

ORIGINAL ARTICLE

# Therapeutic potential of human olfactory bulb neural stem cells for spinal cord injury in rats

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**Study design:** Adult human olfactory bulb neural stem cells (OBNSCs) were isolated from human patients undergoing craniotomy for tumor resection. They were genetically engineered to overexpresses green fluorescent protein (GFP) to help trace them following engraftment. Spinal cord injury (SCI) was induced in rats using standard laminectomy protocol, and GFP-OBNSC were engrafted into rat model of SCI at day 7 post injury. Three rat groups were used: (i) Control group, (ii) Sham group (injected with cerebrospinal fluid) and treated group (engrafted with OBNSCs). Tissues from different groups were collected weekly up to 2 months. The collected tissues were fixed in 4% paraformaldehyde, processed for paraffin sectioning, immunohistochemically stained for different neuronal and glial markers and examined with bright-field fluorescent microscopy. Restoration of sensory motor functions we assessed on a weekly bases using the BBB score.

**Objectives:** To assess the therapeutic potential of OBNSCs-GFP and their ability to survive, proliferate, differentiate and to restore lost sensory motor functions following their engraftment in spinal cord injury (SCI).

**Methods:** GFP-OBNSC were engrafted into a rat model of SCI at day 7 post injury and were followed-up to 8 weeks using behavioral and histochemical methods.

**Results:** All transplanted animals exhibited successful engraftment. The survival rate was about 30% of initially transplanted cells. Twenty-seven percent of the engrafted cells differentiated along the NG2 and O4-positive oligodendrocyte lineage, 16% into MAP2 and  $\beta$ -tubulin-positive neurons, and 56% into GFAP-positive astrocytes.

**Conclusion:** GFP-OBNSCs had survived for >8 weeks after engraftment and were differentiated into neurons, astrocytes and oligodendrocytes, The engrafted cells were distributed throughout gray and white matter of the cord with no evidence of abnormal morphology or any mass formation indicative of tumorigenesis. However, the engrafted cells failed to restore lost sensory and motor functions as evident from behavioral analysis using the BBB score test.

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## INTRODUCTION

Worldwide, an estimated 2.5 million people live with spinal cord injury (SCI), with the possibility of adding 130 000 new cases every year.<sup>1</sup> The main causes of traumatic SCI are motor vehicle accidents, sports and recreational activities, accidents at work and falls at home.<sup>2</sup>

The pathological consequences of SCI are multitude: death of local segmental neurons and glial cells, severance of axons in white matter, formation of fluid-filled cystic cavities and demyelination of spared white matter.<sup>3–5</sup> All these pathological processes, at least to some degree, contribute to the devastating functional outcomes after SCI, warranting the development of a multifaceted combinatorial approach to achieve clinically relevant functional recovery.<sup>6</sup>

The pathophysiological mechanism of SCI comprises both primary and secondary lesions. In the primary lesion, disruption of nerve tracts is followed by a massive influx of peripheral macrophages and T cells and activation of resident microglia, which last for few weeks post injury.<sup>7</sup> The secondary lesion is accompanied by ischemia, edema,

hemorrhage and cytotoxicity and the formation of glial scar around a central cavitation mainly on the site of primary one.<sup>8</sup> The glial scar forms a physical and a chemical barrier against endogenous regeneration of the disrupted nerve tracts.

The confirmation that neurogenesis occurs in specific areas within the adult brain and that neural stem cells (NSCs) reside in central nervous system (CNS)<sup>9</sup> opens new possibilities for cellular therapy. Cell therapeutic intervention may involve the stimulation or grafting of neural progenitor and stem cells (SCs).

SCs are undifferentiated cells that are able to self-renew and differentiate into other cell types.<sup>10</sup> They theoretically hold the potential to treat and cure a broad range of neurological diseases and injuries, ranging from neurodegenerative diseases like Alzheimer's and Parkinson's diseases to retinal diseases, cerebral strokes and SCIs.

Olfactory bulb NSCs (OBNSCs) could provide an innovative avenue of exploration for therapeutic auto transplantation in cases of traumatic or neurodegenerative cord injury. Moreover, autologous

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neuronal material is safe, ethical and could overcome the possibility of immunorejection associated with the use of allogeneic material.<sup>11–16</sup> Both brain and cord-NSCs derived can be propagated *in vitro* and used to restore injured or dysmyelinated spinal cord.<sup>17–19</sup>

Following transplantation, NSCs survive, migrate to lesioned regions, differentiate into specific neurons and promote recovery by enhancing the survival of existing endogenous neurons via indirect mechanisms such as neurotrophic support, immune modulation and enzyme replacement.<sup>20–22</sup>

In previous studies, we had succeeded to isolate NSCs from the adult human olfactory bulb.<sup>11,13,14,23</sup> The promising results obtained with the NSC-based therapy for SCI at the pre-clinical level, coupled with the ability to obtain NSCs from the human olfactory bulb by less invasive methods, prompted us to design the current experiments to assess the therapeutic potential of adult human OBNSCs and their ability to restore normal activity at the behavioral, histological and immunohistochemical level using different SC, neuronal, glial and tumorigenic markers.

## MATERIALS AND METHODS

### Animals

The present study utilized 68 adult female Westar rats (200–250 g body weight). The animals were housed in separate cages with free access to food and water. Females were preferred to males, as the latter develop severe neurogenic pulmonary edema when submitted to SCI.<sup>24,25</sup> Females also develop fewer urinary retention problems after injury compared with males. We followed the procedures adopted by the relevant IACUC at Mansoura University, Egypt.

### Experimental design

The animals were subdivided into four groups: age-matched normal control ( $n=16$ ), lesioned group ( $n=16$ ) whose spinal cord was injured by laminectomy, sham lesion control group ( $n=16$ ), injected with 4  $\mu$ l of cerebrospinal fluid in normal SC, and Engrafted group ( $n=20$ ), which received OBNSC transplantation 7 days after laminectomy.

### Behavioral assessment

Locomotor recovery was assessed using the Basso, Beattie and Bresnahan (BBB)<sup>3,26</sup> locomotor rating scale. Animals were assigned new identification codes after surgery to ensure that behavioral performances were rated in a blind manner.

### BBB open-field locomotion score

The BBB, 22-point (0–21) BBB scale was used to assess hind limb locomotor recovery, including joint movements, stepping ability, coordination and trunk stability.<sup>3</sup>

### SCI rat model

An extended dorsal funiculotomy had been performed according to Pallini *et al.*<sup>27</sup> and Braga-Silva *et al.*<sup>26</sup> In brief, rats were anesthetized using a combination of ketamine (60 mg kg<sup>-1</sup> body weight) and xylazine (20 mg kg<sup>-1</sup> body weight) administered intraperitoneally. The surgery was performed under aseptic conditions; the dorsal area of the rat was shaved from the lower back to the neck, extending 2 cm bilaterally from the midline, with electric clippers. The hair was cut as closely as possible. The body temperature, during surgery, was maintained at 37 °C by a thermostatically regulated heating pad. Lubricant ointment was applied to the eyes to prevent drying. After skin incision, dissection by planes was performed on the spinous process, detaching the spinal trapezius muscle from the spine until the vertebral column was exposed. The spinal cord was exposed between T8 and T9. Injuries were performed using micro scissor to destroy the upper dorso-spinal columns leading two paralysis of both hind limbs. After the injury, the exposed muscles and skin were sutured with 4–0 silk sutures and staples, respectively. Recovery was carried out by transferring injured rats to 30 °C heating pad. The animals were rehydrated by injection of 2.00 cc saline subcutaneously.

### Post-operative care

Bladder expression, gentocin, ascorbic acid and analgesics (butorphenol) were performed twice daily until the restoration of the bladder function. All surgical interventions and postoperative animal care were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996, USA) and were approved by the ethical Committee to the Use of Experimental Animals for Medical Purposes, Mansoura University. All efforts were made to minimize the number of animals used and their suffering.

### Human OBNSC isolation and culturing

Isolation and culture of OBNSC were performed as reported previously.<sup>11,13,14,23</sup> In brief, the olfactory bulbs were collected from adult patients at Neurosurgery, Catholic University, Rome, Italy, after obtaining a written informed consent, and the studies were approved by the Ethical Committee of the Catholic University.

