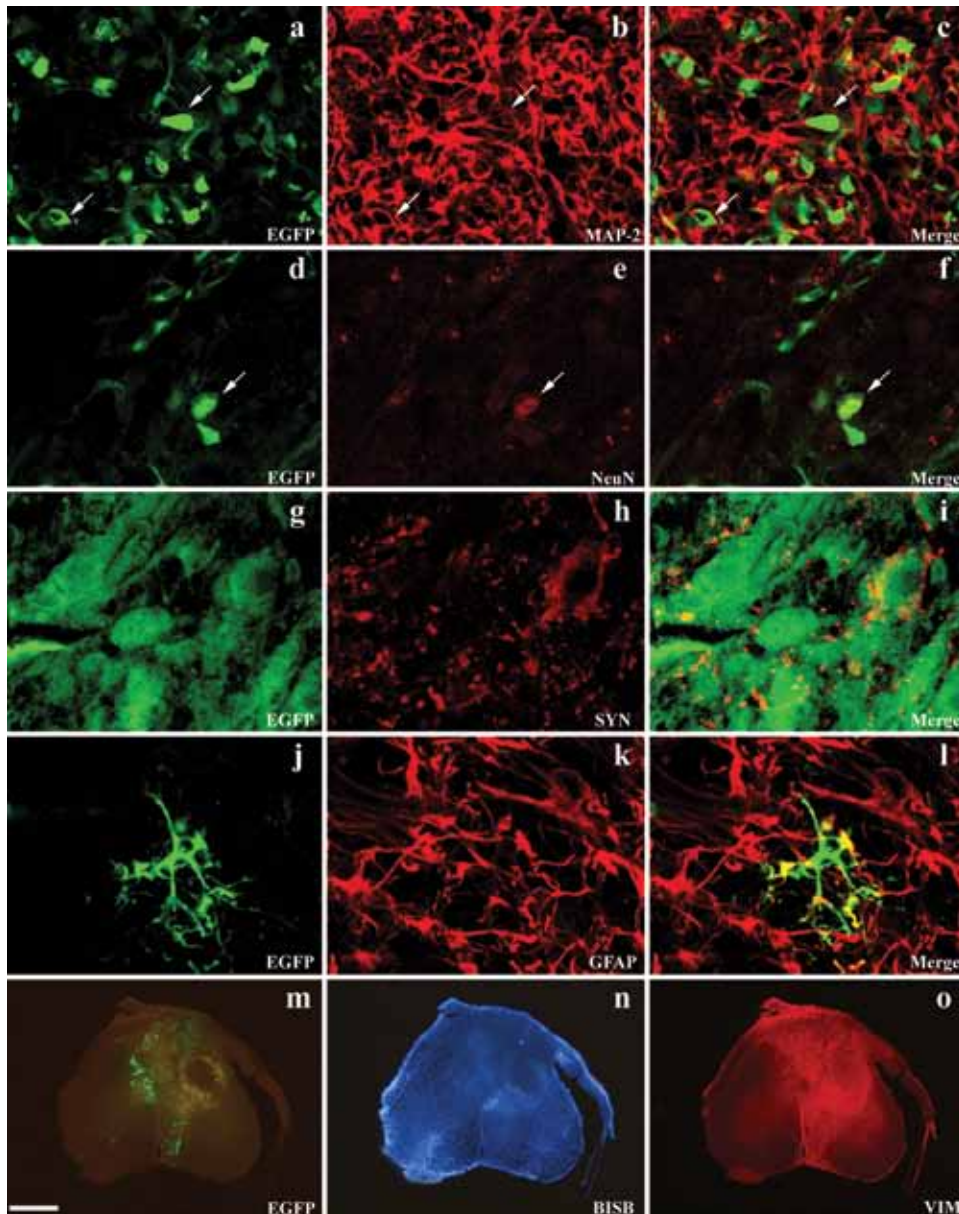
when we analyzed specimens obtained immediately after transplantation, we could find cells only around the injected area (less than 150  $\mu$ m from the site of injection in a cranial or caudal direction).

