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Transplantation of human oligodendrocyte progenitor cells in an animal model of diffuse traumatic axonal injury: survival and differentiation

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Abstract

Diffuse axonal injury (DAI) is an extremely common type of traumatic brain injury encountered in motor vehicle crashes, sports injuries, and in combat. Although many cases of DAI result in chronic disability, there are no current treatments for this condition. Its basic lesion, traumatic axonal injury (TAI), has been aggressively modeled in primate and rodent animal models. The inexorable axonal and perikaryal degeneration often encountered in TAI calls for regenerative therapies, including therapies based on stem cells and precursors. Here we explore the proof of concept of treatments based on transplants of oligodendrocyte progenitor cells (OPCs) destined to replace or remodel myelin and aid with axonal regeneration in a model of TAI. OPCs were derived from the human embryonic stem cell line H9. After OPCs were purified and characterized, they were transplanted into deep sensorimotor cortex next to the corpus callosum of nude rats subjected to TAI based on the impact acceleration model of Marmarou. The time course and spatial distribution of differentiation and structural integration of these OPCs were explored. At the time of transplantation, over 90% of OPCs expressed A2B5, PDGFR, NG2, O4, Olig2 and Sox10, a profile consistent with their progenitor or early oligodendrocyte status. After transplantation, these cells survived well and migrated massively via the corpus callosum in both injured and uninjured brains. OPCs displayed a striking preference for white matter tracts and were contained almost exclusively in the corpus callosum and external capsule, the striatopallidal striae, and cortical layer 6. OPCs progressively matured into MBP (+) and APC (+) oligodendrocytes. The injured environment in the corpus callosum of impact acceleration subjects tended to favor differentiation. Mature transplant-derived oligodendrocytes ensheathed host axons with spiral wraps intimately associated with myelin sheaths. Our findings suggest that OPCs behave in a fashion that is dramatically different that of transplanted neuronal progenitors that is they migrate instead of differentiating locally. Their massive spread along white matter tracts and their avid differentiation to ensheathing oligodendrocytes makes them appealing candidates for cellular therapies of DAI aiming at myelin remodeling and axonal protection or regeneration.

Keywords: traumatic brain injury, diffuse axonal injury, oligodendrocytes, impact acceleration, myelin

Introduction

Axonal injury is the defining feature of diffuse axonal injury (DAI), but is also present in focal contusions (Mac Donald et al., 2007), blast injuries (Mac Donald et al., 2011), chronic traumatic encephalopathy (CTE) (Mckee et al., 2013), and mild injuries (Mittl et al., 1994). Axonal damage in models of DAI is referred to as traumatic axonal injury (TAI), a term often used interchangeably with DAI (Geddes et al., 2000;Wang and Ma, 2010). In the context of DAI, axonal injury causes disconnection of neural circuits at multiple CNS sites (Adams et al., 1985;Gennarelli et al., 1982;Buki and Povlishock, 2006) and leads to a number of neurological impairments, including unconsciousness and persistent vegetative state. These neuropathologies have no satisfactory treatment, besides symptomatic alleviation of various sub-syndromes with physical, occupational, speech and language therapy and various categories of CNS-acting drugs including antispasmodics, antidepressants, and mood stabilizers. Although some training of circuits is anticipated over time and syndromic pharmacotherapies have some effectiveness, most patients with DAI still remain severely symptomatic years and decades later.

Stem cell therapy presents a promising treatment approach for TBI. Some early success in models of ischemic brain injury (Burns et al., 2009) has encouraged the use of stem cell or neural precursor (NP) transplantation, primarily in models of focal TBI (Richardson et al., 2010). Much less is known about the role of stem cell therapies in DAI/TAI. Axonal repair as a primary target of treatment separate from nerve cell regeneration is not as well established in TBI as in spinal cord injury and this is especially true with the problem of myelin repair/remyelination (Kwon et al., 2010). However, there is evidence that demyelination may contribute to degeneration of axons in TAI (Maxwell, 2013;Maxwell et al., 2003) and TAI may be associated with active and ongoing attempts at axonal repair (Singleton et al., 2002). Therefore, adding exogenous OPCs may furnish competent oligodendrocytes that can assist in remyelination/myelin remodeling and prevent axonal degeneration or help myelinate regenerating axons in TAI.

Animal models are invaluable tools in establishing proof of concept that remyelination by exogenously provided oligodendrocytes are possible in TAI settings. Models of inertial acceleration and impact acceleration (IA) are frequently used for experimental studies of DAI/TAI (Wang and Ma, 2010). In the present study we use the IA model of DAI/TAI (Marmarou et al., 1994) and transplant human ESC-derived oligodendrocyte progenitor cells (OPCs) into the deep sensorimotor cortex next to the corpus callosum. Our findings indicate that exogenous human OPCs differentiate into mature oligodendrocytes, migrate extensively along white matter tracts, and begin to myelinate host axons. Our data are consistent with the view that stem cell grafts can serve as effective myelin remodeling tools in TBI scenarios featured by DAI/TAI.

Materials and Methods

Human embryonic stem cell (ESC) culture and differentiation to OPCs. The human ESC line H9 (http://stemcells.nih.gov/research/registry) from WiCell (www.wicell.org; Madison, WI) was maintained according to standard stem cell culture protocols. H9 cells (WA-09; passages 30-41) were grown on mitotically inactivated mouse embryonic fibroblasts (MEFs) essentially as described (Pankratz et al., 2007). OPCs were generated through extensive passaging as neurospheres based on the method of Hu et al (Hu et al., 2009b;Hu et al., 2009a) with minor modifications (**Fig. S1)**. The ventralizing factor SHH (100 ng/mL) along with the caudalizing factor retinoic acid (0.1 μM) were used to initially pattern

neuroepithelial cells; glial differentiation medium (GDM; DMEM/F12, B27 without vitam2009ain A, N1, MEM-NEAA, cAMP, biotin, 60 ng/mL triiodothyronine, 10ng/mL PDGF-AA, IGF1 and NT3) was used for further differentiation. Cells were trypsinized with TrypLE (Life Technologies, Grand Island, NY) at day 84 after induction of differentiation, counted, and plated on p-L-ornithine- and laminin-coated plates. Cells were grown in GDM supplemented with PDGF-AA, IGF1 and NT3 for 12 days, then trypsinized, counted, and resuspended at high concentration, and finally transplanted on day 98 after induction of differentiation.

Characterization of OPCs used for transplantation with immunocytochemistry (ICC)

Two weeks before transplantation on day 84, a time point chosen to correspond to the remaining time in differentiation of OPCs destined for transplantation, OPC neurospheres were trypsinized with TrypLE and counted. Twenty thousand cells were plated on polyornithine and laminin-coated coverslips or Matrigel coated 4-well slide chambers and cultured in GDM supplemented with PDGF, IGF and NT3 for 2 weeks. Cultures were fixed with 4% paraformaldehyde in PBS for 20 min and then subjected to ICC with the oligodendrocytic markers A2B5, PDGFRα, NG2, Sox10, and O4; the neuronal marker TUJ1; astrocyte marker GFAP; and the mesodermal marker BMP4 (Table 1).

Animals and surgical procedures

Ten-week old male nude rats (Crl:NIH-*Foxn1*^{rnu}; Charles River, Wilmington, MA) were used for OPC transplantation. All surgical procedures were carried out according to protocols approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions using gas anesthesia (isoflurane: oxygen: nitrous oxide=1: 33: 66) and aseptic methods. In order to explore the fate of transplanted OPCs and compare differentiation between injured and uninjured scenarios, animals were separated into IA and sham groups. In the IA group, animals were subjected to injury with full artificial ventilation as described in Marmarou et al. (Marmarou et al., 1994). In the present experiments, we employed a severe TBI regimen using a 450g weight that was freely dropped onto the steel disc through a Plexiglass tube from a height of 2 meters. In the sham group, animals received all aspects of the regimen except the injury itself (weight on the steel disc). One week after injury, a time point that appears to optimize survival and differentiation (Yan et al., 2004;Yan et al., 2007), 200,000 live OPCs were transplanted into 2 sites 1 mm apart in the right deep motor cortex next to the corpus callosum (1 mm and 0 mm anterior to bregma, 2 mm lateral to midline and 3 mm ventral to pia) of either injured (n=10) or sham (n=5) animals using procedures that have been detailed in our published work (Yan et al., 2004;Xu et al., 2009;Nasonkin et al., 2009). To explore the progress of differentiation of transplanted OPCs in the TAI environment, animals in the TAI group were allowed to survive for 6 weeks and 3 months. Sham animals with transplanted OPCs were euthanized at 3 months.