The OB tissues were dissociated in Papain 0.1% (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37 °C, cultured with epidermal growth factor (EGF; 20 ng ml<sup>-1</sup>; PeproTech, Rocky Hill, NJ, USA), bFGF (10 ng ml<sup>-1</sup>; PeproTech) and LIF (20 ng ml<sup>-1</sup>; Immunological Sciences, Rome, Italy) in DMEM/F12 (1:1) serum-free medium (Invitrogen, Carlsband, CA, USA).

Primary and secondary neurospheres were dissociated with Accutase, plated at the density of 10<sup>3</sup> cells per cm in a serum-free medium containing EGF and bFGF, and passaged up to P30.

Between P7 and P10, parallel cultures were grown as adherent monolayers, and differentiation assays were performed at 5 days after plating on Matrigel-coated glass coverslips in the absence of EGF and bFGF and in the presence of 1% fetal calf serum (Hyclone) supplemented with cyclic adenosine monophosphate 50 mM, all-trans retinoic acid 5 mM (Sigma-Aldrich) and triiodothyronine 30 nM (Sigma-Aldrich).

For green fluorescent protein (GFP) vector construction, human embryonic kidney (HEK)-293 T cells in log-phase growth were transiently transfected, using standard LipofectAmine reagent (Invitrogen), with GFP-Vector.<sup>28</sup> OBNSCs were transfected in the presence of polybrene solution at 8  $\mu$ g ml<sup>-1</sup> (Sigma-Aldrich), and selection was performed in the presence of G418 (Euroclone) 400  $\mu$ g ml<sup>-1</sup> over time for OBNS/PC selection and maintenance.

### Immunocytochemical assessment

The cells were fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, with phosphate-buffered saline/0.1% Triton-X containing 10% normal goat serum. The primary antibodies were anti-Nestin (1:200, rabbit, Sigma-Aldrich) for detection of undifferentiated NSCs, anti-MAP2 (1:200, rabbit, Sigma-Aldrich) for detection of mature neurons, anti-beta-tubulin III (1:100, rabbit, Sigma-Aldrich) for detection of immature neurons, anti-O4 (1:100, mouse, monoclonal, Sigma-Aldrich) for detection of immature oligodendrocyte and anti-GFAP (1:400, mouse, clone G-A-5, Sigma-Aldrich) for detection of astrocytes.

After washing, the cultures were secondary antibodies labeled with fluorescein iso-thiocyanate, tetra methyl rhodamine iso-thiocyanate and Phycoerythrin, then washed, incubated with 4', 6-diamidino2 phenylinole dihydrochloride (1 mg ml<sup>-1</sup> in methanol, 15 min at 37 °C and finally mounted using Fluorsave (Calbiochem; La Jolla, CA, USA)

### OBNSCs transplantation

At 80% confluence OBNSCs were collected, and dissociated into single cells, and suspended in artificial cerebrospinal fluid (Sigma-Aldrich) at 30 000 cells per  $\mu$ l. A total of 5  $\mu$ l of the cells were injected 0.5 cm rostral to injury site, and the needle was removed after remaining in place for another 5 min, and cyclosporine (Sandimmun, 10 mg kg<sup>-1</sup>) was injected beginning from one day before transplantation and finished on the day of killing.

### Samples collection

Samples ( $n=3$ ) of spinal cord were collected from each group simultaneously at 1, 2, 3, 4, 6 and 8 weeks of OBNSCs transplantation.

### Histological analysis

The tissue was fixed in 10% neutral-buffered formaldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h. The samples were dehydrated in ethanol, cleared in xylene and impregnated and embedded in paraffin. Sections of 5–7  $\mu\text{m}$  were processed and stained with hematoxylin and eosin and Cresyl Echt Violet stains for Nissl substance.

### Immunohistochemistry

At the end of behavioral testing, the animals were anesthetized, and the SC tissues cryoprotected in 30% sucrose were coronally sectioned, and stained with Anti-GFP (1:2000, rabbit, N-Terminal, Sigma-Aldrich) and cell type-specific markers. The following primary antibodies were used: anti-Nestin (1:200, rabbit, Sigma-Aldrich) for detection of undifferentiated NSCs; anti-MAP2 (1:200, rabbit, Sigma-Aldrich) for detection of mature neurons; anti-beta-tubulin III (1:100, rabbit, Sigma-Aldrich) for detection of immature neurons; anti-O4 (1:100, mouse, monoclonal, Sigma-Aldrich) for detection of immature oligodendrocytes; and anti-GFAP (1:400, mouse, clone G-A-5, Sigma-Aldrich) for detection of astrocytes. Fluorescein iso-thiocyanate, (anti-rabbit, anti-mouse, anti-chicken, Sigma-Aldrich), tetra methyl rhodamine iso-thiocyanate (anti-rabbit, Sigma-Aldrich) and Phycoerythrin (anti-mouse, anti-goat, Sigma-Aldrich) were applied, and then the SC tissues were examined under a fluorescent microscope.

### Stereological quantification

Eight weeks post-OBNSC engraftment, the cells were quantified by GFP immunolabeling and neuronal or glial-specific markers. Starting sections were chosen at random, and every sixth section thereafter was analyzed. Cell differentiation was examined by fluorescent microscopic imaging of fluorescent-stained sections to identify the approximate percent of GFP-positive differentiated cells according to their reactivity with previously mentioned neuronal or glial markers. For quantification of the different neuronal and glial subtypes, starting sections were chosen at random, and every sixth section thereafter was double stained with GFP and specific undifferentiated OBNSC (nestin), neuronal (MAP2,  $\beta$ -tubulin) or glial (GFAP, NG2, O4) markers. Labeled cells were counted using ImageJ version 10.2 software (versi 1.46r, NIH, Bethesda, MD, USA), and the values were averaged together to get the final percentage.

### Statistical analysis

The experimental design details, statistics requirement, calculation of sample size and statistical power estimation were accurately estimated based on data from previous similar studies, followed by calculating the power of study. The main research design of the current study is to measure a series of variables from IHC and to test whether those variables are significantly different between the case (engrafted group) and control (non-engrafted groups) group. We have suggested a proper statistical tool for our data, estimating proper sample size from previous literature. On the basis of previous similar experiments, 20–30 SCI AD rat models and control groups should be a proper sample size. For cell counting, BBB score and stereological data few have used a two-way ANOVA and the *post hoc* Tukey's test for analysis of percentage of each cell types following transplantation, and data were presented as means  $\pm$  s.e.m.

## RESULTS

The gray matter of control group was formed of large neuronal cell bodies (Figures 1a and b). The damaged SC displayed the following semi-qualitative pathological criteria: distortion of the central canal, localized petechial hemorrhage (Figures 1c–f and 2a), vacuolation (deleted) and necrosis of neurons (Figures 2b–f), and edematous neuropil (Figure 2f). The lesioned white matter displayed vacuolations, and disruption of neuronal tracts and circuitry (Figure 3a), reactive gliosis represented by the presence of considerable numbers of hypertrophied astrocytes between the vacuolated neuropil (Figure 3b), and activated microglia (Figure 3c). At 6 to 8 weeks post SCI, no pronounced differences were noticed in the lesioned area,

except for an increase in the number of damaged neurons (Figure 3f), hypertrophied astrocytes and gliosis (Figures 1d and e).

Examination of sections of spinal cord of the sham group 6–8 weeks post injury did not show any improvements in the damaged tissue histoarchitecture nor any signs of neuronal regeneration. This was compared with the equivalent 6–8-week lesioned groups (Supplementary Figures S1A–D)

### Isolation and culture of human OBNSCs

In the present study, harvested OBNSCs were genetically engineered to overexpress GFP. The GFP was selected to enable tracing of the engrafted OBNSCs and to distinguish between exogenous and endogenous cells.