### Differentiation of NPs

At each survival interval, NPs were extremely polymorphic, as observed *in vitro*.<sup>4</sup> *In vivo*, some cells displayed a neuron-like phenotype characterized by processes of different lengths, whereas others had fibroblast-like shapes (Fig. 1a and b). No cells positive for nestin, an early neural marker, were detected. MAP-2 (Fig. 2a–c), NeuN (Fig. 2d–f), synaptophysin (Fig. 2g–i), and GFAP (Fig. 2j–l) were each expressed by less than 1% of cells: the differentiation was assessed by colocalization of EGFP (green) and the above-mentioned markers (labeled in red) at the confocal laser scanning microscope.

In sections labeled with antivimentin antibodies, less than 1% of EGFP<sup>+</sup> cells were also positive for vimentin, a sign of possible differentiation into fibroblasts or astrocytes. Moreover host tissue showed a significant increase in vimentin immunoreactivity in the proximity of the injured area, close to the grafted cells (Fig. 2m–o).

No EGFP<sup>+</sup> cells positive for NG2 or MBP (immature and mature oligodendroglial markers, respectively) were found.



**Fig. 2.** Images obtained by means of confocal laser scanning microscopy showing differentiation of NPs within the host spinal cord, assessed by colocalization of EGFP (green) and neuronal and glial markers (labeled in red). a–c: MAP-2 immunoreactivity (arrows). d–f: NeuN immunoreactivity (arrows). g–i: Synaptophysin (SYN) immunoreactivity. The confocal microscope images were obtained in a very thin (1- $\mu$ m) section to show the synaptophysin boutons on EGFP<sup>+</sup> cells and branches. j–l: GFAP immunoreactivity. m–o: Host tissue shows a significant increase in vimentin (VIM) immunoreactivity in the proximity of the SCI, close to the grafted cells. BISB = bisbenzamide. Bar = 20  $\mu$ m (a–l), 450  $\mu$ m (m–o).

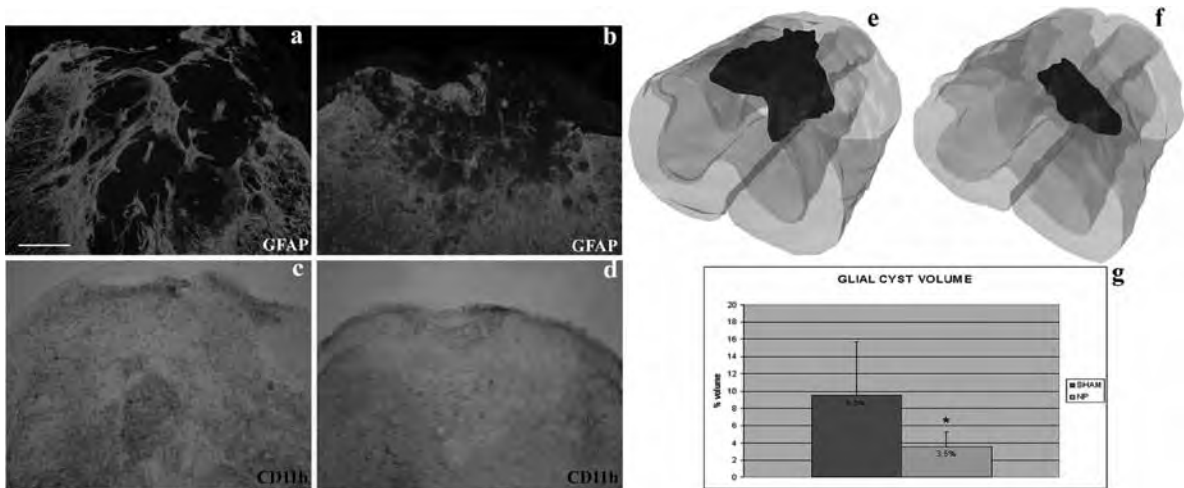
#### Analysis of the Lesion Volume

At 26 days postinjury, the outline of the glial cyst can be clearly visualized due to the increased natural staining of the lesion area (Fig. 1d). A 1700- $\mu$ m spinal cord segment was serially reconstructed at the computer using the NeuroLucida software program. The mean lesion volume, expressed as a percentage of the total volume of the segment analyzed, was 9.5% in saline-injected controls and decreased to 3.5% in NP-treated mice (paired Student t-test,  $p < 0.05$ ) (Fig. 3e–g), thus showing a reduction in the lesion volume in the NP-treated mice to one-third the

value observed in the controls (that is, a 63.2% reduction, Fig. 3a–b and e–g).