Histology, immunohistochemistry (IHC) and microscopy

Brain tissues were prepared from animals perfused transcardially with 4% phosphate-buffered paraformaldehyde. The axonal injury, survival, location and phenotypic fate of OPC grafts were assessed with ABC peroxidase immunohistochemistry (IHC) and dual-label fluorescent IHC in serial coronal or sagittal sections (40 μm) through the brain as described (Koliatsos et al., 1994;Yan et al., 2007;Nasonkin et al., 2009). Axonal injury was studied with well-established TAI markers, including an antibody against the amyloid precursor protein (APP), the monoclonal antibody RMO14 binding to the rod domain of

neurofilaments H and M, and a monoclonal antibody against the 68 kDa light chain neurofilament protein. OPC survival was studied with human-specific nuclei (HNu) or human-specific cytoplasm (SC121) antibody using immunoperoxidase labeling. Differentiation was studied with dual-label immunofluorescence combining HNu or SC121 with other oligodendrocyte markers, i.e. the progenitor and early marker PDGFRα, the early markers O4 and GalC and late markers MBP, PLP and APC. The nuclear mitotic marker Ki67, the early neuronal marker TUJ1 and the astroglial cell marker GFAP were also used in separate colocalization experiments with HNu or SC121 as described (Cummings et al., 2005;Nasonkin et al., 2009;Xu et al., 2006;Yan et al., 2007). All antibody information is listed on Table 1. The Gallyas silver staining method (Gallyas, 1971) was used to evaluate injured and/or degenerating axons and terminals. For this purpose, sections were processed with a commercially available kit (NeuroSilver kit II; FD Neurotechnologies, Ellicott City, MD) as described (Koliatsos et al., 2011).

Stained sections were studied on a Zeiss Axiophot microscope equipped for epifluorescence (Diagnostic Instruments Inc., Sterling Heights, MI) or a Zeiss LSM 510 inverted confocal microscope (Carl Zeiss Inc., Oberkochen, Germany). Confocal microscopic images were captured with pinhole set at 0.8 μm to ensure colocalization of multiple labels at the same resolution level as semithin sections. 3D reconstruction by Z-stack scanning through regions of interest was acquired with LSM software. Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA) was used for montaging and image processing.

Ultrastructural IHC

Myelin formation by transplanted OPCs was assessed ultrastructurally with electron microscopy using standard pre-embedding immunoperoxidase-DAB IHC for the human cytoplasmic antigen SC121 as described (Koliatsos et al., 1994;Nasonkin et al., 2009;Xu et al., 2009;Cummings et al., 2005). Briefly, brain sections prepared as per previous section were treated with a solution containing 4% paraformaldehyde and 0.2% glutaraldehyde for 24 hours. Sections were then rinsed in 0.1M phosphate buffer (pH 7.3) for 3 to 10 minutes, immersed in 1% osmium tetroxide for 15 minutes, dehydrated in graded concentrations of ethanol, embedded in Poly/Bed 812 (Polysciences Inc., Warrington, PA), polymerized at 60°C for 72 hours, and then finally embedded in BEEM® capsules. Half the sections were stained en block in uranyl acetate prior to embedment. Serial ultrathin sections were collected on Formvar coated slotted grids and viewed with a Hitachi H7600 transmission electron microscope equipped with a 2k×2k bottom mount AMT XR-100 CCD camera. Only sections that were not stained with uranyl acetate were used for studying ensheathment profiles originating in OPC transplants.

Quantitation of OPC survival and differentiation

Numbers of surviving OPCs were counted in serial, systematically and randomly sampled coronal sections based on the optical fractionator concept with the aid of a motorized stage Axioplan microscope (Zeiss, www.zeiss.de) equipped with Stereo Investigator (MicroBrightField, www.mbfbioscience.com) as described (Yan et al., 2007). To evaluate the migration and possible final residing location of differentiating OPCs, only the contralateral side of transplantation was examined. OPCs in corpus callosum and cortex were also counted separately for this purpose. Every 12th serial coronal section through the transplant/injury site was selected for stereological analysis. Counting frame was set at 50×50 μm and sampling grid and counting depth were 200×200 μm and 10 μm, respectively.

Differentiation of survived OPCs was estimated in a non-stereological fashion as described (Xu et al., 2006). Briefly, we counted the total number of SC121 (+) cells, as well as cells dually labeled with SC121 and the mature oligodendrocyte marker MBP from our immunofluorescent preparations, on randomly selected fields of cortex and corpus callosum using 40× magnification and avoiding the transplantation site. At least 3 fields in each of 4 serial sections were used from each animal. Numbers of SC121 (+) and double-labeled profiles were pooled from each case and grouped per experimental protocol. Average numbers of single- and double-labeled cells were generated for two TBI groups and one Sham group (n=5 per group). Differentiation rate was expressed as percentage of SC121 and MBP double-labeled cells in the population of SC121 (+) cells.

Migration mapping of oligodendrocyte lineage cells derived from OPC grafts

The positions of all SC121 (+) cells were mapped on every twelfth coronal section through brain levels containing the grafted cells and their lineage using Neurolucida software (MicroBrightField Inc, Williston, VT). Representative cells differentiated from OPCs and their processes were also traced with Neurolucida software.

Statistical methods

Variance between and across samples of numbers of oligodendrocyte-lineage cells classified by experimental history (IA versus sham), migratory destination in brain (corpus callosum versus neocortex), and time point after transplantation (6 weeks or 3 months) was analyzed with two-way analysis of variance (ANOVA) or t-test. In the case of ANOVA, significant differences were further analyzed with post hoc tests to reveal important main effects or interactions. Statistical analyses were performed with STATISTICA 8.0 (StatSoft Inc, Tulsa, OK).

Results

Axonal injury in nude rats using the IA model

Immunocompromised nude rats were used here to avoid immune rejection of human cell xenografts into rodent brain. Because the original IA model was developed in Sprague Dawley (Marmarou et al., 1994) and Wistar (Engelborghs et al., 1998) rats, we first explored whether the same IA settings as the ones used in those strains can cause TAI in nude rats. Induction of TAI was studied with IHC strategies routinely used in TBI studies, i.e. antibodies against amyloid precursor protein (APP), the monoclonal antibody RMO14 binding to the rod domain of neurofilament heavy and medium chains that are exposed after lesion-induced sidearm proteolysis, and a monoclonal antibody against the 68 kDa light chain neurofilament protein (NF68). IHC was used in brain sections from nude rats exposed to a standard severe IA injury (450g weight drop from the height of 2 meter) (Marmarou et al., 1994). Tissues were also processed with a modification of the Gallyas silver method. Twenty-four hours post-injury, APP, RMO14 and NF68 IHC consistently labeled axon pathologies such as undulated axons, axonal swellings and bulbs in the corpus callosum and the corticospinal tract (data not shown). Argyrophilic axonal degeneration based on Gallyas silver staining became evident one week post-injury in the corticospinal tract **(Fig. 1A-B)**, the optic tract and the corpus callosum. Axonal degeneration labeled with Gallyas silver was still present in the corticospinal **(Fig. 1C-D)** and other tracts at 4 weeks and 3 months post injury. These data suggest that the pattern of TAI in nude rates exposed to IA injury is qualitatively similar to the

one described for Sprague-Dawley and Wistar rats and, therefore, the nude rat model is suitable for research into OPC transplantation outcomes in a diffuse TBI background.

Differentiation of human ESCs to OPCs *in vitro*

As per original description (Hu et al., 2009b;Hu et al., 2009a), columnar epithelial cells began to appear and organize into rosettes 10 days after induction of differentiation of embryoid bodies with hES medium without FGF2 (DMEM/F12, Knockout serum replacer, MEM-nonessential amino acid, 0.1 mM β-mercaptoethanol, 4 ng/mL FGF2) for 3 days and then with neural differentiation media (NDM: DMEM/F12, N2, MEM-nonessential amino acid, 2 ug/mL heparin) for 6 days. Neuroepithelial cells were initially converted into Olig2 (+) progenitors in the presence of 0.1 μM retinoic acid on laminin-coated plates for 4 days. The resulting rosette-rich colonies were manually detached and grown into spheres and then continued to be patterned with retinoic acid and SHH for 10 more days. To generate produce pre-oligodendrocyte progenitors, spheres were passaged by Accutase and cultured in NDM supplemented with B27, SHH (100 ng/mL) and FGF2 (10 ng/mL) for 10 days. For further differentiation into OPCs, spheres were cultured in glia differentiation media (GDM: DMEM/F12, N1, B27, MEM-nonessential amino acid, 60 ng/mL T3, 1 μM cAMP, 0.1 μg/mL biotin) supplemented with SHH, PDGF-AA, IGF1 and NT3 for 2 weeks and then dissociated by Accutase and continued to feed with the same GDM without SHH (day 49). Every two or three weeks, the spheres were passaged by the same dissociation method using Accutase and cultured in the same GDM with PDGF-AA, IGF1 and NT3. On day 84 after induction of differentiation, spheres were trypsinized into dissociated OPCs, plated and cultured further as in Materials and Methods. On day 98 cells were detached from plate and resuspended in high concentration for transplantation.

