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Efficient Differentiation of Human Embryonic Stem Cells into Oligodendrocyte Progenitors for Application in a Rat Contusion Model of Spinal Cord Injury

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ABSTRACT

This study utilized a contusion model of spinal cord injury (SCI) in rats using the standardized NYU-MASCIS impactor, after which oligodendrocyte progenitor cells (OPCs) derived from human embryonic stem cell (ESC) were transplanted into the spinal cord to study their survival and migration route toward the areas of injury. One critical aspect of successful cell-based SCI therapy is the time of injection following injury. OPCs were injected at two clinically relevant times when most damage occurs to the surrounding tissue, 3 and 24 hours following injury. Migration and survivability after eight days was measured postmortem. In-vitro immunofluorescence revealed that most ESC-derived OPCs expressed oligodendrocyte markers, including CNPase, GalC, Olig1, O4, and O1. Results showed that OPCs survived when injected at the center of injury and migrated away from the injection sites after one week. Histological sections revealed integration of ESC-derived OPCs into the spinal cord with contusion injury without disruption to the parenchyma. Cells survived for a minimum of eight days after injury, without tumor or cyst formation. The extent of injury and effect of early cell transplant was measured using behavioral and electrophysiological assessments which demonstrated increased neurological responses in rats transplanted with OPCs compared to controls.

KEYWORDS: contusion, embryonic stem cells, motor behavior, oligodendrocyte, somatosensory evoked potential, spinal cord injury

INTRODUCTION

Spinal cord injury (SCI) is characterized by rapid necrosis of neural tissue at the site of injury. This is followed by a delayed secondary degeneration of motor neurons and supporting glial cells which spreads to surrounding gray and white matter regions of the spinal cord (SC). These events culminate in the chronic progressive demyelination of motor neurons suggesting that oligodendrocytes (OLs) should be targeted for remyelination strategies. OLs are a type of glial cells within the central nervous system (CNS) which produce myelin, a macromolecule that insulates neurons. This insulation is essential for the communication of neurons. The regenerative capacity of the adult CNS is limited in its ability to restore damaged tissue. As a result, preclinical trauma research has primarily focused on reducing

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secondary degeneration. However, previous studies suggest that precursors derived from embryonic stem cells (ESCs) potentially can both promote regeneration and reduce secondary degeneration.

Several reports of the therapeutic use of mouse and human ESC-derived neural precursors have been reported in animal models of SCI (Cloutier, Siegenthaler, Nistor, & Keirstead, 2006; Faulkner & Keirstead, 2005; Keirstead et al., 2005; Kerr et al., 2003; Liu et al., 2000). These studies have shown that ESC-derived oligodendrocyte progenitor cells (OPCs) transplanted into rats with diffused motor neuron injury resulted in partial recovery from paralysis by protecting host neurons and facilitating reafferentation of motor neuron cell bodies. Several of these reports by Keirstead and colleagues have specifically shown this phenomenon for human ESC-derived OLs transplanted into rats with mild and severe SCIs. In these studies, transplanted ESC-derived oligodendrocytic populations were able to restore myelination in rats treated seven days after the initial injury, which led to a recovery of motor skills. However, the same treatment did not work on rats that had been injured for 10 months, suggesting that SC therapy in humans during the very early stages of the injury may be more effective.

The purpose of this study was to determine whether ESC-derived OPCs are capable of surviving and integrating into the host tissue when transplanted at short intervals after "moderate" SCI, including 3 hours or 24 hours after infarction. The former time point represents a clinically relevant treatment period for a patient immediately following SCI, while the latter corresponds to the time when most patients are typically stabilized for transplant. Results showed that rats injected with OPCs demonstrated a greater increase in locomotor capabilities and Somatosensory Evoked Potentials (SEP) after one week compared to rats injected with either saline or human fibroblasts. These short-term effects are consistent with OPCs expressing myelin basic protein (MBP) at the site of injection and the presence of OPC filopodial extensions into the white matter. By comparing these early time points, this study begins to address the ability of ESC-derived OPCs to potentially benefit the outcome of CNS injury by promoting survival and growth of host tissue as well as replacing lost cells during the early stages of injury.