#### Quantification of Astrogliosis and Microgliosis

The densities of GFAP- and CD11b-immunopositive profiles were quantified at 3 different rostrocaudal levels. The densities of GFAP- and CD11b-immunopositive profiles were quantified at 26 days postinjury: 13.79%  $\pm$  3.15% and 13.65%  $\pm$  1.35% of the area considered was GFAP<sup>+</sup> in saline-treated controls and NP-treated mice, respectively, and 14.97%  $\pm$  1.53% and 13.96%  $\pm$  1.34% were



**Fig. 3.** Analysis of astrogliosis, microglial activation, and lesion volume. **a and b:** GFAP immunoreactivity. The glial cyst at the level of the lesion site appears completely devoid of astrocytes. Astrogliosis is similar in saline-treated controls (**a**) and NP-treated animals (**b**). **c and d:** CD11b immunoreactivity. The glial cyst is filled with a great number of intermediate and amoeboid microglial cells; also microglial activation is similar in the controls (**c**) and the NP group (**d**). Bar = 200  $\mu$ m (**a-d**). **e-g:** Morphometric analysis of glial cyst: the lesion volume is reduced by 63.2% in NP mice (**f**) compared with controls (**e**).

CD11b<sup>+</sup>, in the control and NP-treated animals, respectively. Therefore, stem cell transplantation did not seem to affect astrogliosis or microglial activation.

In the spinal cords from controls as well as from NP-treated mice, most of the GFAP<sup>+</sup> astrocytes were at the level of the lesion, in particular in the dorsal horns: here the strong astrocytic reaction made it difficult to distinguish the star-shaped profile of these cells (Fig. 3a and b). Instead, 600  $\mu$ m from the lesion in both the rostral and the caudal directions, as well as in the ventral horn in the lesion slice, the intensity of astrocytic activation was noticeably lower, inasmuch as the typical star-shaped profile of astrocytes could be easily recognized. The glial cyst appeared completely devoid of astrocytes (Fig. 3a and b).

Also, microglial activation was most striking at the level of the lesion: in particular, the glial cyst, as well as the surrounding areas, was filled by a great number of intermediate and amoeboid microglial cells (Fig. 3c and d).

Neither astrogliosis nor microglial activation differed significantly between the saline-treated controls and the NP-treated mice. These results were reconfirmed by Western blot analysis (data not shown).

#### Behavioral Tests

To test functional recovery after SCI and NP transplantation, we evaluated injured mice with a battery of behavioral measures: posture assessment, foot-fault test, hindlimb flexion test, sensory test, and grip test. The animals were tested at 4, 7, 12, 19, and 26 days postinjury. Day 0 values correspond to performance before injury. After injury and NP transplantation, in every test, except the grip test, the NP-treated animals showed prompt recovery of motor and sensory functions (Fig. 4).

**Posture.** At 7 days postinjury, the NP-treated mice already exhibited better recovery than the controls (mean score 0.09 vs 0.43): in the following weeks, the NP-treated mice gradually improved, and at 26 days postinjury,

they all showed normal posture (mean score  $0.00 \pm 0.00$  vs  $0.50 \pm 0.33$  in the saline-treated controls,  $p = 0.01$ ).

**Foot-Fault Test.** Immediately after injury, the control mice showed a slightly better functional performance than the NP-treated ones. Beginning at 7 days postinjury, however, the NP-treated animals displayed a consistent recovery; in fact, the number of foot-faults strikingly decreased, and at 26 days postinjury, the mean number of mistakes in the NP group was only  $9.27 \pm 2.06$  (vs  $15.83 \pm 9.45$  in the control group).

