Final Progress Report for the New Jersey Commission on Spinal Cord Research

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Preamble:

This report is the *Final Narrative* summarizing our research supported by the New Jersey Commission on Spinal Cord Research. Unfortunately, Dr. Ira B. Black, PI of record, died January 10, 2006.

"Transplantation of Adult Bone Marrow Stromal Cell-Derived Neurons to Spinal Cord"
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Grant Number 01-3009-SCR-S-0
Completed

1. Goals

Recent studies from our group differentiated adult human and rat bone marrow stromal stem cells (BMSCs) into neurons (Black and Woodbury, 2001; Woodbury et al 2002). *Our ultimate goal was to use these cells in the therapeutic treatment of spinal cord injury.* BMSCs normally differentiate only into mesenchymal cells, including bone, cartilage, muscle, tendon and fat. The differentiation of BMSCs into non-mesenchymal fates had not been demonstrated. Using a relatively simple treatment protocol, the stromal cells were found to be induced to differentiate into a "neuronal" fate *in vitro*. These differentiated cells exhibit neuronal morphological traits, and express a variety of neuron-specific genes (Black and Woodbury, 2001; Woodbury et al 2002). Clonal cell lines, established from single cells, proliferated, yielding both undifferentiated and neuronal cells. Our observations suggest that intrinsic genomic mechanisms of commitment, lineage restriction and cell fate are mutable. Environmental signals apparently can elicit the expression of pluripotentiality that extends well beyond the accepted fate restrictions of cells originating in classical embryonic germ layers.

2. Findings

During this grant period, we attempted to optimize BMSC-derived neurons for transplantations to the injured spinal cord by: (a) defining molecular mechanisms governing neuronal differentiation, (b) delineating the relationship of differentiation to cell division and cell death, (c) perfecting the neuron-inducing medium to maximize the production and health of neurons, and (d) optimizing the neurosurgical transplantation protocols in normal and damaged cords in live rats to maximize recovery of function.

During the course of these investigations we examined over 100 transplantations of either bone marrow stem cells or BMSCs differentiated into neurons into the normal rat spinal cords and into rats that had the dorsal half of the spinal cords transected at T10. At various time points following transplantation, the animals were sacrificed and the spinal cords were immunohistologically processed for cell-type markers to reveal neurons, glia and macrophage-microglia lineages.

Our initial observations of green fluorescent protein (GFP)-transfected BMSCs that were differentiated into "neurons" were quite encouraging. Within a few days of transplantation, the cells displayed many morphological features of neurons including the development of long processes (See Figure 1). In addition, BMSCs that were *not* subjected to differentiation-induction were also found to develop long processes following engraftment (Figure 2).

However, within 1 – 2 weeks post transplantation most GFP labeling disappeared, even with immune suppressing drugs. We hypothesized that the loss of GFP labeling was either due to the death of the transplanted BMSCs or due to a downregulation of the transfected GFP label. To distinguish between these two possibilities, we double labeled the BMSCs with BrdU and GFP (in some cases we used a cell-line expressing cyan fluorescent protein (CFP)). Although GFP labeling continued to disappear within a week of transplantation, numerous BrdU labeled nuclei were detected up to 9 weeks post injection, *particularly in injured spinal cords*. Figure 3 illustrates an example of a transplant of "neuronally" differentiated BMSCs into a rat with a spinal cord injury. The cells were injected on the day of the injury and the animal was allowed to survive 6 weeks. Numerous BrdU-labeled cells were found within and adjacent to the wound. None of the BrdU labeled-profiles expressed neurofilament (medium chain, NF-M). However, numerous NF-M-positive fibers appeared to grow into the graft. This suggests that the graft site may be supportive of axonal growth (see for example Hoffstetter et al 2002). .

In addition, we were unable to consistently find BrdU-labeled cells that also expressed NF-L, Tau, B-tubulin III or NeuN (all neuronal markers). We also did not find co-expression of BrdU and GFAP (a characteristic of astrocytes). Thus the BMSCs did not demonstrate the plasticity reported elsewhere (e.g. Woodbury et al 2002; Munoz-Elias et al 2004). This was true for both differentiated and undifferentiated BMSCs.