MATERIALS & METHODS

Cell Culture

H1 (WA01) cells were purchased from WiCell (Madison, WI) and expanded in ESC growth media (Amit et al., 2000) and differentiated according to modified protocols (Keirstead et al., 2005; Zhang, Wernig, Duncan, Brustle, & Thomson, 2001). Briefly ESC cultures were maintained using supplier protocols. Neural differentiation of ESCs were performed via embryoid body (EB) suspensions with N2B27 medium (Bottenstein & Sato, 1979) supplemented with 200 ng/ml noggin, 20 ng/ml FGF2, and 20 ng/ml FGF4 (all from R&D Systems). EBs were grown for 15 days and then plated on matrigel and grown in N2B27 media supplemented with 20 ng/ml FGF2 for 5 days. Neural progenitors were then split onto matrigel with 0.05% trypsin/EDTA and propagated for 3 weeks in N2B27 media with the addition of 20 ng/ml of PDGFAA (PeproTech) and 20 ng/ml EGF (R&D Systems) until transplantation. Human fibroblasts were derived from human neonatal foreskin (ATCC).

Animals

Rats used in the following study included female Sprague Dawley adult rats weighing approximately 200–220 g and 6–8 weeks of age. All rats were immunocompromised three days prior to cell transplantation with daily injections of cyclosporin A (10 mg/kg/d).

Contusion Model

All experimental procedures in this study adhered to the guidelines delineated in the *Rodent Survival Surgery* guide and approved by the Institutional Animal Care and Use Committees at the Johns Hopkins University. Rats were anesthetized with 7.5 mg/kg ketamine (Phoenix Pharmaceutical) and 60 mg/kg xylazine (Phoenix Pharmaceutical). Laminectomy was performed at thoracic vertebra T8 without perturbing meninges at or below the dura mater. Injury was produced using a NYU-MASCIS impactor consisting of a rod weighing 10 g dropped from a 12.5 mm height to produce "moderate" injury. The NYU-MASCIS impactor model (Gruner, 1992; Young, 2002) is one of the most accepted contusion models for studying SCI, by way of generating injuries which closely parallel human clinical SCI cases (Metz et al., 2000).

Cell Transplantation

Rats were anesthetized as described, and the laminectomy site was re-exposed. Approximately 150,000 cells were injected at a depth of 1.0 mm into the white matter at T7 and T9 and 500,000 cells at a depth of 1.5 mm into the gray matter at the T8

epicenter in 2 μl at a rate of 1 μl/minute using a 10 μl Hamilton syringe. Controls included animals that received D-PBS injections only. Postmortem characterization of transplants was observed eight days following transplantation. Five animals were used per treatment.

Immunohistochemistry

After animal perfusion, SCs from T6-T10 were isolated, incubated overnight in 30% sucrose/DPBS and cryopreserved in O.C.T. freezing compound (Tissue-Tek). Tissue was cut into 8 μm sections and prepared for immunostaining. Staining was performed using antibodies against Oligodendrocyte marker 1 (O1), Oligodendrocyte marker 4 (O4), Galactocerebroside (GalC), A2B5, Nestin, NG2, Sox10, Platelet-derived-growth-factor-receptor-alpha (PDGFR-α), Olig1, MBP, and CNPase using standard protocols along with fluorescent secondary antibodies (all purchased from Millipore). 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclei to determine the percentage of immunopositive cells. Negative controls included isotype primary serum and secondary antibody alone.

Behavioral Tests

The open-field Basso Beattie Bresnahan (BBB) locomotor rating scale is a widely accepted test of locomotor function (Basso, Beattie, & Bresnahan, 1995, 1996). The scale is based on a 0–21-point ranking system. Two well-trained observers blindly scored coordination, paw placement, hind limb movement, trunk stability, and tail placement on a daily basis.

Somatosensory Evoked Potential Measurements

Electrophysiological responses were recorded according to previously reported protocols(Agrawal, Thakor, & All, 2009; All et al., 2009). In brief, 1–2 days prior to injury, transcranial electrodes were implanted as well as a subcutaneous electrode used to stimulate limbs. An optically isolated biopotential amplifier was used to amplify cortical SEP signals.