As described in Materials and Methods, the cell composition of OPC transplants was analyzed in a representative sample of cells destined for transplantation with ICC for protein markers of various neural cell lineages (Alsanie et al., 2013;Iannarelli et al., 2005;Rowitch and Kriegstein, 2010;Richardson et al., 2006)**(Fig. 2)**. Results show that these samples were enriched in OPCs expressing oligodendrocyte-lineage markers including A2B5, PDGFRα, O4, NG2, Sox10 and MBP **(Fig. 2D-J)**. Only a small percentage (less than 10%) expressed the neuronal marker TUJ1, an even smaller percentage (less than 1%) were positive for astrocytic markers (GFAP), and no BMP (+) mesodermal-lineage cells were detected **(Fig. 2A-C)**.

Survival and migration of transplanted OPCs in the rat brain

OPCs were transplanted into the deep sensorimotor cortex of IA- and sham-injured rats. Transplanted OPCs survived very well in the brains of injured and uninjured animals and migrated extensively away from the inoculation site in both groups. We mapped and counted migratory profiles at 6 weeks and 3 months post-transplantation. For the time point of 6 weeks we have data only on IA-injured animals. In the case of 3 months, we have data from both IA-injured and sham animals.

In 6 weeks, there was some migration of transplant-derived SC121 (+) cells into the ipsilateral corpus callosum and external capsule and into the contralateral corpus callosum adjacent to cingulate gyrus. At 3 months, cells had migrated much further **(Figs. 3-4)**. They had densely populated the ipsilateral corpus callosum and entire length of external capsule and reached further into the corpus callosum and external capsule on the contralateral side; some cells had entered the contralateral neocortex **(Fig. 4)**. In many

cases, these cells had migrated 5 mm or more in antero-posterior distance from the inoculation site (**Fig.4).** There was little migration into the gray matter and cortical invasion of OPCs was limited to layers adjacent to corpus callosum (lower layer 6). In the case of migration into the neostriatum (**Fig. 4**), OPCs were localized strictly inside the white matter striae.

Stereological counts of transplant-derived cells contralateral to the injection side provide a good measure of the migratory potential of transplanted OPCs. Cell counts in IA-injured animals in which we have data from both 6 weeks and 3 months show massive progressive migration into the corpus callosum and adjacent cortical layer 6 **(Fig. 5A-B)**. For example, the number of oligodendrocyte-lineage cells in the contralateral corpus callosum is 30 times higher in 3 months compared to 6 weeks **(Fig. 5A).** Two-way ANOVA examining interaction between time and location shows that time tends to advance the position of cells from the corpus callosum into deep cortical layers (**Fig. 5A).** Experimental history (IA versus sham) shows no effect in numbers of oligodendrocyte- lineage cells in corpus callosum or cortex at 3 months. There are about 3-4 times as many oligodendrocyte-lineage cells in corpus callosum compared to cortical layer 6 in both IA-injured and sham animals **(Fig. 5B)**. Two-way ANOVA addressing the interaction between experimental history and location shows no significant effect, i.e. lesion does not promote more advanced migration into deep cortical layers.

At 3 months post-transplantation, a majority of oligodendrocyte-lineage cells around the inoculation site (the triangular region of **Fig. 3**) had round perikaryal profiles and multiple radial processes consistent with type I morphology **(Fig. 6A-C)** (Suchet, 1995). On the other hand, the majority of transplant-derived cells in the corpus callosum were spindle-shaped with parallel processes consistent with type II morphology (**Fig. 6D-F)** (Suchet, 1995). In the gray matter away from the injection site (ipsilateral or contralateral), cytology was mixed.

Very few (less than 1%) human OPCs at the transplantation site or within the main migratory domains (corpus callosum and deep neocortex) were positive for the mitotic marker-Ki67 at 6 weeks or 3 months, in injured or sham animals. This pattern suggests that, at the time points studied here, surviving cells are not proliferative at the original transplant site or in their migratory paths and destinations (**Fig. 7A-B)**.

Differentiation of transplanted OPCs in the rat brain

At 6 weeks and 3 months post transplantation, under either IA or sham conditions, no neurons and very few astrocytes were derived from transplanted OPCs (**Fig. 8A-B**). The majority of transplanted cells were identified as PDGFRα (+) or MBP (+) profiles in both the inoculation site and migratory pathways/destinations. MBP immunoreactivity was expressed in both round and spindle-shaped oligodendrocyte profiles derived from the transplant (**Fig. 8C-H**). At 3 months, most transplant-derived cells around the transplantation site, in corpus callosum and deep cortical layers were also APC (+)(**Fig. 8I-K**).

In the area surrounding the transplantation site of IA-injured animals, cell counts of PDGFRα (+) profiles show that 74.6±9% of graft-derived cells are PDGFRα (+) at 6 weeks post transplantation; this number is significantly reduced to 49.4±11% at 3 months. Conversely, the percentage of MBP (+) oligodendrocytes derived from OPCs is significantly higher at 3 months (67.8±12%) compared to 6 weeks (37.1±9%) (**Fig. 9A, left**). A similar pattern is seen in the corpus callosum (**Fig. 9A, right**), but trends in this case do not reach statistical significance. In the area surrounding the transplantation site, there are no significant

differences in PDGFRα (+) or MBP (+) cell rates between sham and injured animals (**Fig. 9B, left**). In the corpus callosum, rates of MBP (+) cells are higher in injured animals (**Fig. 9B, right**).

Ultrastructural IHC for the human cytosolic epitope SC121 was used to disclose transplant-derived oligodendrocyte processes and the involvement of such processes in the ensheathment of host-derived axons or the formation of myelin. In semi-thin preparations accompanying thin sections, SC121 (+) processes co-localized with toluidine blue-stained myelin sheaths (**Fig. 10A**). Using thin sections, we found numerous SC121 (+) cytoplasmic projections juxtaposed to or ensheathing unlabeled (host) axons. Ensheathment was featured by complex configurations, including the presence of outer and inner cytoplasmic tongues and close juxtapositions with compact myelin (**Fig. 10B-C**). It was not possible to ascertain whether compact myelin belonging to the same host axons as SC121 (+) sheaths was continuous with the latter in our preparations (**Fig. 10B-C**).

Discussion

General Points

Our findings indicate that the IA model of Marmarou can be effectively replicated in the nude rat background. Using the nude-rat IA model, OPC transplants survive well in the deep sensorimotor cortex and behave in a fashion very different from NPs i.e. they migrate massively and show almost exclusive affinity for white matter tracts, especially the corpus callosum and adjacent white matter in deep cortical layers. The progressive migration of transplanted OPCs is accompanied by progressive maturation into MBP (+) and APC (+) oligodendrocytes that ensheath host axons. Our findings provide further support to the notion that ESCs and neural stem cells can be coaxed to specific fates that continue to progress to fully differentiated progenies after transplantation into the adult CNS. These progenies behave in a fashion that is strikingly similar to indigenous differentiated neural cells. Given the very low level of proliferation (less than 1%) of transplanted cells as early as 6 weeks post-transplantation and their prompt differentiation into mature oligodendrocytes, the possibility of overgrowth and, thence, tumorigenic risk is very low.

The use of ESCs such as line H9 was based on a number of considerations including: thorough characterization and inexhaustible supply of the parent line (Thomson et al., 1998;Xu et al., 2005;Zhang et al., 2001;Yan et al., 2005); great versatility to differentiate to any neural cell type in sufficient quantity for transplantation (Hu et al., 2009b;Hu and Zhang, 2009;Li et al., 2005;Weick et al., 2011;Yan et al., 2005;Zhang et al., 2001;Zhang et al., 1998;Nguyen et al., 2014); and well-established methods for *in vitro* manipulation to fate determination prior to transplantation. The choice of human ESCs is based on availability and access considerations, the greater translational value of such cells, and a long experience in our laboratory using human cells as transplants in rodent hosts (Nasonkin et al., 2009;Nasonkin and Koliatsos, 2006;Xu et al., 2012;Xu et al., 2006;Xu et al., 2009;Xu et al., 2011;Yan et al., 2006;Yan et al., 2004;Yan et al., 2007).