The transplantation of BMSCs elicited a strong and immediate inflammatory response, even in animals that were treated with the immune suppressing drug cyclosporine. This was evidenced by intensive ED-1 immunostaining within the graft (See Figure 4). ED-1 is a specific marker for activated microglia and monocytes/macrophages in rats. Using the lectin IB4, which also marks rat microglia and macrophages, we were able to detect large numbers of inflammatory cells invading the graft 72 hrs and 1 week after transplantation (Figure 5). In addition, the graft became rapidly encapsulated by reactive astrocytes, which delineated a boundary between the graft and the host (Figure 6).

This strong immune response and rapid clearance of the injected BMSCs was mirrored in a parallel study conducted by Dr. Black and his colleagues in which BMSCs were injected into the brains of rats (Coyne et al 2007). Within the brain, Coyne et al (2007) found BMSC grafts became rapidly infiltrated by ED-1 positive microglia and macrophages. By 14 days after

grafting, the GFP labelling was absent and the graft site was encapsulated by GFAP positive astrocytes. Similar to our findings, BMSC transplants were rapidly rejected by the host, even under an immune suppression regimen.

Similar to our observations in the spinal cord, Coyne et al (2007) were able to detect BrdU labelling within the brain many weeks following the transplantation of BrdU-labelled BMSCs. However, they present considerable evidence of a transfer of BrdU from the graft to the host, calling into question the validity of BrdU as a label for distinguishing host from graft.

Literature Cited

Black IB and Woodbury D. 2001. Adult rat and human bone marrow stromal stem cells differentiate into neurons. Blood Cells Mol Dis 27:632-636.

Coyne TM, Marcus AJ, Woodbury D, and Black IB. 2007. Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. Stem Cells 24:2483-2492.

Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, and Olson L. 2002. Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. Proc Natl Acad Sci U S A 99:2199-2204.

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Woodbury D, Reynolds K, and Black IB. 2002. Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J Neurosci Res 69:908-917.

3. Implications for Future Study and Clinical Treatment

Our approach, using adult stem cell-derived neurons confers several potential future advantages: (1) Use of the patient's own cells for autologous transplantation eliminates the danger of immunorejection and the need for toxic immunosuppressive agents; (2) The self-renewing BMSCs and the neurons grow vigorously in culture providing a vast reservoir of source material; (3) Neuronal differentiation is achieved by environmental manipulation only, without altering the genome, eliminating the need for immortalization and minimizing the probability of neoplastic transformation; (4) The use of adult cells circumvents the ethical concerns associated with the use of embryos.

Future studies will have to address the issue of the strong immune response observed following BMSC transplants into the spinal cord and the brain (Coyne et al 2007). One possible approach may be to use autologous transplants. Another area that needs to be addressed is the degree to which BMSCs may support axonal growth. We (see Figure 3), and others (Hofstetter et al 2002) have observed severed axons regenerating into the graft.

4. Plans for Continuation of this Work

We are presently examining the molecular mechanisms governing differentiation and self-renewal in BMSCs and in stem cells from other sources. Future studies will address the strong immune response elicited by BMSCs. Dr. Crockett and Dr. Kouichi Ito, Department of Neurology have recently submitted a grant proposal to the New Jersey Commission on Spinal Cord Research to examine to the degree to which the immune system may be modulated to allow for BMSC survival and differentiation following spinal cord injury.

5. Publications

Although a large number of technical issues prevented the successful completion of the original goals of the project, several publications and scientific reports were generated as a result of Dr. Black's efforts to define and refine the potential therapeutic use of adult bone marrow stromal cell-derived stem cells.

Black, I.B. and Woodbury, D.: Adult rat and human bone marrow stromal stem cells differentiate into neurons. Blood Cells Mol. Dis., 27(3), 632-636, 2001.

Muñoz-Elias, G., Woodbury, D.L., and Black, I.B.: Differentiating marrow stromal stem cells express neuronal gene products and morphologies during cell division. Soc. Neurosci. Vol. 27, 2001.

Woodbury, D., Reynolds, K., Crockett, D.P., and Black, I.B.: Adult bone marrow stromal stem cells express germline, ectodermal, endodermal and mesodermal genes prior to neurogenesis. Soc. Neurosci. Vol. 27, 2001.