RESULTS

Several studies have shown the production of oligodendrocyte precursors from human ESCs (Faulkner & Keirstead, 2005; Izrael et al., 2007; Kang et al., 2007; J. Q. Zhang et al., 2006). Furthermore, developmental studies in the mouse and humans demonstrate that several markers have been shown to characterize stages in oligodendrocytic development. Markers of early neuroectodermal induction include Nestin, A2B5, and Sox10 as well as Pax6, Lim1, Islet1, and Sox1. We chose to study the former three markers as the latter group is also expressed in early endoderm formation. Oligodendritic cell fate begins when Nestin$^+$ and A2B5$^+$ neuroectodermal progenitors differentiate into glial-restricted precursors that express PDGFR-α and NG2. These cells give rise to progenitors which differentiate into oligodendrocyte and astrocyte progenitors. Preoligodendrocytic progenitors begin to express Olig1, O4, PDGFR-α, and NG2 while later pro-oligodendrocytic progenitors display O1, CNPase, and GalC (Cai, Pang, Xiao, & Rhodes, 2001; Holland, 2001; Jakovcevski & Zecevic, 2005a; Jakovcevski & Zecevic, 2005b; Magnus & Rao, 2005; Miller, 1996; Tekki-Kessaris et al., 2001; S. C. Zhang, Ge, & Duncan, 2000). In this study, the majority of cells (>90%) expressed these markers uniformly, while GalC and CNPase were variable in both expression intensity and number of cells expressing these markers (~50% and 80% positive cells respectively, Figure 1).

In addition to later progenitor markers, mature OLs also express MBP. Previous studies have shown that MBP$^+$ and mature OLs are produced after growth factor withdrawal and the introduction of 3,3′5-triido-L-thyroine (T3). In this study, we performed a detailed immunocytochemical analysis to confirm the developmental state of OPCs derived without T3. Our results show that OPCs cultured with FGF2 and PDGFR-α after several weeks express later markers of oligodendrocyte differentiation except for MBP. Negative controls included secondary antibody only (data not shown). Importantly, OPCs did not express markers of undifferentiated pluripotent cells such as Oct4, Nanog, and Sox2 (data not shown). Cellular proliferation rapidly decreased after two months, so that most cultures had ceased dividing after three months. These cells never expressed MBP in culture.

After transplantation, results showed the integration of ESC-derived OPCs into the rat SC with contusion injury, without disruption of the parenchyma (Figure 2). Cells survived for the duration of experiments without tumor formation. Injected OPCs did not express markers of undifferentiated pluripotent cells (data not shown). On the other hand, OPCs did express human-specific Nestin, which along with human specific nuclear antigen expression, was used to discriminate between human and host tissue. When cells were injected into the gray matter (Figure 2(A)), OPCs appeared to migrate from the site of injury and Nestin$^+$ cellular extensions were seen in the white matter surrounding the injection site. In ad-