In adulthood, the sources of usable stem cells or neural progenitors in CNS are limited to a few forebrain niches and the yield or repair potential of such niches is low. For example, in mouse models of MS, the limited recruitment of endogenous OPCs into demyelination sites does not suffice for effective remyelination (Boyd et al., 2013). Therefore, supplementation of such limited stem cell pools with exogenous progenitors is a reasonable first step for a cellular therapy. Besides providing sources of fully

differentiated nerve cells competent to replenish lost cells, transplanted progenitors also release neuroprotective molecules (Xu et al., 2006;Koliatsos et al., 2008;Xu et al., 2006) and, importantly, may induce endogenous stem cells/progenitors cell to proliferate and differentiate as auxiliary niches, thereby improving the efficacy of self-repair mechanisms (Xu et al., 2012).

Generation of OPCs from human ESCs

In the body of *in vivo* studies reported here, we used OPCs that were prepared from human ESC line H9 with methods described by Hu and colleagues with minor modifications (Hu and Zhang, 2009;Hu et al., 2009a;Hu et al., 2010). Our experience with culturing and differentiating H9 cells and then characterizing the derived OPCs *in vitro* is very similar to the original description of Hu and colleagues; OPCs derived in this manner express PDGFRα, NG2, O4, and Sox10, a pattern consistent with a classical OPC identity (Alsanie et al., 2013). Methodological issues concerning OPC derivation are very important, because the use of highly concentrated OPCs for grafting is key for achieving the desired outcomes, i.e. myelination of host axons, within a limited time period.

Differentiation of human ESCs into OPCs is a longer and more arduous process than the one leading to neuronal progenitors. In work reported here we initially used two methods (Nistor et al., 2005 and Hu et al, 2009) for deriving and characterizing OPCs prior to transplantation. In our hands, the method of Hu and colleagues appeared to be more successful in generating viable transplants, although this observation was not confirmed by a more systematic study. Major differences between the two methods are: trophic factors used; extracellular matrix used; enzyme used to passage the cells; length of time in 3D culture; and timing of OPC harvest. In previous published work, the method of Nistor and colleagues generated OPCs with good viability after transplantation (Keirstead et al., 2005;Nistor et al., 2004;Sharp et al., 2010;Cloutier et al., 2006;Hatch et al., 2009a), but the transplantation site of these authors (spinal cord) was different from ours (neocortex). Interestingly, the same team of investigators reported that their OPCs did not survive past 2 weeks after transplantation in animal models of MS (Hatch et al., 2009b). Our *in vivo* outcomes using OPCs prepared as per Hu and colleagues are consistent with the ones reported by that team on shiverer mice (Hu et al., 2009a).

Issues related to *in vivo* differentiation of OPC transplants

Two important trends in OPC maturation were the progressive phenotypic differentiation in the transplantation area and the higher rate of maturation of OPCs in the corpus callosum of injured subjects. With respect to the former, we have observations only from IA-injured animals, but we speculate that there is a similar differentiation trend in control animals. We also postulate that differentiation trends in the corpus callosum are not too dissimilar to these in cortex around the transplantation site and that differences in significance may be caused by the fact that OPC maturation may be earlier in the white matter (Vigano et al., 2013). Regarding the latter, it would appear that injury may contribute to OPC differentiation; the difference between corpus callosum and cortex around the transplantation site may be due to the fact that cortex is not a primary site of injury in the IA model that preferentially affects white matter tracts.

The role of injured or otherwise pathological environment as a niche for differentiation deserves further commentary. Environments associated with acquired neurological injury such as stroke and spinal cord injury have been shown to promote endogenous stem cell differentiation (Burns et al., 2009) (Moreno-Manzano et al., 2009;Yang et al., 2006) and this effect may be mediated, in part, by trophic and cytokine signals such as stem cell factor (Sun et al., 2004), stromal cell-derived factor 1α (Robin et al., 2006), fibroblast growth factor-2 (FGF-2) (Tripathi and McTigue, 2008), vascular endothelial growth factor (VEGF) (Vaquero et al., 1999), ciliary neurotrophic factor (CNTF), and CXC chemokine receptor 4 (Chi et al., 2006). Some of these factors are known to act as tropic cues for migration or to specify the definitive phenotype of endogenous or exogenous stem cells (Fricker et al., 1999;Flax et al., 1998;Kim, 2004;Erlandsson et al., 2004;Widera et al., 2004;Imitola et al., 2004;Zhang et al., 2003;Sun et al., 2004).

The invasion of migrated OPCs and their differentiated progenies into deep cortical layers matches native cortical myelination patterns: these patterns show an overall denser myelination in lower cortical layers and variable myelination in superficial layers. It is also interesting that, at least up to 3 months post-transplantation, the differentiated progeny of transplanted OPCs does not advance to more superficial layers. This distribution is identical to that of native mature oligodendrocytes: in contrast to OPCs that are radially spread across cortical layers, oligodendrocytes favor deep layers and follow the deep-to-superficial-layer myelin gradient (Tomassy et al, 2014).

The evident purpose of OPC transplants as cell therapies is the replenishment of damaged or destroyed myelin sheaths. Although molecular myelin markers such as MBP may be telling on the myelin-forming potential of OPCs and their progenies, they do not directly show that MBP (+) cells are forming myelin. The presence of structurally mature myelin can be only ascertained ultrastructurally. Using ultrastructural IHC or EM combined with histochemistry, previous studies have demonstrated the ability of specific progenies of neural stem cells or OPCs to ensheath (Yan et al., 2004) or increase the thickness of abnormal myelin sheaths in hosts (Hu et al., 2009a;Cloutier et al., 2006;Cummings et al., 2005;Tirotta et al., 2010;Keirstead et al., 2005;Wang et al., 2013), but labeling of exogenously derived myelin is technically difficult. In the present study, ultrastructural IHC divulges that transplant-derived cells ensheath host axons in intimate proximity to myelin sheaths, but the host-versus-transplant identity of the myelin itself is difficult to ascertain. The human cell marker SC121 is a cytosolic marker and, therefore, it is found in oligodendrocyte cytoplasmic projections and ensheathing tongues but not in the membranous myelin sheath itself. Unstained preparations that were used here have not been particularly useful primarily because of this reason. Furthermore, existing myelin antibodies cannot resolve between human and rodent myelin and this problem limits their usefulness in confocal microscopy or ultrastructural IHC.

Ultimately, the proof of concept that remyelination or myelin remodeling via OPC transplants can be beneficial in TAI/DAI will depend on the demonstration that such exogenous OPCs afford functional benefits. In the case of IA injured rodents, such benefits can be sought out in a number of behavioral domains, perhaps the most prominent of which is motor control that depends on the intactness of the corticospinal tract. Experiments addressing functional repair with exogenous OPCs have been successfully performed in models of spinal cord injury (Kwon et al., 2010;Richardson et al., 2010) and demyelination (Givogri et al., 2006;Wang et al., 2013;Windrem et al., 2008;Pluchino et al., 2003).

Stem cell transplantation as experimental therapy for TBI

Some success in models of ischemic brain injury (Burns et al., 2009) has encouraged the use of stem cell/NP transplantation in models of focal TBI (Richardson et al., 2010;Shindo et al., 2006). However, because of the complexity of TBI and its animal models, there is a need to identify specific repair targets based on key pathological mechanisms. Such repair tasks include replacing dead neurons, supporting injured neurons, and protecting axons or assisting with axonal repair/regeneration. The problem of neuronal injury/death is encountered both in focal injury (Clark et al., 2000;Smith et al., 2000) and in the course of TAI (Povlishock and Katz, 2005;Singleton and Povlishock, 2004). Neuronal cell death in focal TBI is acute and has necrotic components, whereas in TAI/DAI it is slow with apoptotic features and may be associated with retrograde and transsynaptic effects (Buki and Povlishock, 2006;Povlishock and Katz, 2005;Greer et al., 2011). Although axonal repair/remyelination as a therapeutic target separate from neuronal regeneration is best established in spinal cord injury (Kwon et al., 2010), there is evidence that demyelination may contribute to degeneration of axons in TAI (Maxwell, 2013;Maxwell et al., 2003). Therefore, contributing exogenous OPCs in the case of TAI may assist in remyelination and prevent axonal degeneration and disconnection within brain circuits.