Woodbury, D., Reynolds, K., and Black, I.B.: Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J. Neurosci. Res., 96, 908-917, 2002.

Kramer, B.C., Woodbury, D., and Black, I.B.: Adult rat bone marrow stromal cells express genes associated with dopaminergic neurons. Soc. Neurosci. Vol. 28, 2002.

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Muñoz-Elias, G., Woodbury, D., and Black, I.B.: Marrow stromal cells, mitosis and neuronal differentiation: stem cell and precursor functions. Stem Cells, 21:437-448, 2003.

Woodbury, D. and Black, I.B.: Long-term survival, migration and phenotypic expression of marrow stromal cells transplanted into the adult rat brain. Soc. Neurosci., Vol., 29, 2003.

Kramer, B.C., Marcus, A.J., Coyne, T.M., Reynolds, K., Woodbury, D., and Black, I.B.: A human amniocyte cell line differentiates into putative neurons in vitro. Soc. Neurosci., Vol., 29, 2003.

Muñoz-Elias, G., Marcus, A.J., Coyne, T.M., Woodbury, D., and Black, I.B.: Adult bone marrow stromal cells in the embryonic brain: engraftment, migration, differentiation, and long-term survival. J. Neurosci., 24 (16), 4585-4595, 2004.

Coyne, T.M., Marcus, A.J., Kramer, B.C., Woodbury, D., and Black, I.B.: Transplantation of marrow stromal cells to the adult brain: Long-term survival and region-specific gene expression. Soc. Neurosci., Vol. 30, 2004.

Marcus, A.J., Coyne, T.M., Reynolds, K., Woodbury, D., and Black, I.B.: Adult bone marrow stromal cells in the embryonic brain: Long-term six months survival and subregion-specific differentiation. Soc. Neurosci., Vol. 30, 2004.

Kramer, B.C., Woodbury, D., and Black, I.B.: Cloning and transfection of nurr-1 into adult rat bone marrow stromal cells reveals a potential nurr variant. Soc. Neurosci., Vol. 30, 2004.

Differentiated BMSCs Exhibit Extensive Processes after Transplantation into the Cervical Spinal Cord of Intact Rats