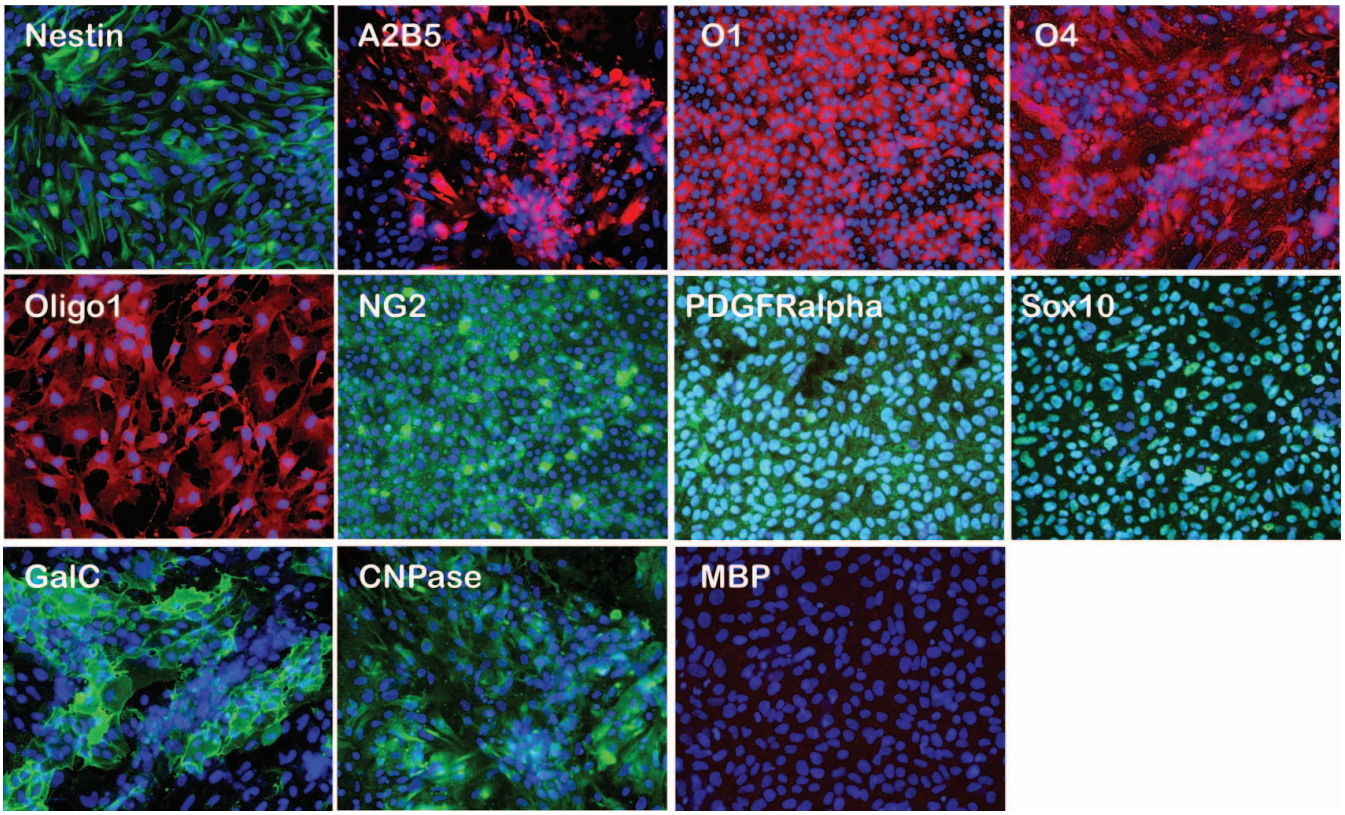

FIGURE 1. Indirect immunofluorescent expression of markers of early neural progenitors and the oligodendroglial lineage in human embryonic stem cell derived OPCs after two weeks in PDGF- and EGF-supplemented N2B27 media. Markers of early NPCs included Nestin, Olig1, A2B5, and Sox10. Markers of early OPCs included O1, O4, and PDGFR-α. Later OPC markers included NG2, CNPase, GalC, and mature oligodendrocytes, MBP. DAPI (blue) was used to stain nuclei.

dition to the OPC markers, CNPase (Figure 2(C)) and Olig1 (Figure 2(D)), transplanted OPCs also eventually expressed the mature marker MBP (Figure 2(E)). Host OLs were also seen at injection sites into the white matter as demonstrated by Olig1 staining (Figure 2(D)). Similar results occurred whether cells were injected immediately after injury or one day following injury. Moreover, quantitative data depicted in Figure 3 show that approximately 30% of the cells survived one week after injury whether injected into the gray or white matter regardless of injection time. Importantly, a similar proportion of cells appeared to survive in the laminectomy-only control group, suggesting that the environment after injection may not be as hostile as originally believed for the survival of these cells. Indeed, clinical trials involving transplantation of human mesenchymal stem cells into damaged heart tissue following infarctions have shown survival of injected cells weeks following injection (Osiris)(Pittenger & Martin, 2004).

BBB analyses demonstrated increases in locomotor activity in both treatments after OPC injection (Figure 4). It is interesting to note that motor recovery in rats took a day longer when cells were injected 24 hours after injury compared to 3 hours. In addition, analyses performed on rats injected with OPCs or PBS without contusive injury demonstrated that OPC injections did not cause further injury (data not shown). Injections with human fibroblast cells (HFs) enhanced the effects of injury demonstrated by significant decreases in hindlimb locomotion. This occurred with no rebound in motor ability after several days like those seen in controls with saline injections or with injections of OPCs suggesting that HF injections facilitated further damage in the tissue.

SEP recordings demonstrated an initial decrease in peak-to-peak amplitudes one day after injury similar to those shown previously for this level of injury (Figure 5) (Agrawal et al., 2009). Subsequently, OPC-treated rats showed significantly higher amplitudes compared to rats injected with saline alone. Increased amplitudes in SEP may be the result of axonal remyelination of spared fibers and/or reorganization of neuronal circuits. However, it remains to be determined whether increases in SEP as well as BBB measurements are due to an indirect effect of surviving endogenous OPCs in the host or from myelinating transplanted cells.