There is no published work on stem cell-based therapies for models of TAI/DAI. However, the field can borrow from spinal cord injury that invariably involves trauma in long tracts (Sharp et al., 2010;Keirstead et al., 2005;Iwanami et al., 2005;Neirinckx et al., 2014;Fujimoto et al., 2012;Ogawa et al., 2002;Nori et al., 2011). Experimental cell therapies in animal models of spinal cord injury have utilized various stem cell preparations including neurospheres (Nori et al., 2011) and OPCs (Keirstead et al., 2005;Sharp et al., 2010;Sun et al., 2013), and there is an ongoing clinical trial using neural stem cells (NCT01772810, 2014). In a landmark study, OPCs have been found to remyelinate and restore locomotion after contusional spinal cord injury in rodents (Keirstead et al., 2005). In contrast to spinal cord injury where long tracts course in relatively circumscribed areas, DAI involves disparate white matter tracts (Povlishock, 1992;Smith et al., 2003) and it is not possible to transplant cells into all these sites. Therefore, transplantation route (systemic, ventricular, and parenchymal) and location of transplant (in the case of parenchymal delivery) are critical. The choice of transplantation site may be based on factors such as concentration of axonal pathology or sites of injury responsible for critical symptoms. The choice of transplantation into deep sensorimotor cortex in the present study was based on the expectation that this site would provide oligodendrocytes for both the corpus callosum and the corticospinal tract, which are affected in the IA injury (Foda and Marmarou, 1994;Marmarou et al., 1994). Our findings indicate that there was little, if any, invasion of cells into the internal capsule by 3 months, but the extensive migration of OPCs via the corpus callosum and descending tracts and their remarkable differentiation into mature oligodendrocytes predicts a broader remyelination potential with longer survival times. Of course, transplantation sites can also be optimized to the desired functional outcome or involve multiple locations as we have shown in models of motor neuron disease (Xu et al., 2012;Xu et al., 2009;Xu et al., 2011).

Conclusions and Future directions

In conclusion, the findings in this study support the idea that human ESC-derived OPCs can serve as a competent source of mature oligodendrocytes to myelinate CNS axons after TAI, and provide proof of concept that regenerative strategies targeting myelin remodeling can be further considered in TBI models in the future. In view of the fact that stem cell therapies are being progressively introduced in clinical trials of neurodegenerative and traumatic diseases of the CNS

(Hess et al., 2013;Riley et al., 2013;Brazzini et al., 2010;Kwon et al., 2010) (NCT01494480, 2014;NCT01217008, 2014;NCT01895439, 2014;NCT02254863, 2014;NCT01772810, 2014), these timely results should encourage further translational work targeting the problem of axonal degeneration in the context of DAI and specifically interventions designed to regenerate or remodel the myelin sheath.

Abbreviations: APC, adenomatous polyposis coli protein; APP, amyloid precursor protein; BMP-4, bone morphogenetic protein-4; CC, corpus callosum; CST, corticospinal tract; CTE, chronic traumatic encephalopathy; DAB, 3,3'-diaminobenzidine; DAI, diffuse axonal injury; ESC, embryonic stem cell; GFAP, glial fibrillary acidic protein; GDM, glia differentiation media; HuN, human nuclei protein; IA, impact acceleration; ICC, immunocytochemistry; IGF1, insulin-like growth factor 1; IHC, immunohistochemistry; MBP, myelin basic protein; MEFs, mouse embryonic fibroblasts; MS, multiple sclerosis; NDM, neural differentiation media; NP, neural precursor; NT3, neurotrophin 3; OPC, oligodendrocyte progenitor cell; PDGFRα, platelet-derived growth factor receptor α; PLP, myelin proteolipid protein; SHH, sonic hedgehog; TBI, traumatic brain injury; TAI, traumatic axonal injury; TUJ1, type III-tubulin epitope J1

Competing interests

No competing financial interests exist.

Authors' contributions

LX, configured the details of experimental plan, performed all animal surgeries and IHC staining, analyzed in vivo data and prepared the first draft of manuscript.

JR, established and maintained human OPC cultures and characterized them prior to transplantation.

HH, performed ultrastructural IHC.

AM, performed stereological counts of PDGFRα and MBD (+) cells derived from the OPC transplant.

AA, helped with stereology.

ER, performed mapping and stereological counting of migrated OPC-lineage cells.

BJC, participated in the design of the project.

VEK, principal investigator, designed the project, troubleshot the experiments and prepared the final manuscript.

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VEK, M.D. is an expert in neural injury and repair, neurodegenerative disease, and traumatic brain injury.

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Figure Legends

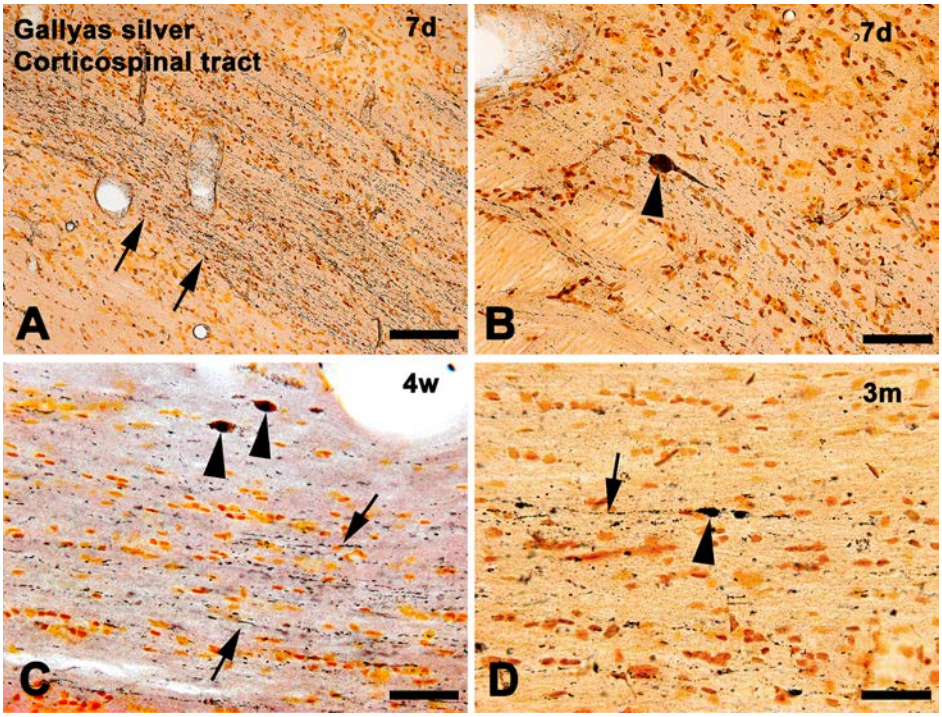


Figure 1. Traumatic axonal injury in IA-injured nude rats as demonstrated by Gallyas silver staining. One week after exposure of rats to severe IA injury, argyrophilic axonal degeneration is pronounced in the corticospinal tract (A) and, in some cases, is featured by classical axon bulbs (B). At one (C) and three (D) months after injury, degenerating axons are still evident in this region. Scale bars: 50 μm

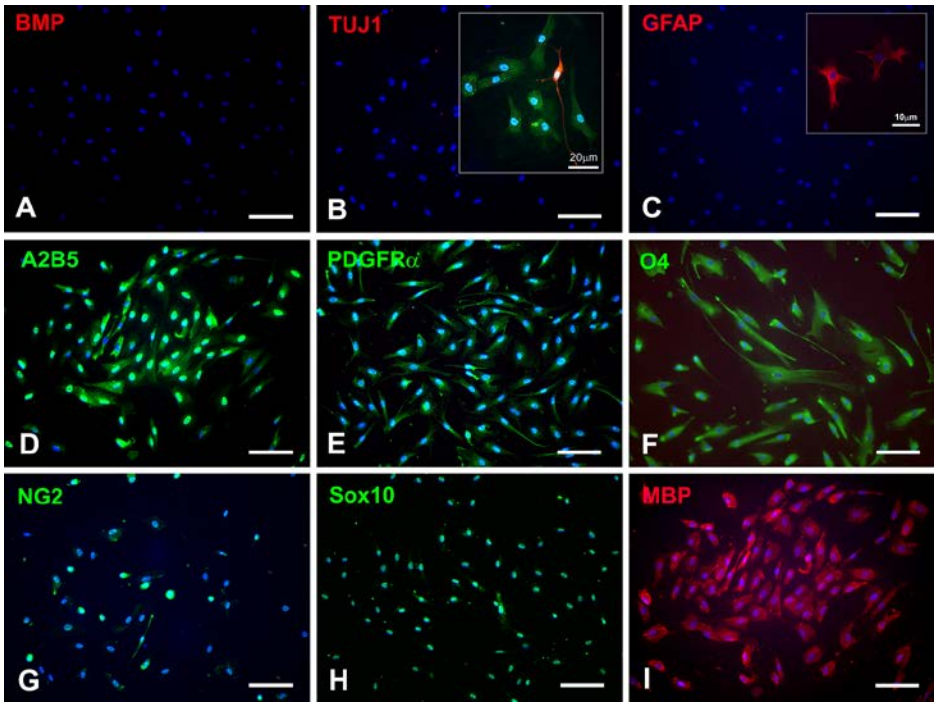
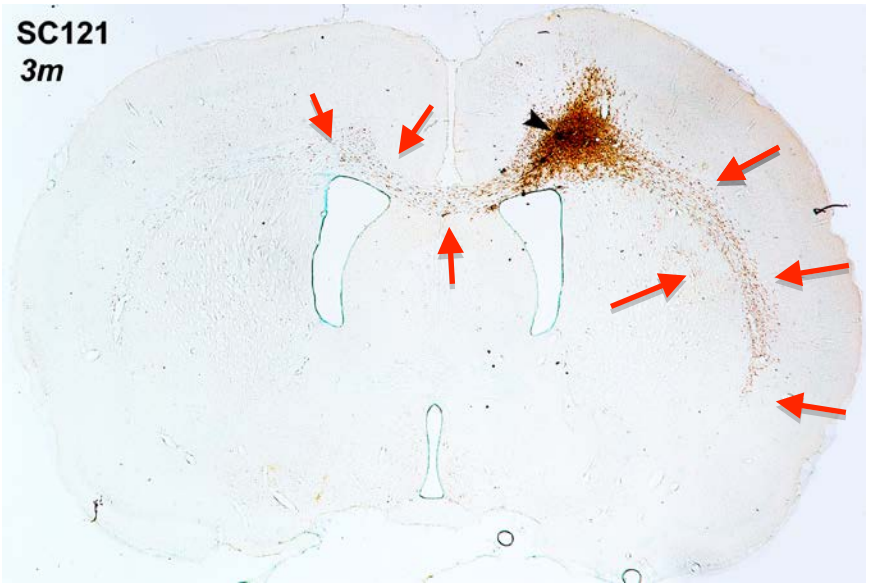