**Hindlimb Flexion.** The NP-treated mice already displayed a remarkable recovery at 4 days postinjury, when their mean score was  $0.73 \pm 0.40$  (vs  $1.33 \pm 0.45$  in the control group). The NP-treated mice showed progressive recovery at each time point until 26 days postinjury, when all NP-treated mice manifested complete recovery (score 0.00); in contrast, at 26 days postinjury, the mean score for the control group was  $1.50 \pm 1.00$  ( $p = 0.005$ ).

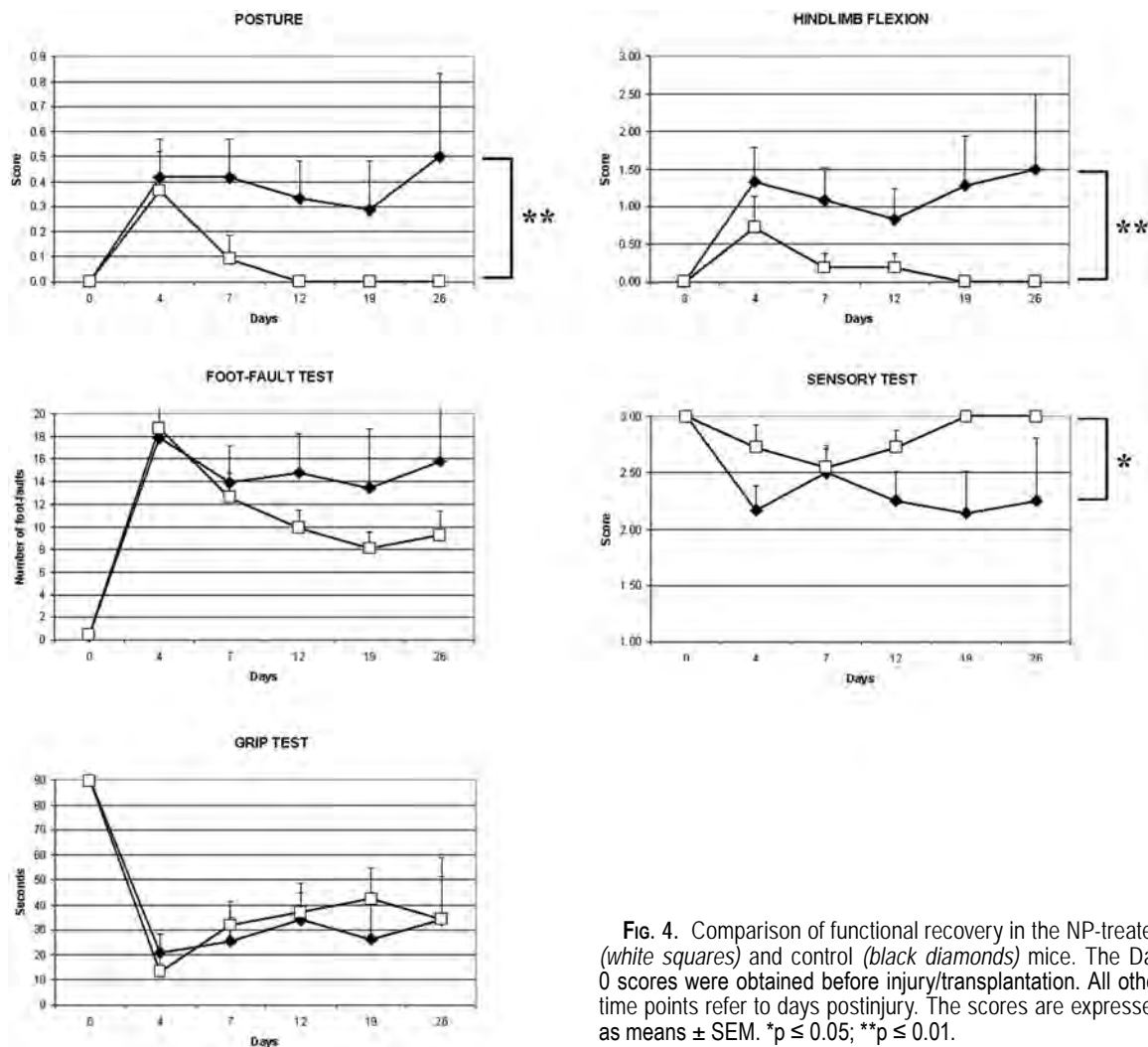
**Sensory Test.** As for the hindlimb flexion test, at 26 days postinjury, the NP-treated animals showed a complete recovery (score 3.00), whereas the saline-treated controls had a mean score of  $2.25 \pm 0.55$  ( $p = 0.02$ ).