### Assessment of the multipotent potential of GFP-OBNSCs

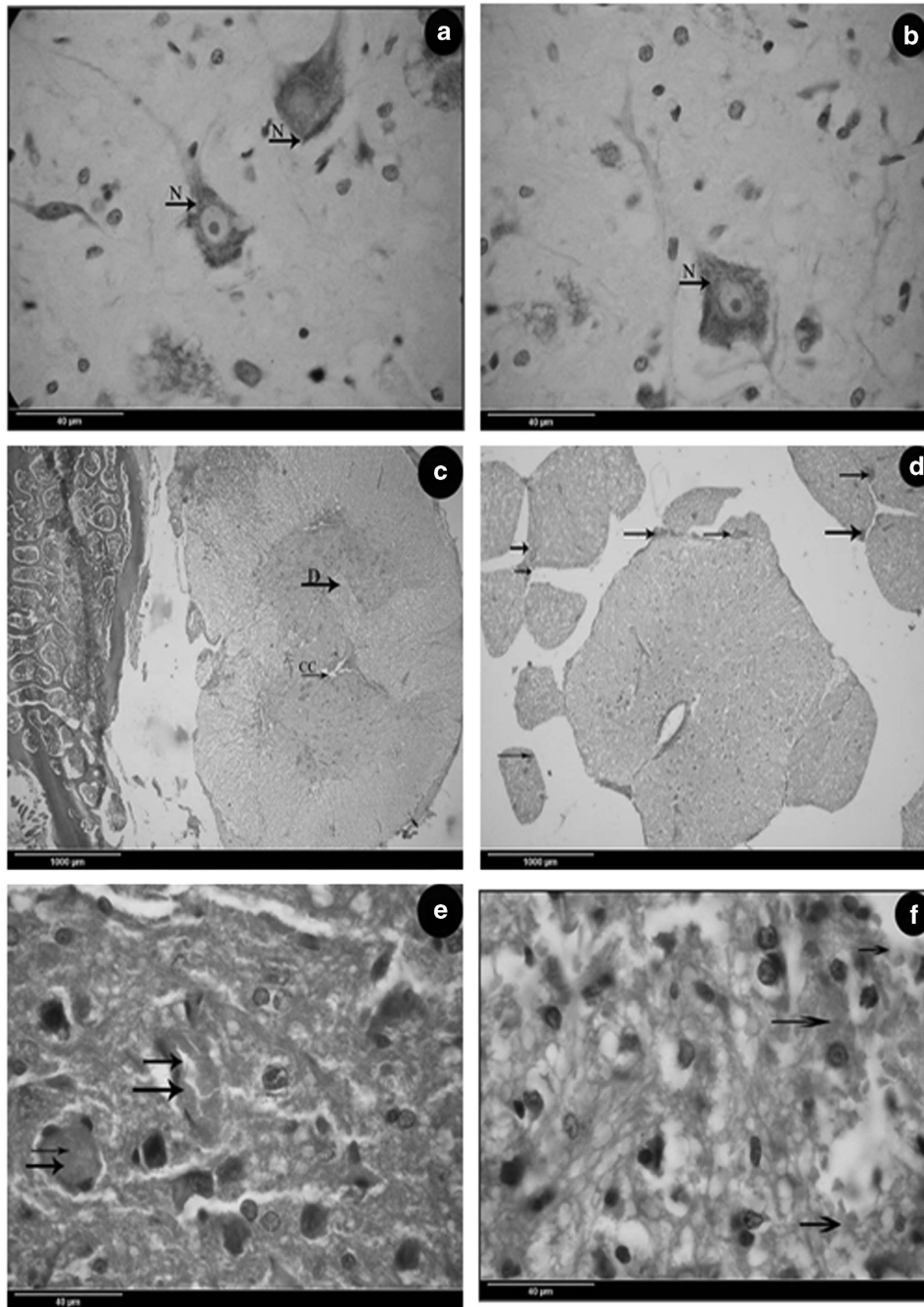
Before the transplantation experiment, we intended to investigate the multipotent potential of our GFP-OBNSCs and to test their ability to differentiate into different neuronal and glial elements *in vitro*.

By culturing GFP-OBNSCs in a serum-free chemically defined medium, containing the SC mitogens, the GFP-OBNSCs were maintained in an undifferentiated state and only proliferated into clusters of cells known as neurospheres (Supplementary Figure S2A). Two days after passaging, cellular small clusters known as neurospheres were collected (Supplementary Figure S2A). The neurospheres continued to increase in diameter to reach about 200–250  $\mu\text{m}$  at day 7 post culture. They were collected from cultured plates at 80% confluence, digested with acutase for 1–2 min at 37.5 °C water bath and triturated into single cells (Supplementary Figure S2B) by 10–15 up and down pipetting using a heat polished Pasteur pipette. The cells were counted using a hemocytometer for determining the total number, and their viability was assessed using the Trypan blue exclusion assay (Supplementary Figure S2C). The GFP-OBNSC multipotentiality was investigated through differentiation assays. To assure that the cells remained undifferentiated, expression of nestin (an undifferentiated NSC marker) was inspected. Large number of cells remained undifferentiated (Supplementary Figure S2D). The immunocytochemical examination showed a negative immunoreactivity for GFAP and for MAP2. Between 12 and 15 days post differentiation, cells gave rise to neuronal and glial lineages. In all, 45 to 55% of the cells were differentiated into astrocytes (Supplementary Figures S2E–F and Figure 4a), MAP2 mature neuronal marker (25–30%; Figures 4a–c) and  $\beta$ -Tubulin III-positive immature neuronal marker (6%; Supplementary Figure S2A; Figure 4d). These findings indicated that the GFP-OBNSCs possessed a highly proliferative and differentiable nature.

### Engraftment of GF-OBNSCs into the SCI rat model

The potential of our GFP-OBNSCs for *in vitro* proliferation and differentiation into astrocytes and oligodendrocytes had encouraged us to assesses the possible therapeutic potential of GFP-OBNSCs for SCI at the pre-clinical level. Microscopical inspection revealed that OBNSCs were able to survive in the lesion hostile site for at least 8 weeks post implantation. Moreover, no signs of immunorejections were recorded during the 8 weeks.

The use of GFP-immunostained sections enabled us to differentiate between exogenous (derived from GFP-OBNSCs) and endogenous neuronal and glial cells. To confirm the ability of engrafted GFP-OBNSCs to survive, proliferate and differentiate in the lesion environment, we utilized fluorescent immunohistochemistry (IHC)