Figure 2. Characterization of OPCs used for transplantation 99 days after induction of differentiation. No mesodermal lineage cells were detected (A: BMP) and very little neural (B: TUJ1) and astrocyte (C: GFAP) markers were expressed in the OPCs. Most cells (90-95%) were positive for early and late OPC and pre-oligodendrocyte markers (D: A2B5, E: PDGFRα, F: O4). Fifty % of cells were NG2 positive (G) and most cells were positive for the transcriptional factor Sox10 (H). Cells were also positive for the oligodendrocyte marker MBP (I) although they did not have MBP (+) processes. Insets in (B) and (C) show typical rare neuronal and astrocytic profiles in these OPC cultures. Inset in (B) is taken from a culture that was double immunostained for NG2 (green) and TUJ1 (red). Scale bar: 20 µm



ADD arrows to highlight human cell distribution

Figure 3. Extensive migration of transplant-derived cells 3 months post-transplantation. This representative section from an IA-injured rat shows that SC121 (+) cells (brown) have migrated extensively from the transplantation site (arrowhead) along the corpus callosum on both sides. Methylene green was used as counterstain.

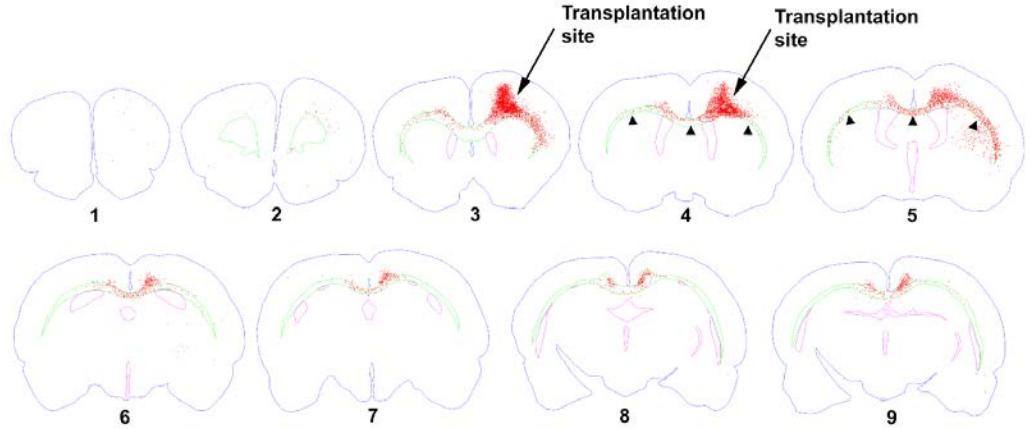


Figure 4. Migration map of OPC transplant-derived cells from a representative case of an IA-injured rat 3 months post-transplantation. Images were acquired and processed from serial coronal sections (40 µm; every 24th) using Neurolucida software (MicroBrightField Inc, Williston, VT). Distance between sequential sections is 0.96 mm. Distance between migrating cells in section 9 and edge of transplantation site (section 4) is 4.8 mm.

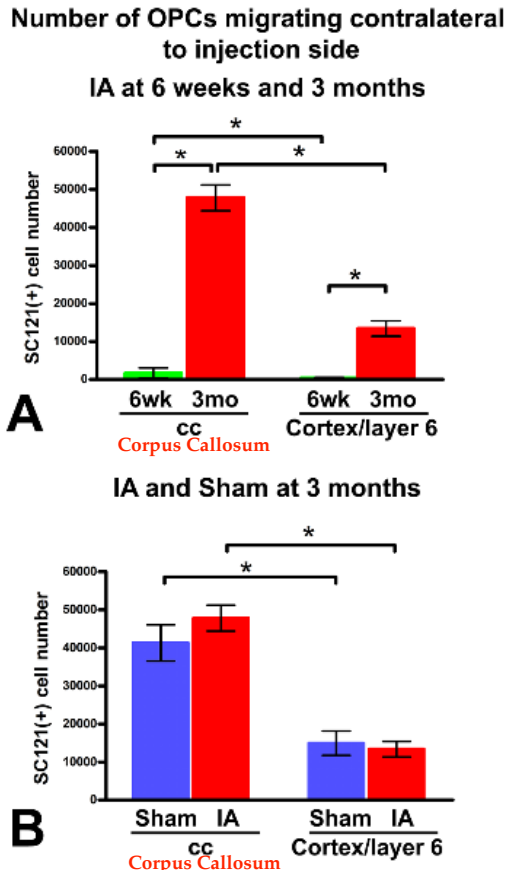


Figure 5. Stereological counts of transplanted OPCs in the contralateral hemisphere 6 weeks and 3 months post-transplantation. Bar diagrams in panel A reflect migratory patterns at two time points after transplantation in two brain regions in IA animals. Bar diagrams in panel B illustrate migratory tendencies in two locations (corpus callosum and deep cortex) based on experimental history (IA versus sham) at 3 months post-transplantation. In (A), difference in cell numbers between 6 weeks and 3 months is significant by *t* test ($p<0.05$) in both corpus callosum and deep cortex (layer 6); two-way ANOVA shows that there is interaction between time and location, i.e. time tends to favor deep cortical over callosal location ($p<0.05$). In (B), there are no differences in cell numbers between sham and IA-injured subjects in corpus callosum or deep cortex at 3 months post-transplantation. In both groups of subjects, there are more cells in corpus callosum than deep cortex by *t* test ($p<0.05$). Two-way ANOVA shows that there is no interaction between experimental history and location, that is injury does not seem to influence the location of cells in one site over the other. cc, corpus callosum; IA, impact acceleration

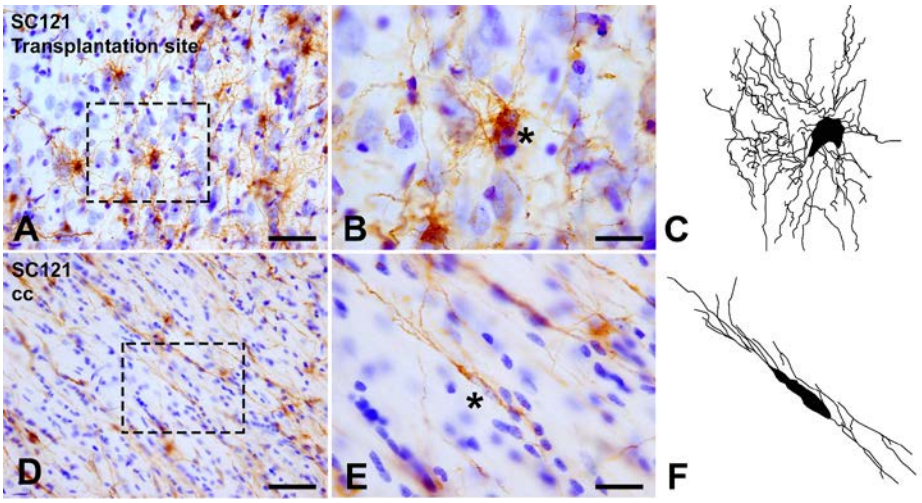


Figure 6. Some cytological features of OPC transplant-derived cells 3 months post-transplantation. Around the transplantation site, a large number of transplant-derived SC121 (+) cells (brown) have round features with extensive and radially arrayed processes (A-C). In the corpus callosum (cc), the majority of SC121 (+) cells are spindle shaped with long parallel processes (D-F). B and E are enlargements of bracketed areas in A and D, respectively. C and F are Neurolucida tracings of representative cells from B and E indicated with asterisks. Scale bars: A and D, 50μm; B and E, 20 μm