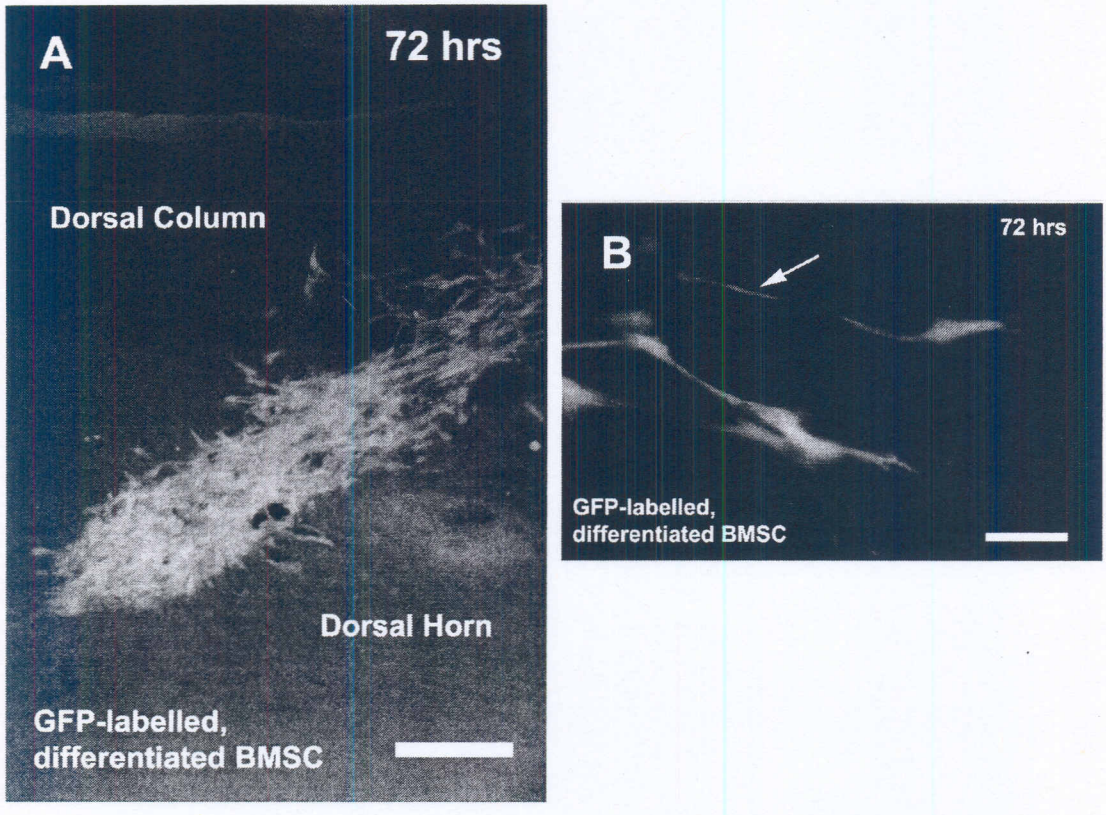

Figure 1 Photomicrographs of differentiated, Green-fluorescent protein (GFP)-labelled BMSCs injected into the cervical spinal cord (C6/C7) of an uninjured rat. The rat was sacrificed 72 hrs after transplantation. Scale Bar = 100 μm. **A**. Low-power view of an injection site Note some cells appear to be migrating away from the injection site. **B**. High-magnification view of differentiated BMSCs that possess extensive neuron-like processes (arrow) Scale Bar = 50 μm.

Undifferentiated BMSCs Develop Extensive Processes *in vivo*

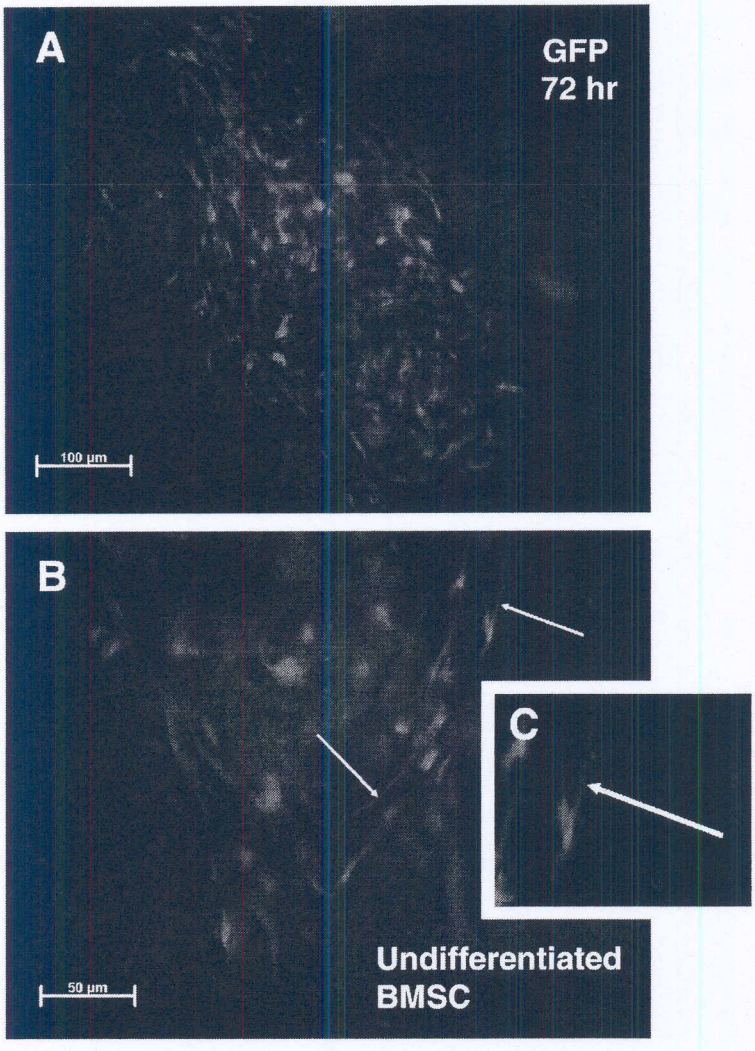

Figure 2 Photomicrographs of undifferentiated, green-fluorescent protein (GFP)-labelled BMSCs injected into the cervical spinal cord (C6/C7) of an uninjured rat. The rat was sacrificed 72 hrs after engrafting the cells. These cells were not exposed to the neuron induction medium. **A.** Low-power, sagittal view of the injection site. **B.** The ventral portions of the injection site at higher magnification. Many of the cells possessed long, neuron-like processes (Arrows point to two examples). **C.** High magnification view of a single cell with processes (same cell as upper right arrow in B).