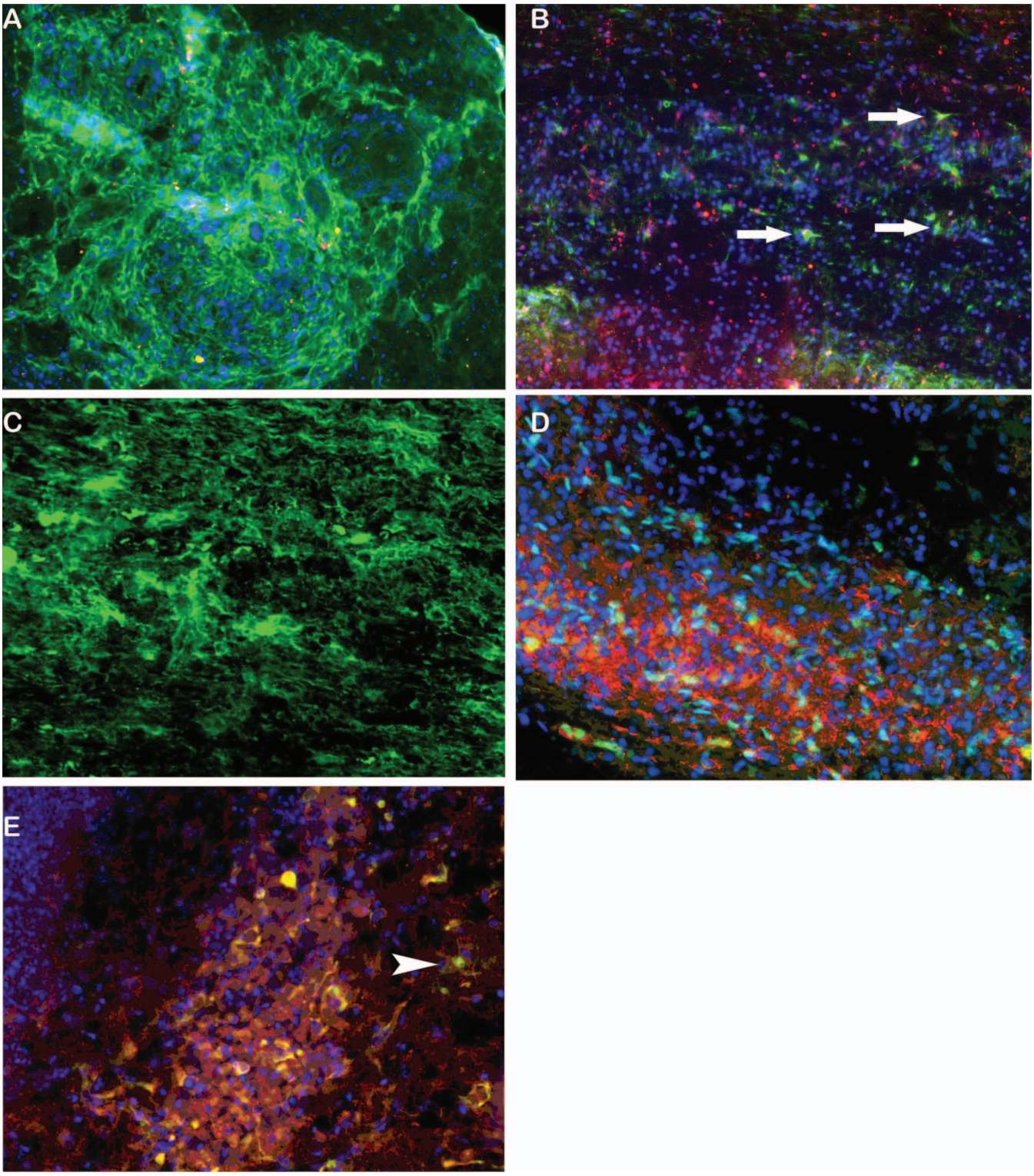

FIGURE 2. Immunohistological characterization of ESC-derived OPCs injected 3 hours and 24 hours after contusion in rat spinal cord. Tissue was isolated after a week in the cords. (A) Human-specific nestin antibody expression (green) surrounding the injection site at the epicenter of injury into the gray matter and (B) nestin+ oligodendritic processes, extended into white matter ~1 mm from the center of injection (arrows). (C) CNPase (green) expression around the injection sites and (D) Olig1 (red) expression in human cells (human-specific nuclear antigen: green) into white matter demonstrates restructuring of OPCs in the area by human and host cells. (E) Overlap (yellow) of human-specific nestin (green) and myelin basic protein (red) expression of human OPC-derived cells in white matter. Majority of cells expressed MBP (yellow) though some cells could be seen expressing nestin only (arrowheads). DAPI (blue) was used to stain nuclei. $N = 20$ rats studied.