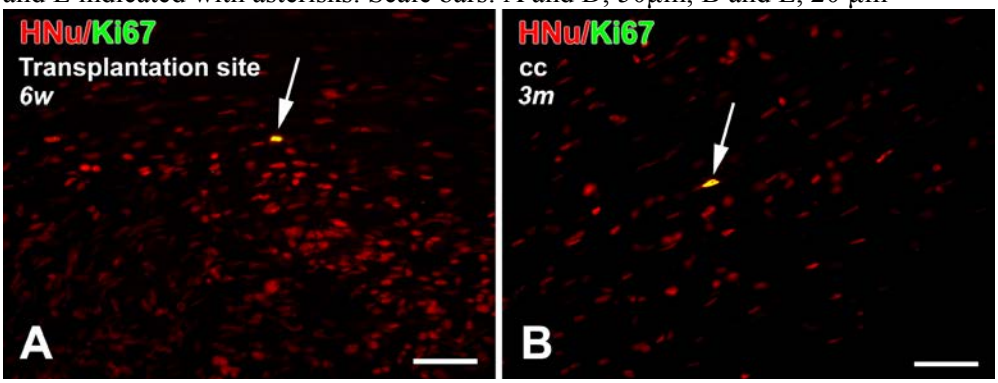


Figure 7. Proliferative activity of OPCs at 6 weeks and 3 months post-transplantation. Only rare HNu (+) transplant-derived cells (red) are mitotic Ki67 (+) cells (green) at the transplantation site at 6 weeks (A) or, after migration, in the corpus callosum at 3 months (B). Images are from a representative IA-injured rat. Scale bars: 50 μm

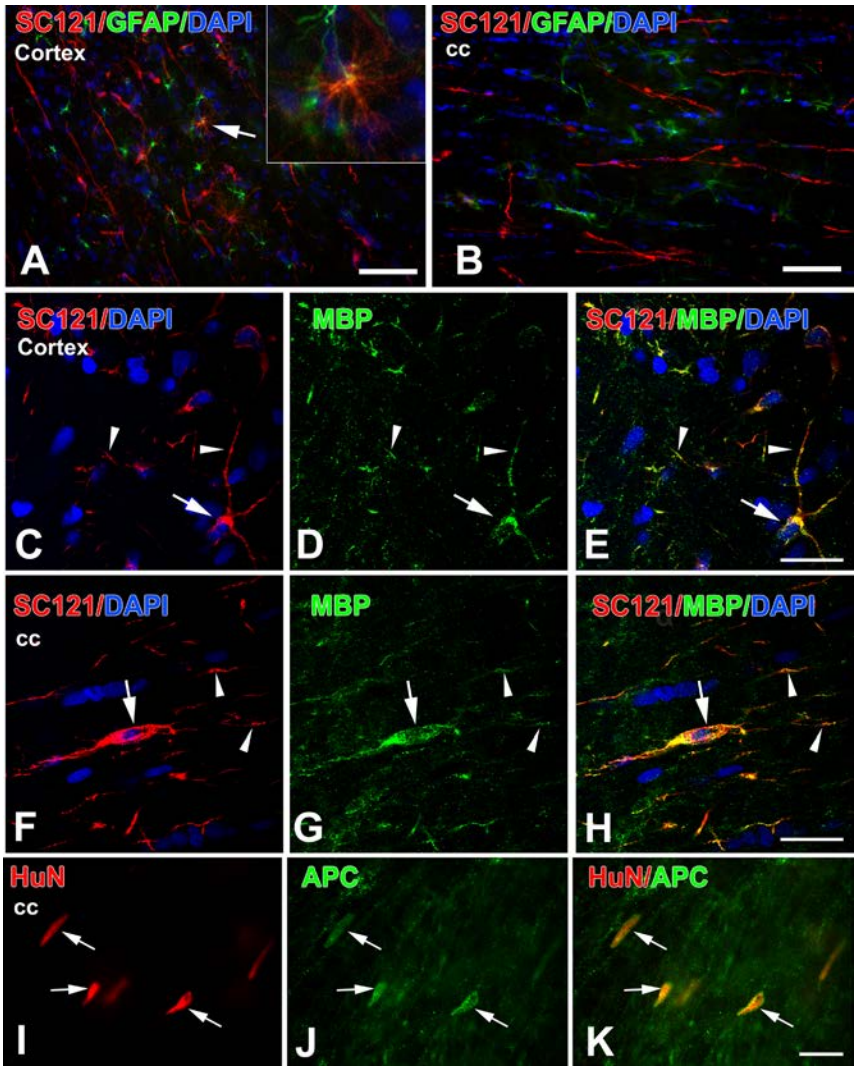


Figure 8. Differentiation of transplanted OPCs 3 months post-transplantation. All images are from representative IA-injured animals. At 3 months, we found no SC121 (+) cells expressing TUJ1 (+) neuronal phenotypes; only rare SC121 (+) OPCs (red) had differentiated into GFAP (+) astrocytes (green) at the transplantation site (arrow, A). Inset in A is a magnification of the astrocytic profile indicated by arrow in main panel. In the corpus callosum (B), no OPCs (red) are immunoreactive for GFAP (green). Panels C-H are confocal images to show that various types of cells derived from the OPC transplant (round in C or spindle-shaped in F, red) become mature MBP (+) oligodendrocytes (green in D and G); both cell bodies (arrows) and processes (arrowheads) of transplant-derived cells are immunoreactive for MBP. Panels I-K show APC (+) cell bodies of transplant-derived cells in the corpus callosum. Panels E, H and K are merged images of panels C-D, F-G and I-J, respectively. Scale bars: A and B, 50 μm; C-K, 20 μm

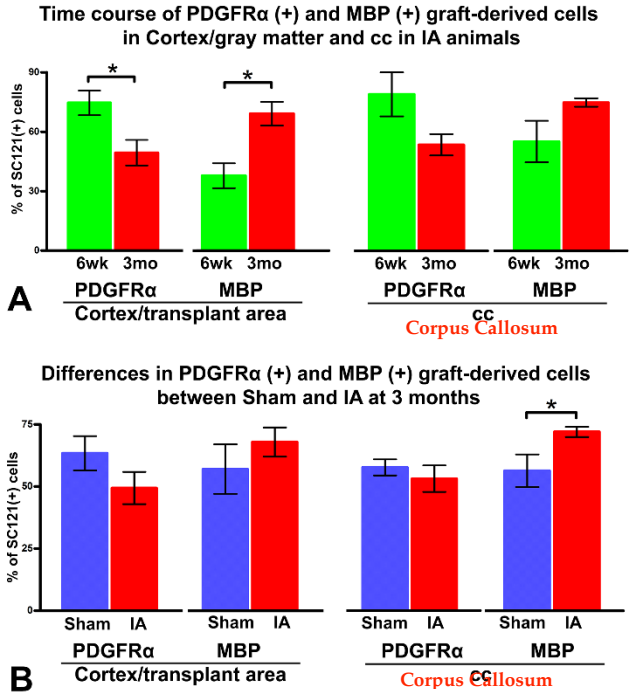


Figure 9. Stereological counts of PDGFRα- and MBP-immunoreactive cells derived from OPC transplant at 6 weeks and 3 months post transplantation in IA-injured and sham rats. Bar diagrams in panel A reflect differentiation patterns at two time points after transplantation in two brain regions (corpus callosum versus cortical transplantation area) in IA animals. Bar diagrams in panel B illustrate differentiation pattern in two locations (corpus callosum versus transplantation area) based on experimental history (IA versus sham) at 3 months post-transplantation. In (A), IA rats at 3 months have more MBP (+) and fewer PDGFRα (+) transplant-derived oligodendrocytes than at 6 weeks post-transplantation; counts were performed at the transplantation site. In (B), IA rats at 3 months have more transplant-derived, MBP (+) oligodendrocytes compared to sham in the corpus callosum.
cc, corpus callosum; IA, impact acceleration