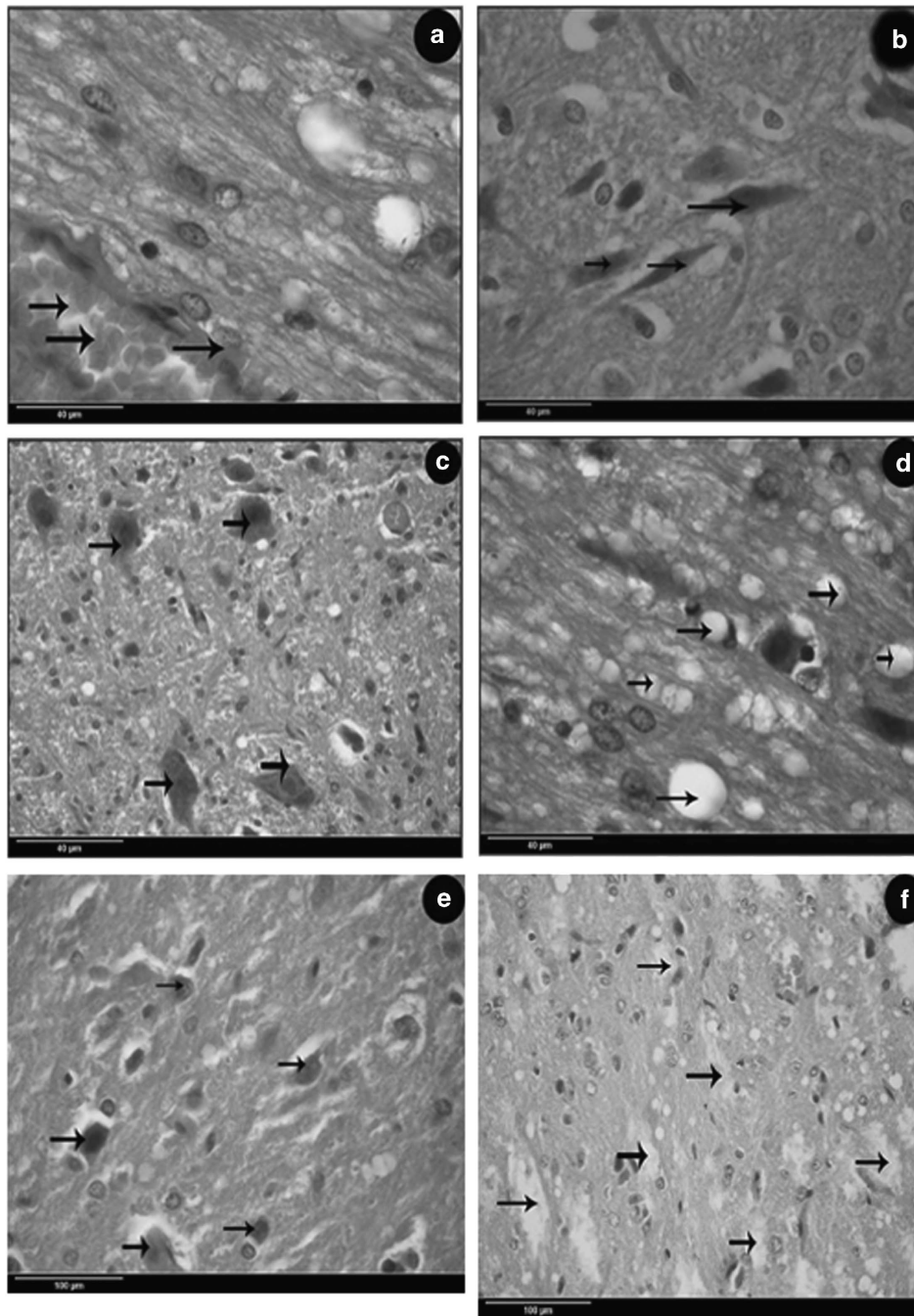
**Figure 1** (a) Photomicrograph of a section of rat's spinal cord of the control group showing Nissl granules as basophilic mottled structures in the cytoplasm of neuronal cell bodies and dendrites (N), cresyl violet stains. (b) Control group showing Nissl granules appear as basophilic mottled structures in the cytoplasm of neuronal cell bodies and dendrites (N). Cresyl violet stain. (c) One-day post injury showing distortion in central canal (CC) and loss of integrity of gray matter (D), H&E stain. (d) One-day post injury showing extensively fragmented spinal cord with localized petechial hemorrhages (arrows), H&E stain. (e) Two weeks post injury showing severe congestion of blood capillaries (arrows), H&E stain. (f) Two weeks post injury showing hemorrhagic parenchyma with extravazation of RBCs of injured spinal cord (arrows), H&E stain. A full color version of this figure is available at the *Spinal Cord* journal online.

against GFP and different neuronal and glial markers to be able to differentiate between exogenous (our GFP-OBNSCs) and endogenous (non-human, non-GFP-positive) cells following engraftments.

Confocal inspection confirmed the presence of a considerable number of endogenous non-GFP astrocytes forming an elaborate network within the different regions of white and gray matter. Non-GFP-positive endogenous cells expressing GFAP (Figure 5a), and O4 (Figure 5b) were also encountered within different regions of gray and

white matter. Endogenous O4 and GFAP-positive cells did not express GFP. Using the IHC protocol described above, we succeeded in revealing the presence of GFP-expressing cells in different gray and white regions, a considerable distance away from injection site indicating its ability to migrate within lesioned area of the SC tissue.

Two weeks following transplantation (Figures 5c–e), GFP-immunoreactive cells were encountered within different regions of the spinal cord. It was very interesting and worth mentioning that

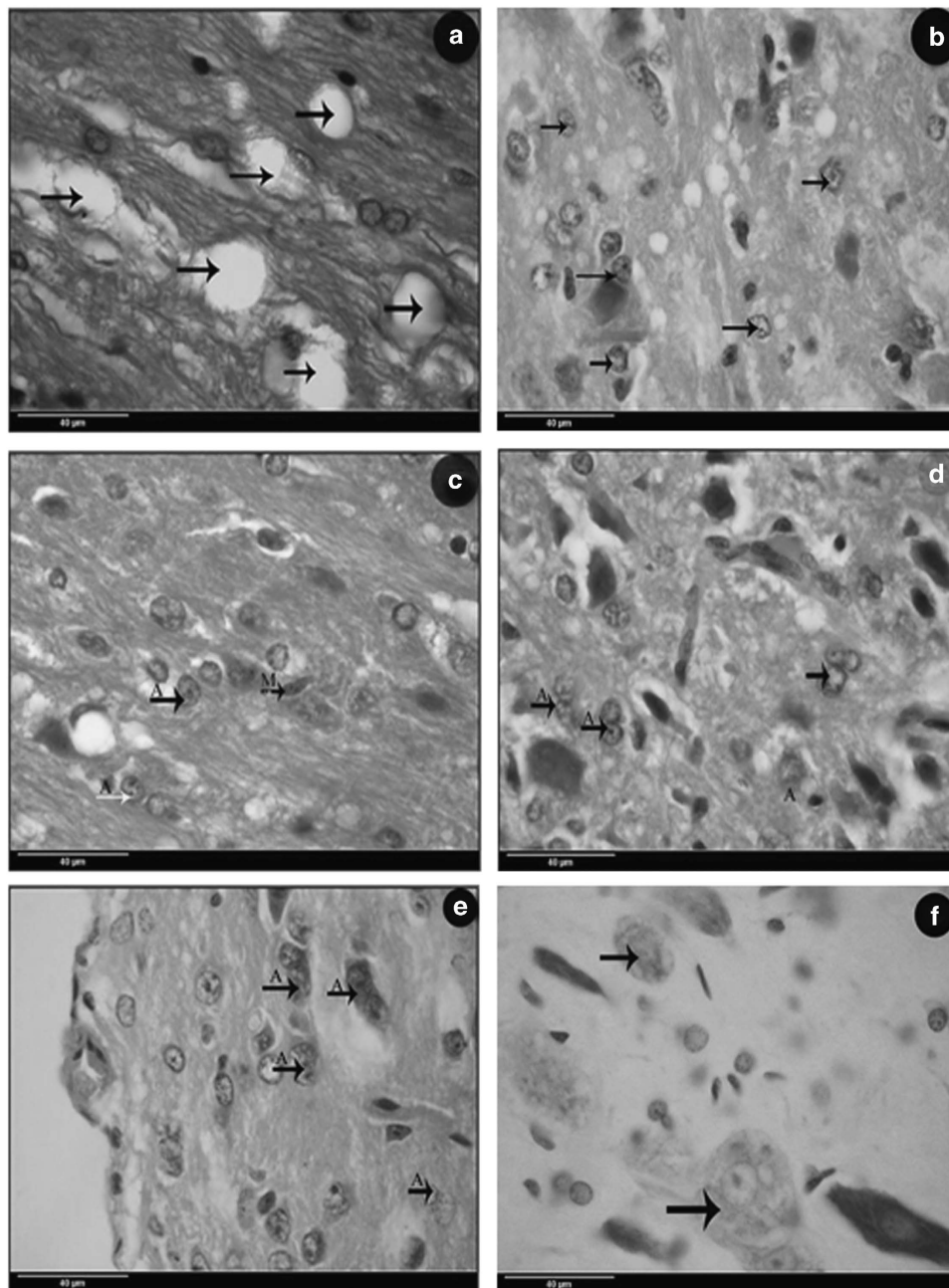


**Figure 2** (a) Two weeks post injury showing congestion and extravasation of red blood cells from blood capillary (arrows), H&E stain. (b) Two weeks post injury showing dark, shrunken necrotic neuron with eosinophilic cytoplasm, and pyknotic nuclei (arrows), H&E stain. (c) Two weeks post injury showing dark, shrunken neuronal cell bodies with loss in nuclear morphological details (arrows), H&E stain. (d) Four weeks post injury showing gray matter with many vacuolations (arrows). H&E stain. (e) Four weeks post injury showing necrotic neurons (arrows) within the gray matter. H&E stain. (f) Four weeks post injury showing edematous neuropil (arrows). H&E stain. A full color version of this figure is available at the *Spinal Cord* journal online.

most of the identified GFP-positive cells were encountered within the white matter. Few GFP-positive cells were seen in the gray matter of lesioned animals. Beside the positive GFP cells that were revealed in areas of white matter, negative GFP cells were also seen within the different regions of gray and white matter. Four weeks later (Figures 5f and 6a–d), enzyme IHC and fluorescent IHC with GFP showed the ability of engrafted OBNSCs to differentiate into neural and glial cells. Negative cells were interpreted as endogenous neuronal or neuroglia

cells. Fluorescent immunohistochemical analysis showed that the GFP-nestin cells were attracted to lesion areas (Figures 7a1, 2,3), and the GFP-GFAP-positive astrocytes formed rows between damaged and surviving nerve tracts.