**Grip Test.** This test evaluated the mice's endurance in hanging onto a grid with their backs parallel to the surface. Unlike in other tests, the 2 groups performed similarly in this test. Between 7 and 19 days postinjury, the NP-treated mice showed mild improvements in comparison with the controls (Fig. 4); however, at 26 days postinjury, there was minimal difference between the groups, with the NP-treated animals hanging onto the grid for a mean duration of  $34.40 \pm 16.78$  seconds and the saline-treated controls,  $33.92 \pm 24.91$  seconds.

## Discussion

Several groups of authors have investigated the dif-

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**Fig. 4.** Comparison of functional recovery in the NP-treated (white squares) and control (black diamonds) mice. The Day 0 scores were obtained before injury/transplantation. All other time points refer to days postinjury. The scores are expressed as means  $\pm$  SEM. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ .

differentiation and integration of neural precursors derived from the embryonic neural tube following grafting in the lesioned spinal cord, but did not consider their effects on recovery.<sup>6,21,23</sup> Those who investigated recovery mainly addressed autonomic outcomes, such as bladder control and erection.<sup>49,50</sup> Our study is the first to consider the anatomical and functional outcome of transplantation of neural tube precursors in the compression model, and it can be compared with a similar study in the contusion model.<sup>30</sup>

We have evaluated the transplantation of NPs as a possible therapeutic approach in a model of spinal cord compression in the mouse. We show that NPs are able to survive for a long time, integrate, reduce glial cyst volume, and promote functional recovery.

### Survival and Differentiation of Transplanted NPs

We have previously studied the effects of delayed NP grafting on axonal support and sprouting;<sup>4</sup> here, we have tested the effects of acute transplantation, since some authors<sup>26,27,34</sup> report positive results in this same therapeutic time window, transplanting different stem cell types into injured spinal cord. Performing the graft immediately

after injury, we observed a considerable rate of survival (0.2%–2% of  $10^5$  grafted cells) at 26 days postinjury, although our estimate is probably low due to the section thickness and to the presence of cell clusters in which individual profiles were difficult to resolve. These data are in agreement with what we observed in the delayed transplantation experiments,<sup>4</sup> suggesting that both strategies can be effective.

In the present study, only a small percentage of NPs were positive for neuronal markers (MAP-2 and NeuN); some injected cells also expressed synaptophysin, a marker of synaptic vesicles, supporting integration of grafted cells into the circuits of the host injured spinal cord. Our results are in agreement with those obtained by others in the intact adult spinal cord,<sup>16</sup> in the lateral funiculus injury model,<sup>22</sup> or in the standard moderate contusion injury model,<sup>30</sup> even though we had a lower number of differentiated grafted neuronal precursors (< 1%). In keeping with our findings, Cao et al.<sup>6</sup> showed that in the injured spinal cord the differentiation of neuronal-restricted precursor cells that occurs in the normal adult rat spinal cord is inhibited.

Nevertheless, a limited number of neuron-committed

transplanted cells together with undifferentiated transplanted cells are sufficient to support surviving motoneurons by secretion of neurotrophic factors and immunomodulatory molecules.