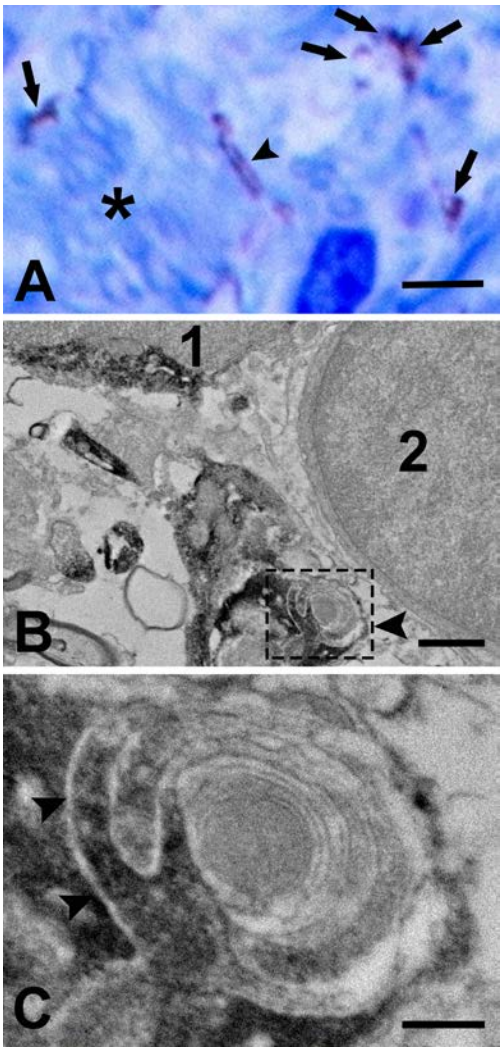


Figure 10. Ensheathing profiles issued by transplant-derived oligodendrocytes based on ultrastructural IHC. In A, an accompanying semi-thin section through the corpus callosum counterstained with toluidine blue shows the co-localization of SC121(+) brown processes with blue myelin sheaths in longitudinal (arrowheads) or transverse (arrows) axonal profiles. Colocalization profiles are purple. Asterisk shows a group of SC121 (-) axons. In B, two SC121 (+) processes (arrowheads) are shown to ensheath unlabeled axons. These profiles are adjacent to one SC121 (+) cell (#1) and also one unlabeled cell (#2). Cells 1 and 2 have the appearance of oligodendrocytes. Panel C is a magnification of framed area in (B) and shows detailed ultrastructural features of ensheathment by transplant-derived oligodendrocytes. SC121 (+) tongue processes (arrowheads) are wrapped around a myelinated axon. Myelin sheath on the inside appears to be unlabeled. Scale bars: A, 5 µm; B, 1 µm; C, 500 nm

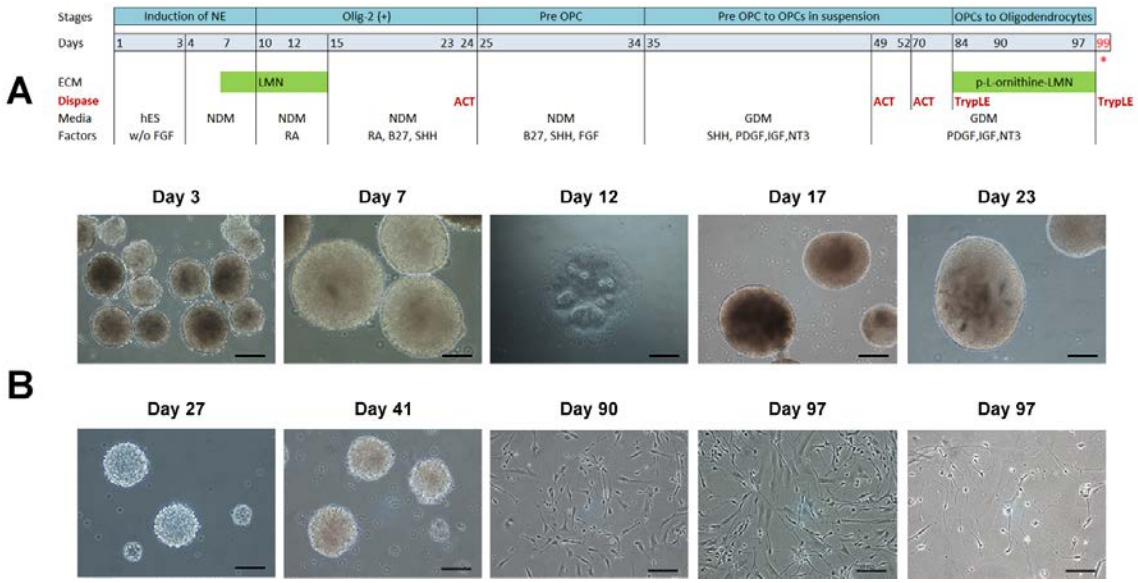


Figure S1. A schematic illustration (A) of the method used to prepare OPCs for transplantation and representative cell morphology observed (B). Method was based on **Hu and Zhang, 2009, Hu et al., 2009, and Hu et al., 2010.** Human embryonic stem cell H9 colonies were detached by dispase on day 1 to prepare embryoid bodies (EBs) that were subsequently cultured for 3 days in hES medium (B: day 3) without FGF and for 3 days in NDM. EBs (B: day 7) were plated on Laminin-coated plates and cultured with NDM for 3 days and NDM with RA for 5 days (B: day 12). One Day 15 colonies were manually detached and cultured as spheres (B: day 17) for 10 days in NDM with RA, B27 and SHH. On day 24, big spheres (B: day 23) were dissociated by Accutase (ACT) into small spheres (B: day 27) and cultured with NDM containing B27, SHH and FGF for 10 days. On day 35, medium was switched to GDM with SHH, PDGF, IGF and NT3 and cultured for 2 weeks (B, day 41). On day 49 and 70, cells were passaged with Accutase treatment and treated with GDM with PDGF-AA, IGF1 and NT3 for the remaining of the protocol. On day 84 spheres were trypsinized and plated on p-L-ornithine and laminin coated plates or coverslips and cultured for 2 weeks in the same medium. GDM with PDGF-AA, IGF1 and NT3 for transplantation and ICC (Figure 2), respectively. On day 99 (*), the cells were trypsinized with TrypLE, counted, resuspended in high concentration and used for transplantation. The representative morphology of the OPCs on Day 90 and Day 97 are shown in B. Scale bars in B: Day 12, 500 µm, Day 3 and 17; 200 µm, all others; 100 µm

Table 1. Primary antibodies used for immunocytochemistry, immunohistochemistry and ultrastructural immunohistochemistry.

Target Phenotypes	Target Proteins/Epitopes	Host	Dilution	Vendor
Human cell markers	Human Nuclei Protein antibody (HNu)	Mouse	1:1,000	Millipore, Billerica, MA
	Human cytoplasm-specific antibody (SC121)	Mouse	1:3,000	StemCells, Inc., CA
Neuron (*in vivo*)	Type III-tubulin epitope J1 (TUJ1)	Rabbit	1:400	Covance, Berkeley, CA
Neuron (*in vitro*)	Type III-tubulin epitope J1 (TUJ1)	Mouse	1:100	Sigma, Saint Louis, MO
Mitotic marker	Ki67 antigen (NCL-Ki67p)	Rabbit	1:400	Novocastra Labs, Newcastle, UK
Progenitor/early oligodendrocyte	Olig2	Goat	1:50	Santa Cruz, Santa Cruz, CA
	Platelet-derived growth factor receptor α (PDGFRα)	Rabbit	1:100	Santa Cruz, Santa Cruz, CA
	A2B5	Mouse	1:200	Millipore, Billerica, MA
	NG2	Rabbit	1:100	Millipore, Billerica, MA
	O4	Mouse	1:100	Millipore, Billerica, MA
Late oligodendrocyte	Myelin Basic Protein (MBP)	Rabbit	1:200	Abcam, Cambridge, MA
	Adenomatous polyposis coli protein (APC)	Rabbit	1:200	Novus Biologicals, Littleton,CO
	Myelin proteolipid protein (PLP)	Chicken	1:200	Novus Biologicals, Littleton,CO
Astrocyte (*in vivo*)	Glial fibrillary acidic protein (GFAP)	Rabbit	1:400	Dako, Carpinteria, CA
Astrocyte (*in vitro*)	Glial fibrillary acidic protein (GFAP)	Rabbit	1:100	Millipore, Billerica, MA
Mesodermal marker	Bone morphogenetic protein-4 (BMP4)	Mouse	1:300	Millipore, Billerica, MA

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