Examination of GFP-stained sections at 6 to 8 weeks (Figures 6a,b,e, f and 7a,b; Supplementary Figures S3A,B) post engraftment revealed the presence of a considerable number of GFP-MAP2-positive neurons (Figure 7c). Moreover, the number of GFP-GFAP-positive

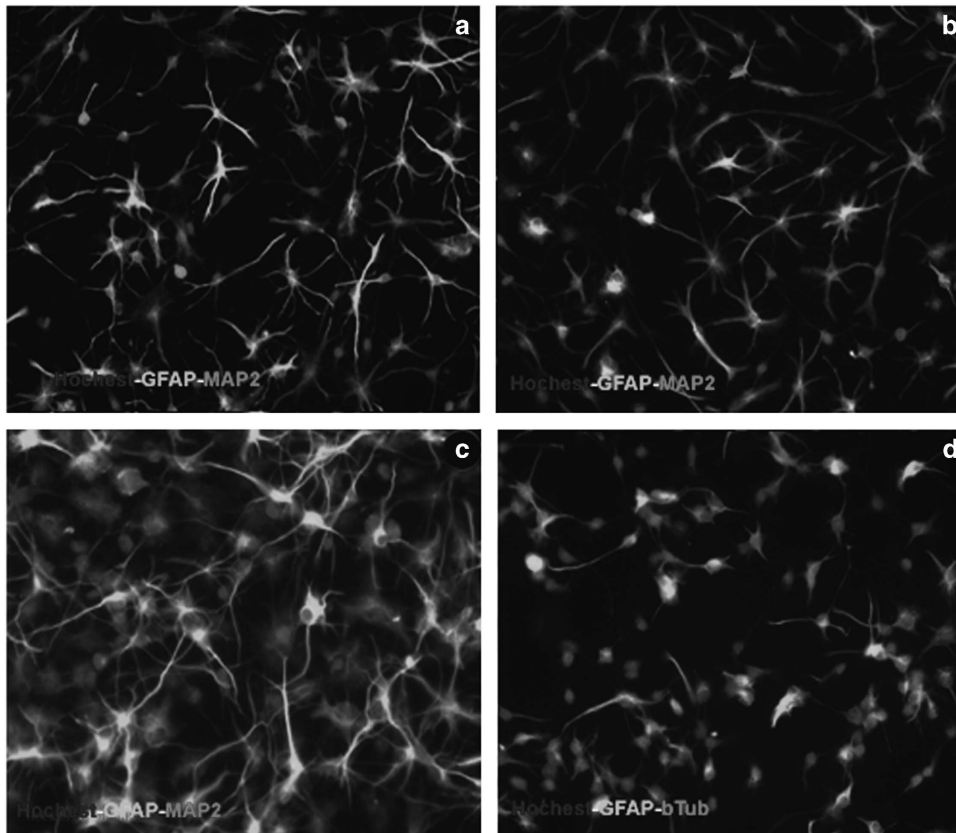


**Figure 3** (a) Four weeks post injury showing vacuolated white matter and disruption of neuronal tracts (arrows), H&E stain. (b) Four weeks post injury showing edematous neuropil with large number of hypertrophied reactive astrocytes (arrows), H&E stain. (c) Four weeks post injury showing gray matter with large pale nucleus of astrocytes (A) and dark elongated nucleus of microglia cells (M), H&E stain. (d) Six weeks post injury showing edematous gray matter with hypertrophy and twining of astrocytes (A), H&E stain. (e) Six weeks post injury showing astrocytosis with proliferative astrocytes (A), H&E stain. (f) Six weeks post injury showing lyses of Nissl granules inside neuronal cell bodies (arrows), Cresyl violet stain. A full color version of this figure is available at the *Spinal Cord* journal online.

astrocytes formed a more extensive network of GFP-GFAP-positive cytoplasmic processes within the different regions of white matter (Figure 7b). From the initial engraftment of 150 000 GFP-OBNSCs per animal,  $47 \pm 78\%$  GFP-OBNSCs present 8 weeks following transplantation (Supplementary Figure S3C), which represent 0.3-fold decrease in the initial engrafted cells.  $55 \pm 7.1\%$  of GFP-positive astrocytes,  $27 \pm 4.8\%$  were O4-positive oligodendrocyte, and  $6 \pm 4.1\%$  differentiated into GFP- $\beta$  tubulin or GFP-MAP2-positive neurons (Supplementary Figure S3D).

#### Testing of locomotor recovery

Recovery during those 7 days to a plateau was below the range of 10 points. No further improvement was noticed. One month after OBNSCs engraftments, the locomotor dysfunction was reduced with slight movements of a joint. The locomotor evaluation on the BBB test was above 12 points. Three months post treatment, no further improvements in BBB score were recorded. This might indicate that OBNSCs transplantation helped slightly alleviate the severity of the injury but with no significant improvement



**Figure 4** (a) Fluorescence image ( $\times 20$ ). The differentiation potential of GFP-OBNSC was assessed by examining their reactivity against different neuronal and glial cell molecular markers between passages 12 and 15. Differentiated GFP-OBNSCs exhibited positive immunoreactivity for GFAP astrocytes marker (45–55%; green) and MAP2 mature neuronal marker (25–30%; red). The nuclei were stained blue with Hoechst. (b) Fluorescence image ( $\times 20$ ). The differentiation potential of GFP-OBNSC was assessed by examining their reactivity against different neuronal and glial cell molecular markers. Differentiated GFP-OBNSCs exhibited positive immunoreactivity for GFAP astrocytes marker (45–55%; green) and MAP2 mature neuronal marker (25–30%; red). The nuclei were stained blue with Hoechst. (c) Fluorescence image ( $\times 40$ ). The differentiation potential of GFP-OBNSCs was assessed by examining their reactivity against GFAP (green) and MAP2 (red), immature neuronal marker (6%; red). (d) Fluorescence image ( $\times 20$ ). Differentiated GFP-OBNSCs exhibited positive immunoreactivity for  $\beta$ -Tubulin III, (red). The nuclei were stained blue with Hoechst. A full color version of this figure is available at the *Spinal Cord* journal online.

in hind limb movement ( $P \leq 0.001$ ) (Supplementary Figures S4A,B and S5A).

## DISCUSSION

We have developed a rat model by performing laminectomy at the level of T9. Classical laminectomy has always been associated with a high mortality rate in rats, either from shock induced by severe pain or due to severance of vital major sympathetic or parasympathetic pathways arising from the SC. In many cases, death occurred mainly because of gastro-intestinal impaction as the result of paralysis of major nerves arises from the damaged spinal cord or because of urinary bladder infections such as cystitis or paralysis and urinary retention. Boockvar *et al.*<sup>29</sup> demonstrated that the survival percentage of transplanted animals in CNS injury studies is often as low as 50–60% at the termination of the study.