In addition to neuronal differentiation, some transplanted cells also expressed the glial marker GFAP, which is always distributed near the perimeter of the glial cyst; similarly, host astrocytes, absent inside the glial cyst, give rise to the glial scar surrounding the lesion site, which produces local signals that prompt both host astrocytes and NPs to participate in glial scar formation. The role of reactive astrocytes is not yet well understood: recently they have been shown to protect tissue and to preserve motor functions after neurotrauma through production of antiinflammatory cytokines and neurotrophic factors.<sup>12,33,41,46</sup> They secrete a wide variety of trophic molecules including NGF, CNTF, FGF, BDNF (brain-derived neurotrophic factor), and GDNF (glial cell line–derived neurotrophic factor):<sup>32,51</sup> in particular GDNF supports one of the most potent motoneuron survival and regeneration factors.<sup>3,55</sup>

In contrast, when we transplanted NPs into another model of SCI far away from the lesion site, we failed to observe glial differentiation, a finding that supports the relevance of local signals produced at the injury site.<sup>4</sup> Since the undifferentiated NPs were positive for nestin but not for mature neuronal or glial markers *in vitro*, we hypothesized that undifferentiated NPs can be stimulated to differentiate into neurons when in contact with injured tissue.<sup>4</sup>

#### *Analysis of Astrogliosis, Microgliosis, and Lesion Volume*

In the present study, Western blot and immunohistological analysis revealed that astrogliosis and microglial activation did not differ significantly between saline-treated controls and NP-treated animals. Moreover a small number of grafted NPs differentiated into GFAP<sup>+</sup> cells.

As mentioned above, reactive astrocytes can play a dual role after SCI and can be both harmful and beneficial, especially in the subacute phase.<sup>40</sup> Because in the chronic phase astrocytes can also represent a chemical barrier, our findings suggest that NPs do not contribute to the recruitment of host astrocytes for glial scar formation: a similar result has been observed by Hooshmand and colleagues,<sup>19</sup> who used human CNS stem cells grown as neurospheres (hCNS-SCns) in a murine model of contusion.

Besides the potential beneficial effect of astrocytes, some authors have proposed that reactive microglia could also play an important role in limiting neurodegeneration and improving synaptic recovery following CNS damage; reactive microglia are also able to release neuroprotective factors such as BFGF (basic fibroblast growth factor) and NGF,<sup>5</sup> and also endogenous endocannabinoids,<sup>10</sup> which can attenuate neuronal damage and protect against excitotoxicity. Activated microglia also participate in the stripping of synapses following axotomy, which seems to improve survival and recovery of function of affected neurons, thus having a neuroprotective effect.<sup>8</sup> Moreover, microglial cells are phagocytic and can remove apoptotic cells and debris at the lesion site, promoting remyelination and repair.<sup>36</sup>

In light of these considerations, we can consider our results concerning astrogliosis and microgliosis worthy of further investigation. Also, the fact that the percentage of astroglial and microglial cells that were activated was similar in the saline-treated and NP-treated mice could explain the slight improvement shown by the saline-treated mice (although this improvement was not sufficient to result in a significant recovery of performance).

With morphometric analysis, we observed that the lesion volume was reduced by 63.2% following NP transplantation. Therefore, NPs transplanted close to the lesion site contribute to a reduction in glial cyst volume. Our results are in agreement with those of Agudo and colleagues,<sup>1</sup> who demonstrated that transplantation of Schwann cell precursors after spinal crush injury in rats partially prevents the formation of the glial scar by means of extensive spread of Schwann cell precursors into the surrounding injured tissue, where cells appeared intimately integrated with local astrocytes.

In our experiments, NPs were distributed mainly around the lesion, but a small percentage also penetrated the glial scar, similar to what was observed by Mitsui et al.<sup>30</sup>—that is, that neuronal- and glial-restricted precursors transplanted in contused rats survived and filled the lesion sites.