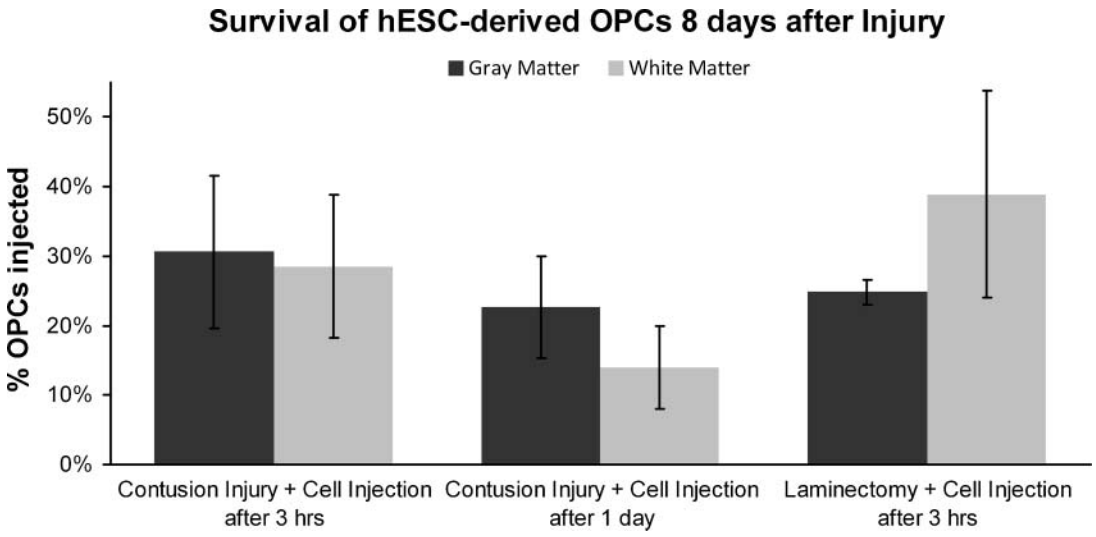

FIGURE 3. Prevalence of ESC-derived OPCs 8 days after injury. Comparison of OPCs injected into the gray versus white matter 3 hours or 1 day after injury. Cell survival was also compared when no injury was performed (laminectomy only).

DISCUSSION

We employed a successful differentiation protocol to isolate relatively pure populations of OPCs for transplant. These populations were negative for undifferentiated markers, such as Oct4, Nanog, and Sox2. Greater than 95% of these cells expressed Sox10, A2B5, PDGFR-α, NG2, O4, O1, and Nestin. However, the marker of mature OLs, MBP was not detected until after transplantation. Interestingly, the cell bodies of transplanted ESC-derived OPCs survived at the site of injury into the gray matter as well as the white matter as seen by human-specific Nestin$^+$ staining and O4 staining. Nestin$^+$ staining of integrated human OPCs was also observed at distances millimeters away from their white matter injection sites.

The variable expression demonstrated by GalC and CNPase, two markers which precede MBP expression in this study, distinguish unique stages in pro-oligodendrocyte differentiation. These results illustrate the need for a better understanding of the stages of OL development. In fact, very little is known regarding the number of steps involved in development of the oligodendrocyte lineage in humans. This is compounded by the limited number of studies in