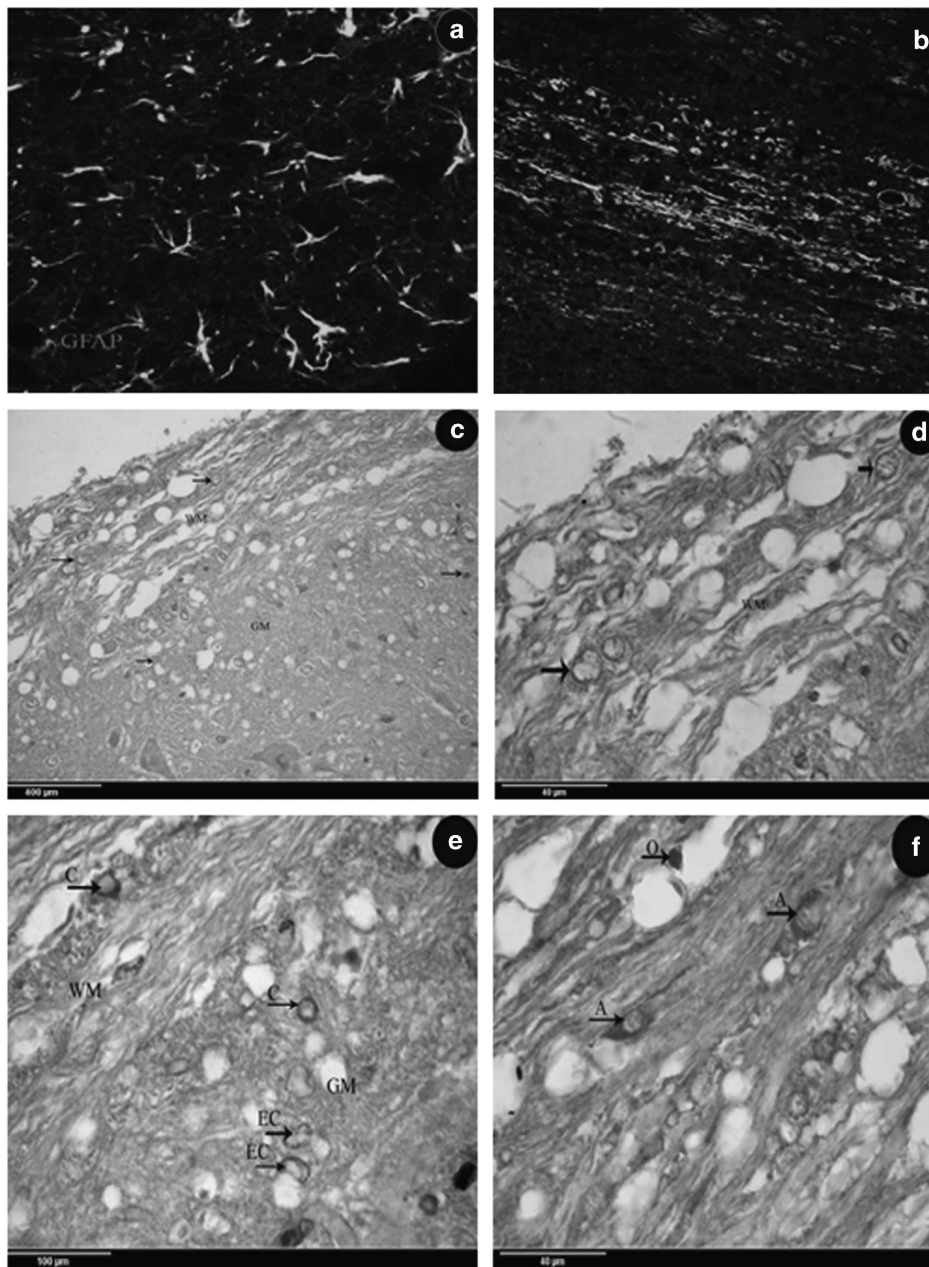
We used control groups to confirm that any tissue or functional restoration was the result of OBNSC transplantation and not a consequence of spinal cord self-neuroregeneration. Normal control and sham-operated groups showed no spontaneous neuroregeneration. Moreover, examination of sections of spinal cord of the sham group after 6 to 8 weeks showed no improvements in the damaged tissue histoarchitectures or signs of neuronal regeneration. Any

restoration or repair observed could thus be ascribed to the transplantation procedure alone.

In the present study, we used genetically engineered OBNSCs to overexpress human GFP. The rationale for these genetic modifications was to provide neurotrophic support for our cell line and to enable us to trace the resultant GFP-OBNSCs following transplantation. Other growth factors such as basic fibroblast growth factors have been reported to activate some downstream signals, essential for the neuroprotective effect, such as PI3K/Akt and ERK1/2 pathways. In particular, the PI3K/Akt pathway is very important for mediating neuronal survival under conditions of glucolipotoxicity and inflammation.<sup>30,31</sup> In the present study, GFP-OBNSCs were isolated, grown as neurospheres, and able to differentiate into neuronal and glial cells. Similar findings were previously reported by Marei *et al.*<sup>11,13,14,23</sup> who have demonstrated the capacity of OBNSC to transform *in vitro* giving rise to neurons, oligodendrocytes and astrocytes.

### GFP-OBNSCs successfully engraft and migrate

Previous studies demonstrated that xenograft engraftment of NSCs is very difficult to achieve. Furthermore, many studies fail control host immunorejection (Salazar *et al.*<sup>32</sup>). In the present study, to trace and assess the survival, proliferation and differentiation potential following