BrdU-labeled Nuclei were Detected Several Weeks after Engraftment

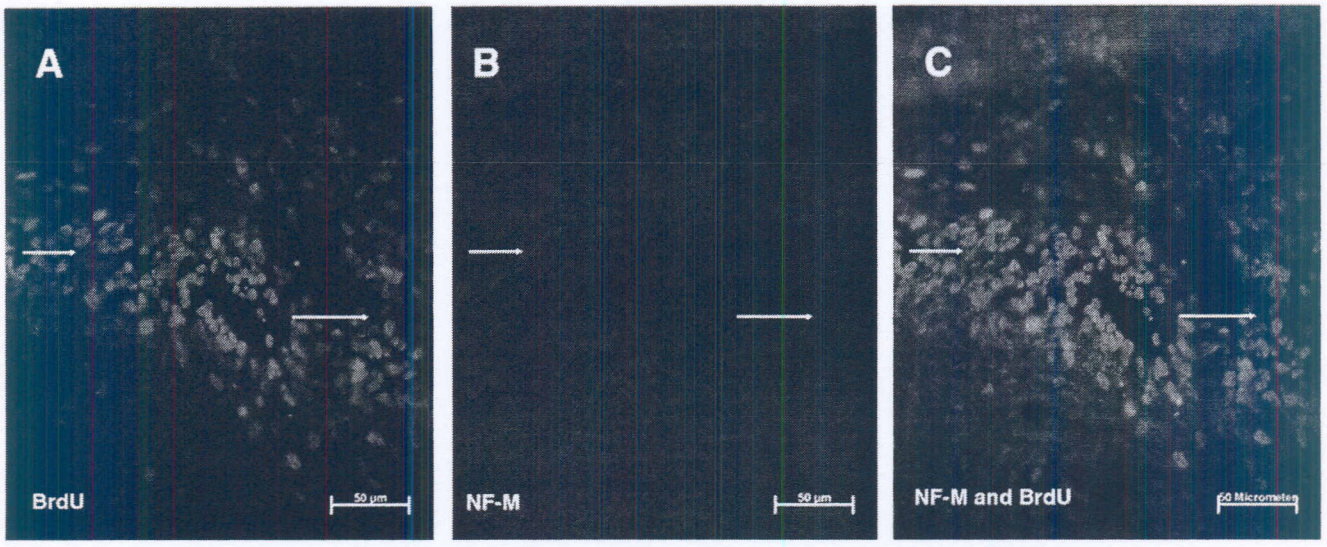

Figure 3 Detection of BrdU labelled nuclei six weeks post transplantation. NF-M fibers were found within the graft. **A.** BrdU labelled BMSCs detected by antibodies to BrdU and viewed under a Cy2 filter set. The cells were injected into the injured spinal cord (dorsal hemisection) at the time of injury. Survival time = 6 weeks. **B.** Immunoreactivity for neurofilament medium chain (NF-M) viewed with a Cy3 filter set. **C.** Overlay of **A** and **B**. The arrows point to NF-M positive fibers originating from the host, apparently growing into the graft. None of the BrdU-labeled cells appeared to express NF-M. Scale bar = 50 µm.