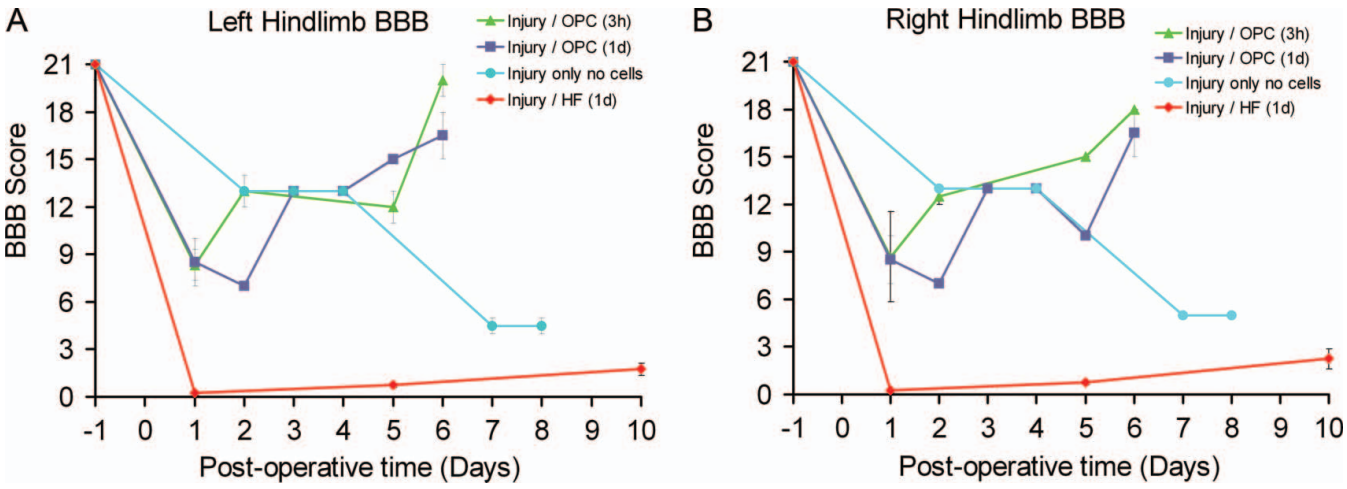

FIGURE 4. Behavioral assessment one week following moderate contusive injury (12.5 mm) and cell transplantation: BBB scoring of (▲) injury without cells (control), (◇) injury with human fibroblast injections 24 hours after injury (●) injury and OPC injection 3 hours and (■) 24 hours after injury as analyzed from the (A) left hindlimb and (B) right hindlimb. ($N = 3$–5 per treatment).

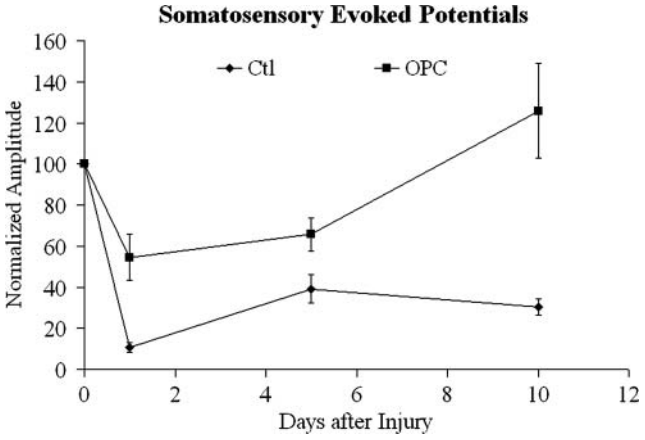

FIGURE 5. Somatosensory Evoked Potentials showing changes in peak-to-peak amplitude of the SEP (mean ± SE) normalized to baseline (before injury). After injury, rats injected with OPCs demonstrated increases in the SEP amplitude compared to controls ($N = 5$ per treatment).

human fetal tissue and few glial stage-specific markers. One marker, our results suggest, that may be useful for this purpose is Olig1. In mouse and human studies, Olig1 expression has been reported in both the nucleus and cytoplasm of the OL lineage depending on developmental stage. These studies showed nuclear expression in less differentiated progenitors and cytoplasmic expression in more mature OLs (Arnett et al., 2004; Jakovcevski & Zecevic). Similarly, toxic models of CNS demyelination have demonstrated that Olig1 proteins are expressed in the nucleus of cells in early remyelinating lesions of the corpus callosum, brainstem, and SC of adult mice. This pattern is mimicked in postmortem brain tissue from human patients with multiple sclerosis. In both cases, OLs of these lesions were characteristic of less differentiated NG2+, MBP–, OPCs involved in repair (Arnett et al., 2004; Woodruff, Tekki-Kessaris, Stiles, Rowitch, & Richardson, 2001).

Results from our study compared to other differentiation schemes also demonstrate a clear difference in the localization of Olig1 expression. Here, we found that Olig1 was robustly expressed in the cytoplasm of cells that also express NG, CNPase, and PDGFR-α. These results suggest they are fully differentiated progenitors of the OL lineage compared to other studies which showed nuclear localization (Keirstead et al., 2005). Since both studies reported using the same antibody to detect Olig1, differences in the differentiation state of the OPCs among these studies may be explained by variations in differentiation protocols (Hatch, Nistor, & Keirstead, 2009).

Another important parameter in cell-based therapy is the time between infarct and treatment. In the case of SCI, the associated inflammatory response following tissue necrosis creates a hostile environment for injected cells. This is followed by the invasion of fibroblasts into the lesion site and initiation of the scarring process, resulting in an impendent to migrating OPCs and OLs. Thus, it seems intuitive that an expedited injection time increases the chance for transplanted cells to overcome this obstacle and shorten the recovery time. Results from the only other published work on ESC-derived OPC transplantation in SCI have demonstrated the effects of transplantation 7 days and 10 months following mild or severe SCI (Cloutier et al., 2006; Faulkner & Keirstead, 2005; Keirstead et al., 2005). These studies showed that transplanted ESC-derived oligodendrocytic populations were able to restore insulating tissue and promote motor recovery in rats treated 7 days after the initial injury, but not in rats that had been injured for 10 months.