Finally, we showed that some grafted NPs were positive for neuronal markers (MAP-2 and NeuN) and expressed synaptophysin, suggesting that they could integrate into local host circuits. We can also suppose that NPs stimulate the formation of new circuits, as other authors suggest that stem cells are fundamental in optimizing the plastic response in severed tissue.<sup>18</sup>

#### *Functional Outcome*

Behavioral tests performed in our experiments evaluated posture, resistance force, sensory function, and hindlimb coordination, suggesting that NP transplantation determines an earlier, steadier, and significant improvement in performance in comparison with saline injection. The exception was the grip test, which did not show any significant differences between the 2 experimental groups at 26 days postinjury; this is probably due to the complexity of this test, which requires coordination, endurance, strength, and the involvement of numerous muscles and joints, unlike the other tests, which address more specific functions. Our results support the common view that a combined scoring method should be used to determine effective recovery.<sup>37</sup> Our studies have demonstrated that NPs are able to integrate into the host tissue and in part to differentiate into MAP-2 and NeuN<sup>+</sup> cells, suggesting their ability to support the surviving motoneurons following injury. Moreover the combined transplantation of neuronal and glial precursors generates a microenvironment in which glial cells support survival and differentiation of neuronal cells.<sup>22</sup> Additionally, as previously mentioned, neural stem cells can constitutively secrete neurotrophic factors and immunomodulatory molecules, which are fundamental to supporting surviving host motoneurons and axonal regeneration and CNS repair.<sup>25</sup>

# Transplant of neural precursors in the lesioned spinal cord

This secretion of neurotrophic factors and immunomodulatory molecules can explain the behavioral recovery. Mitsui and colleagues<sup>30</sup> have suggested that NP grafting following SCI can assure local protection and improve motor functions. Therefore transplantation of NPs offers therapeutic benefits because of their ability to reestablish neuronal circuits and create an environment favorable to healing at the injury site.<sup>22</sup>

In the present study, we have displayed a reduction of glial cyst volume in NP-treated animals. Moreover, we found that NPs were distributed close to the perimeter of the lesion site and occasionally penetrated it. The reduced glial cyst volume and the presence of NPs supplying trophic factors could explain the better recovery of function in NP-treated mice compared with controls: in fact, neural stem cells are able to promote survival of motoneurons, protect against excitotoxic insults,<sup>24</sup> contribute to neurogenesis,<sup>38</sup> provide neuroprotection,<sup>47</sup> and promote remyelination of injured axons.<sup>20</sup> Our observations correlate behavioral improvement with the reduction in glial cyst volume: NPs surround the lesion site, almost giving the impression of forming a ring around it that limits its expansion.

## Conclusions

Our results confirm that NPs can offer protection to the host spinal cord: in fact, they survive several weeks in the absence of immunosuppression and integrate into the injured spinal tissue even though transplanted directly into the hostile lesion site: importantly, NPs reduce the glial cyst volume, giving neuroprotection, and consequently promote functional recovery after compression. These results are in accord with our previous finding that NPs promote serotonergic sprouting and behavioral improvement in a murine model of lumbar hemisection with delayed transplantation.<sup>4</sup> Although the immediate cell transplant is not necessarily translatable to clinical practice, due to the unpredictable outcome of SCI in the early phase, this study provides evidence for a potential role of cell transplantation in preventing glial cyst formation.

## Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper. This work was supported by the Compagnia di San Paolo, Regione Piemonte, Associazione Girotondo Onlus and Associazione Italiana Mielolesi (nonprofit organizations) grants to A.V. and represents partial fulfillment of the doctoral thesis requirements of Drs. Boido and Garbossa. Dr. Boido is the recipient of a fellowship from Associazione Girotondo Onlus.

Author contributions to the study and manuscript preparation include the following. Conception and design: Boido, Garbossa, Vercelli. Acquisition of data: Boido. Analysis and interpretation of data: Boido, Vercelli. Drafting the article: Boido. Critically revising the article: Vercelli. Reviewed final version of the manuscript and approved it for submission: Vercelli. Study supervision: Vercelli.

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Manuscript submitted January 8, 2010.

Accepted January 18, 2011.

Please include this information when citing this paper: published online April 1, 2011; DOI: 10.3171/2011.1.SPINE10607.

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