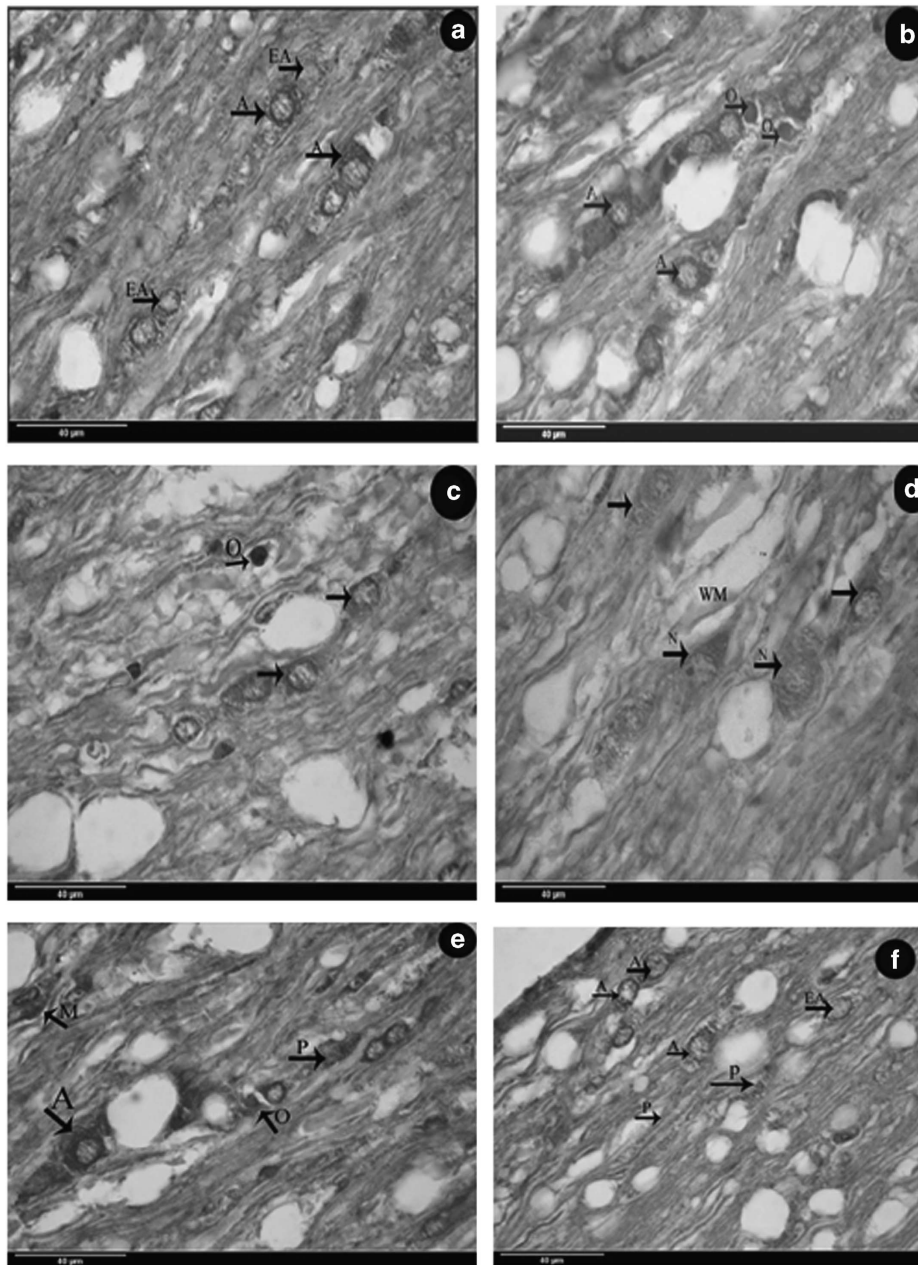


**Figure 5** (a) A higher magnification is showing extensive astrocytosis and gliosis as evident by the presence of high number of GFAP-positive (green) cells. (b) Photomicrograph of confocal microscopy double (deleted) immunostained with O4 (green) and GFP (red; deleted) of the spinal cord section 6 weeks following engraftment of GFP-OBNSCs showing the presence of a high number of O4-positive (green) oligodendrocytes. (c) Enzyme immunocytochemical staining of paraffin sections for GFP 2 weeks post engraftment of GFP-OBNSCs showing GFP-positive cells (arrows) distributed in the white matter (WM) and gray matter (GM). (d) Enzyme immunocytochemical staining of paraffin sections for GFP 2 weeks post engraftment of GFP-OBNSCs shows GFP-positive cells with dark brown granules in their cytoplasm (arrow) in the white matter (WM). (e) Enzyme immunocytochemical staining of paraffin sections for GFP 2 weeks post engraftment of GFP-OBNSCs shows GFP-positive cells (C) in the white gray matter. Note also the negative GFP cells with morphological criteria suggestive for endogenous cells (ECs). (f) Enzyme immunocytochemical staining of paraffin sections for GFP 4 weeks post engraftment of GFP-OBNSCs shows GFP-positive cells (brown) in the white matter. The GFP-positive cells exhibited morphological criteria characteristic for astrocytes (A). Note also endogenous oligodendrocytes (O) that give negative reaction for GFP. A full color version of this figure is available at the *Spinal Cord* journal online.

transplantation of GFP-OBNSCs at different time intervals designed for the present study (2, 4, 6, 8 weeks), sections from spinal cord were processed for double IHC for GFP and different neuronal (MAP2,  $\beta$  tubulin) and glial (GFAP, NG2, O4) markers. We have calculated the survival rate of engrafted GFP-OBNSCs based on calculation of the total GFP-positive cells that were identified in the different regions of the SC tissue at 4 weeks post engraftment. The

stereological quantification of the transplanted cells revealed that the survival rat ranged between 30% of the 150 000 per 5  $\mu$ l initially transplanted GFP-OBNSCs. In this regard, other studies revealed the survival of 0.1 to 37% of the initial transplanted cells detected at the time of euthanization.<sup>33–41</sup> A study transplanting human NSCs into athymic nude rats quantified 275% more cells than initially transplanted.<sup>42</sup>

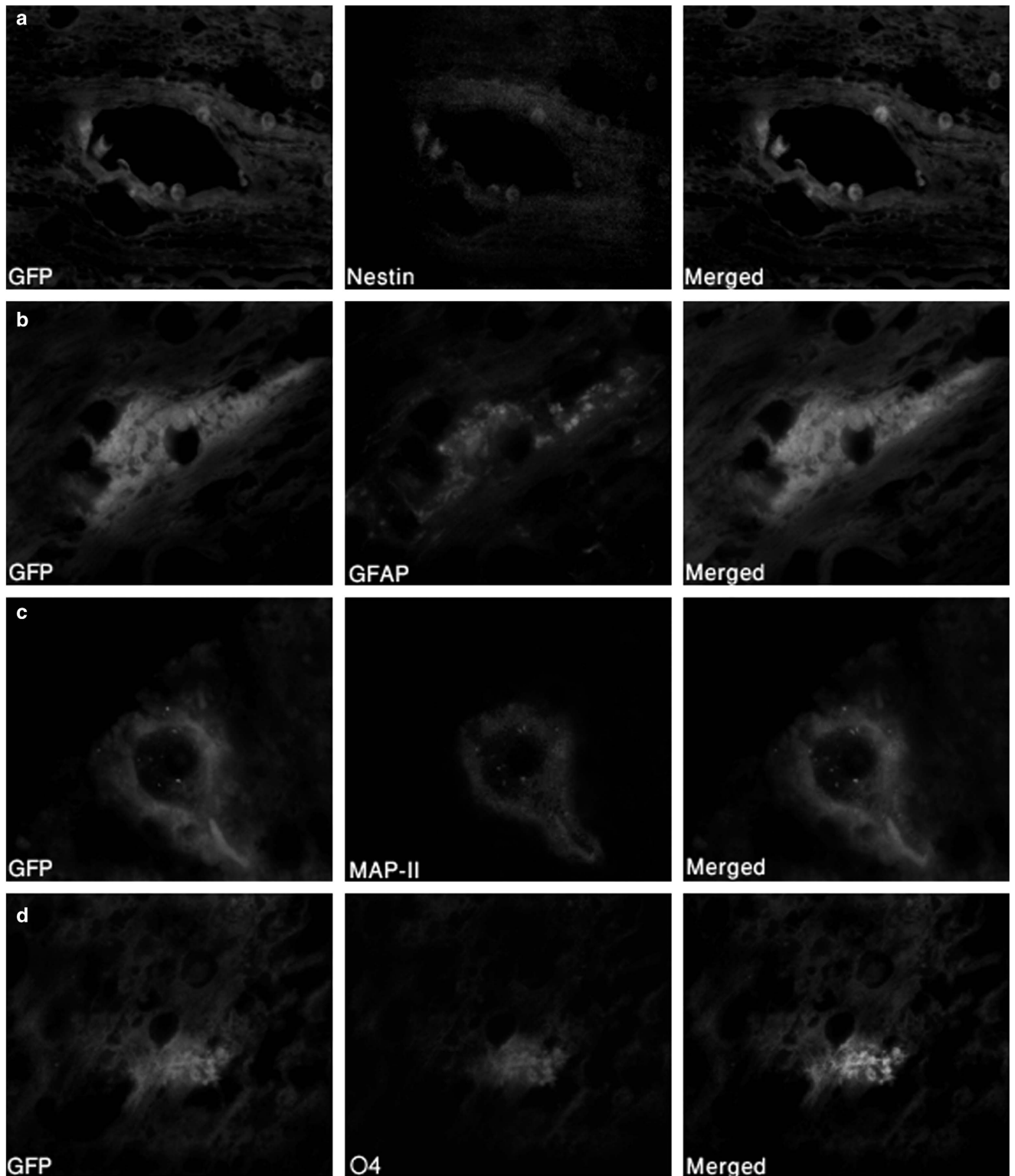




**Figure 6** Enzyme immunocytochemical staining of paraffin sections for GFP. (a) Four weeks post engraftment of GFP-OBNSCs showing GFP-positive cells (brown) in the white matter (WM). The GFP-positive cells exhibited morphological criteria characteristic for astrocytes (A). Note also, the GFP-negative endogenous astrocytes (EA). (b) Four weeks post engraftment of GFP-OBNSCs showing GFP-positive cells (brown) in the WM. The GFP-positive cells exhibited morphological criteria characteristic for astrocytes (A) and oligodendrocytes (O). (c) Four weeks post engraftment of GFP-OBNSCs showing GFP-positive cells (brown) in the WM. The GFP-positive cells exhibited morphological criteria characteristic for astrocytes (arrow) and oligodendrocytes (O). (d) Four weeks post engraftment of GFP-OBNSCs showing GFP-positive cells (brown) in the WM. The GFP-positive cells exhibited morphological criteria characteristic for astrocytes (arrows) and neuron (N). (e) Six weeks post engraftment of GFP-OBNSCs showing GFP-positive cells (brown) in the WM. The GFP-positive cells exhibited morphological criteria characteristic for astrocytes (A) with GFP-positive cytoplasmic processes (P), microglia (M) and oligodendrocytes (O). (f) Six to eight weeks post engraftment of GFP-OBNSCs showing GFP-positive cells (brown) in the WM. The GFP-positive cells exhibited morphological criteria characteristic for astrocytes with GFP-positive cytoplasmic processes and EA. A full color version of this figure is available at the *Spinal Cord* journal online.