One Week following Transplantation of BMSC into the Uninjured Spinal Cord, the Graft is Invaded by ED-1+ Macrophages

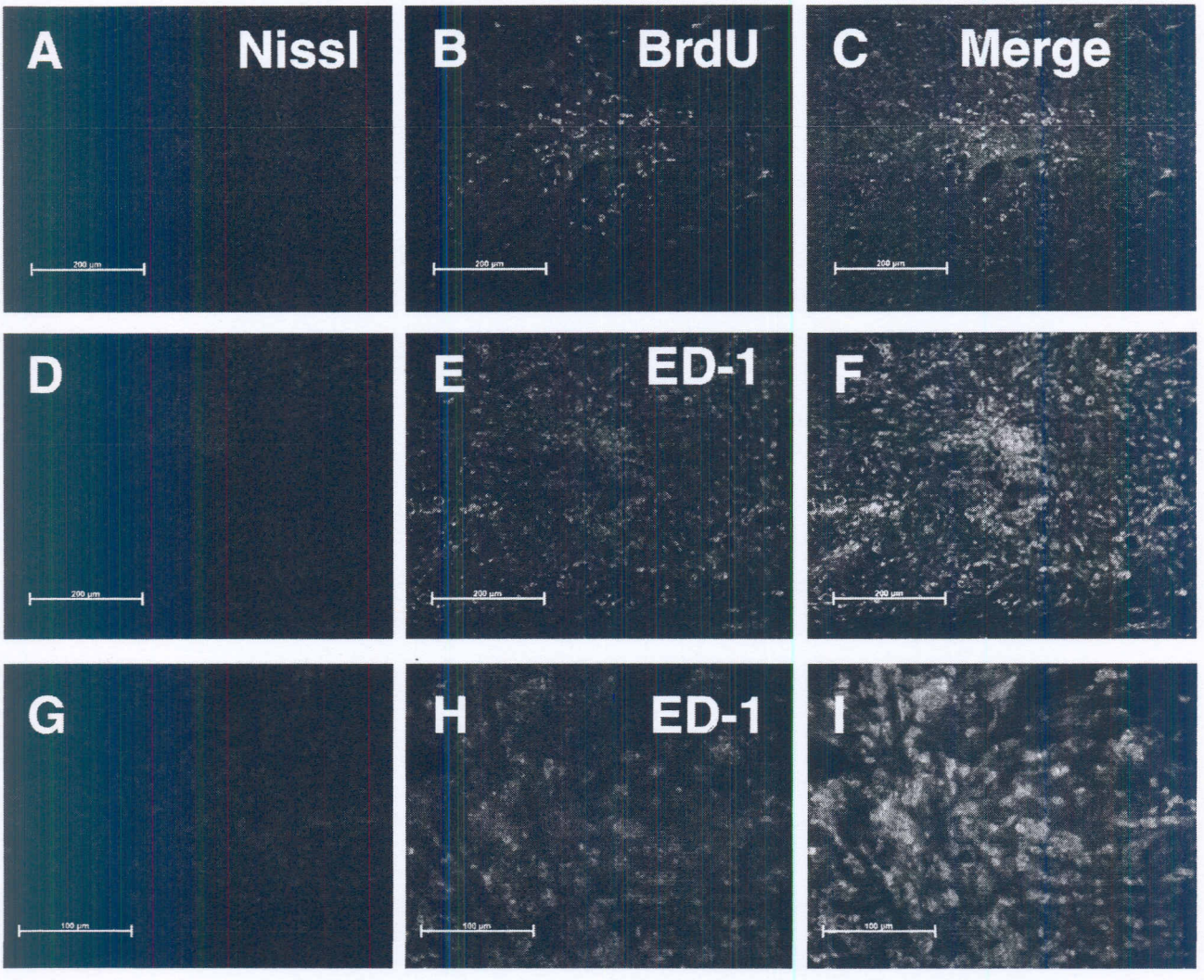

Figure 4 Antibodies against the macrophage marker, ED-1, revealed an intense immune response within the graft of BMSCs one week following transplantation. The rat was treated with immune suppressing cyclosporine. **A**, **D**, and **G** illustrate Nissl staining with NeuroTrace Red. **B** shows BrdU immunoreactivity to reveal the grafted cells. **E, H** depict immunostaining for ED-1. **C**, **F** and **I** are merged images. **G, H** and **I** are high magnification view of the graft depicted in **D**, **E** and **F.** Scale Bars A –F = 200 μm; Scale Bars G – I = 100 μm.