Despite variations in transplant methodology and SCI models that were utilized between studies, similar trends in locomotor recovery occurred during the first week. For instance, in both studies BBB scores demonstrated the greatest increase within the first week after OPC injections and at similar rates. BBB scores after 3 hours and 24 hours increased most significantly, from ~9 to 16 scoring for the first week following injections regardless of the time of injection compared to ~ 5 to 12 after 7 days reported earlier by Keirstead (Keirstead et al., 2005). These results suggest that transplantation may be applied sooner than previously expected. In contrast, injections with HFs caused greater tissue damage than saline injections. Although HFs survived for only a few days, the SC architecture was clearly disrupted supporting a specific role of OPCs in improving locomotor scores after SCI.

In fact, results from the work of Keirstead and colleagues almost led to the first FDA approved human clinical trials using ESC-derived tissue. This trial involved injecting 2 million ESC-derived oligodendrocytic progenitor cells into patients 7–14 days after injury. However, before this trial was able to begin, the FDA suspended the approval after the company reported a higher than expected incidence of cysts in some of the animal subjects. Although no teratomas were detected in these animals, the trial now awaits further testing for approval. Of significance, the approval of this trial sponsored by the Geron Corp. occurred in lieu of very little published literature on human ESC-derived OPC transplantation in animals and only a few studies for human stem cell therapy using fetal neural stem cells for Parkinson's disease (Freed et al., 2001; Olanow, Freeman, & Kordower, 2001). Indeed the issue of tumor formation is made

evident by the recent report of multifocal brain tumor formation in a young patient with inherited ataxia telangiectasia (Amariglio et al., 2009). This occurred four years after the first of two injections of fetal neural stem cells. In this case, the quality of stem cells was not reported raising concerns of proper quality controls as well as raising an important issue regarding the age of the patient at the time of therapy. Conversely, it is important to note that xenotransplantation may limit tumor formation through incompatible cytokine-receptor interactions with the microenvironment across species. For this reason, allogenic studies using rat OPCs would be helpful.

While our study is consistent with other reports the mechanism of locomotor recovery is unknown. Even though we report here that transplanted human ESC-derived OPCs expressed MBP after one week, it is not clear whether secretions by these cells also prevent demyelination of the host tissue. Indeed multiple studies have shown short-term effects of stem cell derived transplants in reducing inflammation and necrosis in both neural and cardiac damaged tissue. Another possibility is that the ESC-derived OPCs promote remyelination of the host cells. While the results suggest myelination occurs with cell transplantation, it is more likely that improved motor scores after only a week are due to an indirect effect of injected cells on the host tissue as demyelination is a gradual process, which occurs weeks after the injury (Totoiu & Keirstead, 2005).

Indeed, other studies have also shown that stem cell-based therapies appear to promote recovery not by long-term survival but through short-term effects on the host tissue. Evidence for this has been shown in human clinical trials using mesenchymal stem cells (MSCs) which have been utilized to treat heart infarctions and neurodegenerative diseases (Hare & Chaparro, 2008; Smith, Barile, Messina, & Marban, 2008a, 2008b). While cardiac models have produced limited long-term potential, MSCs for neurodegenerative diseases have produced mixed results with no functional or long-term integration observed. However, these MSC-derived transplants have shown neural protection of degenerating neurons, migration into damaged tissue, decreases in inflammation, and reduction of tissue injury in mouse models of multiple sclerosis, amyotrophic later sclerosis, Alzheimer's, Huntington's disease, and Parkinson's disease (Torrente & Polli, 2008). It remains to be seen whether this will hold true for cells derived from human ESCs.

Finally, another important factor in stem cell studies is the use of sensitive monitoring methods to measure any potential, beneficial outcomes following treatment. For instance, motor- and somatosensory-evoked potentials provide a more sensitive in vivo evaluation compared to behavioral testing by measuring the electrophysiological capacity of the motor and sensory pathways along the SC, unlike BBB which relies on observational changes in gross locomotion (Blight & Young, 1989; Fehlings, Tator, & Linden, 1989; Grundy, 1983; Li et al., 2008; Misra, Kalita, & Kumar, 2008; Nuwer, 1998). Our SEP results demonstrate that OPCs preserve or enhance somatosensory output after injury as shown by higher amplitudes in OPC-treated animals, and is complemented by increasing BBB scores indicative of locomotor pathway function. These results lend support for a long-term study using a combinatorial approach already under investigation by the authors to determine the efficacy of SCI OPC therapy including tumor risk assessment.

Declaration of interest: There are no conflicts of interest with the authors.

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