We assessed survival of transplanted GFP-OBNSCs where we demonstrated that the survival rate was about 30% of the transplanted cells. This may indicate that the transplanted cells are capable of at least some limited proliferation inside this tissue. Using double-fluorescent IHC for GFP, and different neuronal and glial markers, we assessed the differentiation potential of our GFP-OBNSCs following transplantation. In this regard, 27%

of the engrafted cells differentiated along the GFP-O4-positive oligodendrocyte lineage, nearly as many as 16% differentiated into GFP- $\beta$ tubulin-positive neurons, and about 56% of the cells were differentiated into GFP-GFAP-positive astrocytes. Comparable results were previously reported by Piao *et al.*<sup>43</sup> and Jakovcevski and Zecevic.<sup>44</sup> No tumor or signs of immunorejections were recorded during the 8 weeks. This argues that if the method



**Figure 7** Fluorescent double-immunohistochemical staining of paraffin sections for GFP and different neural stem (nestin), neural (MAP2) and glial (GFAP, O4) markers. (a) GFP-Nestin immunoreactive undifferentiated OBNSCs 2 weeks post engraftment show migration toward lesion area. Magnification  $\times 40$ . (b) Differentiation of OBNSCs into GFP-GFAP-positive astrocytes 4 weeks post engraftment  $\times 100$ . (c) Differentiation of OBNSCs into GFP-MAP2P-positive neurons 4 weeks post engraftment  $\times 100$ . (d) Differentiation of OBNSCs into GFP-O4-positive oligodendrocytes 4 weeks post engraftment  $\times 100$ . A full color version of this figure is available at the *Spinal Cord* journal online.

can be improved for greater transplant survival and differentiation over an indefinite period, the likelihood of problems arising because of transformation of the grafts to a malignancy may be very low.

After two weeks, the great numbers of engrafted GFP-OBNSCs that displayed positive immunoreaction to undifferentiated neural SC marker (nestin) confirm the undifferentiated nature of the transplanted cells and hence an increased ability to survive in the lesioned tissue. This is in agreement with the results reported by Salazar *et al.*<sup>32</sup> who demonstrated that cells found in the white matter exhibited oligodendrocyte-like morphology, whereas those in the gray matter exhibited neuronal morphologies. The implication of the formation of GFP-O4-positive oligodendrocytes in the white matter and GFP-MAP2, and GFP- $\beta$  tubulin-positive neurons in the gray matter is that the migration of the undifferentiated SC into specific areas subjects them to local factors that determine the lineage they follow, enhancing the probability that they will become functional replacements for lost cells.

Two weeks following transplantation, most of the identified GFP-positive cells were encountered mainly in the white matter, with few, if any, GFP-positive cells in the gray matter of lesioned animals. This might reflect the differences in the local architecture of the cord that restricted migration into the gray matter compared with the white matter. Some GFP- $\beta$  tubulin-positive neurons were seen in the gray matter.

Four weeks after transplantation, the tissue sections did display different neuronal and glial cells associated with specific markers. An interesting finding was that GFP-GFAP-positive astrocytes (presumably derived from transplanted cells) tended to form rows between damaged and undamaged nerve tracts. Engrafted GFP-OBNSCs did differentiate into  $\beta$ -tubulin and MAP2-positive neurons, of which a considerable number was encountered in the gray matter. This implies that the transplanted cells have multiple potential fates, perhaps dependent on the location into which they are initially injected and/or to which they may migrate.

NSCs may be responding to mitogen present in the host microenvironment (Salazar *et al.*<sup>32</sup>). Other studies have reported that NSCs predominantly differentiate into astrocytic (Pallini *et al.*<sup>27</sup>). In the present study, 56% of transplanted hCNS-SCns differentiated into astrocytes, indicating the presence of many variables that may promote astrocytic differentiation.

#### Restoration of normal SC histoarchitecture following GFP-OBNSCs

Examination of GFP-stained sections 6 weeks after engraftment identified the presence of a considerable number of GFP-MAP2 and GFP- $\beta$ -tubulin-positive neurons. Moreover, the number of GFP-GFAP-positive astrocytes had increased forming a more extensive network of GFP-GFAP-positive cytoplasmic processes within the different regions of white matter. Using confocal microscopy, and a double immunofluorescent labeling technique for GFP and different specific neuronal and glial cell markers, we noted the presence of a considerable number of endogenous non-GFP astrocytes forming an elaborate network within the different regions of white and gray matter. Non-GFP-positive endogenous cells expressed that GFAP and O4 were also encountered within different regions of gray and white matter. Endogenous O4 and GFAP-positive cells did not express GFP. We can speculate that the neuron-like cells derived from the graft may have been close enough to normal to elicit the differentiation of endogenous glial elements from existing endogenous progenitors *in situ*.

Our findings agree with those of Salazar *et al.*<sup>32</sup> who elucidated that engrafted NSCs in white matter predominantly differentiated into oligodendrocytes. This observation might suggest that graft-derived cells might be able to remyelinate spared host axons. The same author added that  $\beta$ -tubulin III-positive NSCs were found within the gray matter.

Our results strongly indicate that engrafted cells can in fact produce relatively normal neurons and glial elements. The survival of the grafted cells is required to sustain locomotor recovery, suggesting that cell replacement is likely a key mechanism of the action of grafts in restoring function. Neuronal differentiation of transplanted hNSC could promote restoration of disrupted circuitry by formation of bridge or bypass connections.

Neuronal differentiation could also provide trophic support to enhance neuroprotection.

To overcome the need of bone morphogenetic protein, Cao *et al.*,<sup>17</sup> NSCs were engineered to express noggin, a bone morphogenetic protein antagonist, as a way to obtain better differentiation into neurons and oligodendrocytes. This strategy did not enhance the ability of NSCs expressing noggin to differentiate into neurons and oligodendrocytes.

Neurotrophin-3-NSC-derived cells enhanced the axonal sprouting that was previously observed. NPCs were then tested *in vivo* by transplanting them immediately following a C3 dorsal column transection injury.

#### GFP-OBNSC's ability to restore sensory and motor function

To determine whether our engraftment protocol could improve the locomotor functions in the rat model, we assessed the potential improvement in locomotor function using the BBB scale. Three months post treatment, no improvement in the BBB score was recorded. This might indicate that, although OBNSCs transplantation helped moderate the severity of the injury and facilitated recovery, there was no significant improvement in hind limb movement ( $P \leq 0.001$ ). These findings were in conflict with those of Salazar *et al.*<sup>32</sup> who demonstrated that NSCs transplanted into early chronic spinal cord-injured NOD-scid mice survived, proliferated and differentiated primarily into oligodendrocytes and neurons and improved behavioral recovery.

#### Mechanism of action

Several mechanisms have been suggested for the possible mechanism by which NSC-based therapy could restore lost locomotor function. Transplanted NSCs might mediate recovery by increased sparing of white matter around the lesion.<sup>45</sup> Another mechanism for recovery was suggested by Boockvar *et al.*<sup>29</sup> who feel that NSCs act to decrease the lesion size.

Despite the marked ability of the engrafted GFP-OBNSCs to remain viable and preserve their ability to differentiate, forming the SC tissue, we did not observe improved locomotor recovery in the BBB test. We believe that this can be attributed to the following: (i) the short time window used for this study; restoration of damaged nerve tracts and neuronal circuitry may require a longer time than 6 to 8 weeks; (ii) the hostile SC environment that follows injury, including extensive astrocytosis and gliosis; this may act to compromise the ability of engrafted cells to restore damaged nerve tracts.

The exact mechanism of recovery is not known, but integration of GFP-OBNSCs and remyelination of host axons is one possibility, and we suggest that GFP-OBNSCs were able to differentiate and integrate with the host cells. In this regard, wild-type human NSC differentiated

into astrocytes or oligodendrocytes but not into the neurons after transplantation.<sup>46,47</sup>

The ability to differentiate into oligodendrocyte, astrocytes and neurons means that the contributions of neuronal and glial elements to locomotor recovery need further investigations. Previous studies have suggested that SCI transplantation is more effective subacutely rather than in a chronic state.<sup>33</sup>

Several studies have provided explanation why NSC transplantation might in some case fail to induce a significant improvement in the locomotor functions. The reasons for failure were explained on the basis of formation of a cystic cavity and a glial scar, demyelination that occurs after<sup>48</sup> and secretion of inhibitory molecules by astrocytes, making it difficult for injured neurons to regenerate across the injury site. The main class of inhibitory molecules include chondroitin sulfate proteoglycans,<sup>49</sup> which have been shown to inhibit axonal regeneration in 3D settings.<sup>50</sup>

## DATA ARCHIVING

There were no data to deposit.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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