One Week following Transplantation of BMSC into the Uninjured, Cyclosporine-treated Spinal Cord, the Graft is Invaded by Griffonia Simplicifolia (IB4)+ Macrophages

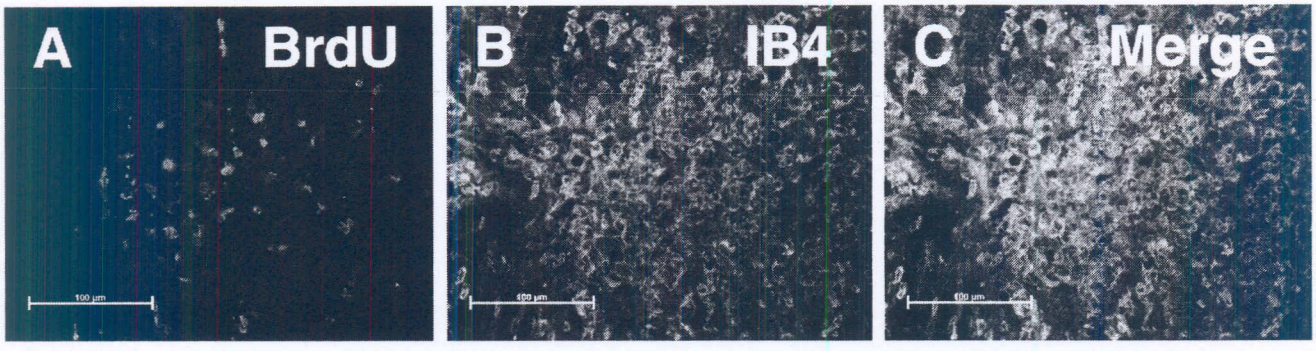

Figure 5 We also used the lectin Griffonia simplicifolia (IB4) to detect macrophages invading the graft. Here we illustrated BrdU-labelled undifferentiated BMSCs transplanted into a cyclosporine-treated rat. The rat was sacrificed 1 week following transplantation of the cells. **A**. BrdU immunoreactivity. **B.** IB4 binding. **C**. Merged image of **A** and **B**. Scale Bars = 100 μm.

The BMDC Graft becomes Encapsulated by Astrocytes.

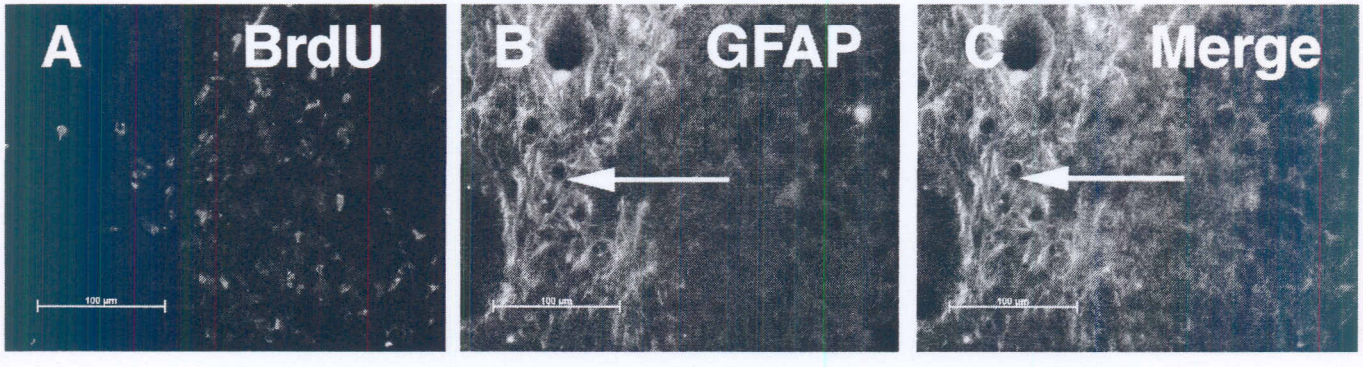

Figure 6 The grafted cells become encapsulated by GFAP+ astrocytes. **A.** BrdU immunostaining to reveal undifferentiated BMSCs transplanted into a cyclosporine treated rat, 1 week prior to sacrifice. **B.** GFAP immunostaining of reactive astrocytes (arrow) encapsulating the graft. **C.** Merged image of **A.** and **B**. Scale Bars = 100 μm.