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Human neural stem cell differentiation following transplantation into spinal cord injured mice: association with recovery of locomotor function

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Stem cells are under intense investigation as potential therapeutics for central nervous system (CNS) injury and disease. However, several reports have suggested that stem cells grown as neurospheres and transplanted into an injured environment preferentially differentiate into astrocytes, contributing to glial scar. Further, the causal relationship between functional recovery and cell transplantation has not been empirically investigated in early studies. Using severe combined immunodeficient (scid) mice to minimize xenograft rejection, we report that prospectively isolated human fetal CNS-derived stem cells grown as neurospheres (hCNS-SCns) survive, migrate and express differentiation markers for neurons and oligodendrocytes after long-term engraftment in spinal cord injured (SCI) NOD-scid mice. Only rarely do these cells differentiate into glial fibrillary acidic protein (GFAP)-positive astrocytes, with no apparent contribution to glial scar. hCNS-SCns engraftment was associated with recovery of locomotor function. After long-term engraftment and stable behavioral plateaus in recovery were achieved (4 months post-transplantation), locomotor improvements were abolished by selective ablation of human cells with diphtheria toxin (DT). These data suggest that hCNS-SCns survival is required for locomotor recovery, possibly via differentiation and integration of human cells in the mouse host or continuous supply of trophic or other support necessary for gains in host cell function. [Neurol Res 2006; **28**: 000–000]

Keywords: Spinal cord injury; human fetal stem cells; xenograft transplantation; functional assessment; histological analysis

INTRODUCTION

Pluripotent embryonic stem cells (ESCs) are under intense investigation as potential therapeutics for central nervous system (CNS) injury and disease. However, ESCs also have the potential for uncontrolled division and tumorigenesis after transplantation[1,2]. Fate commitment to selected cell lineages or types and/or genetic addition of a molecular cell death switch before ESC transplantation may address this issue. An alternate strategy is to generate tissue-derived, expandable stem cell lines[3–6]. These cells have greater lineage restriction and therefore reduced propensity for teratoma formation, and may require less manipulation *in vitro* before transplantation.

Rodent CNS-derived stem/progenitor cells can grow either as a monolayer under serum-free conditions[7–9] or as free-floating clusters of cells, termed neurospheres[5,10]. After long-term expansion, stem cells generated by either method retain multilineage differentiation potential *in vitro* and *in vivo*[8,11,12]. Critically, human CNS stem cells can also be propagated as neurospheres, enabling the creation of banks of human neural stem cells[13]. This strategy is improved by direct isolation of human CNS stem cells using fluorescence-activated cell sorting for cell surface antigens, yielding a highly purified cell population. Uchida *et al.* have reported that neurospheres derived from human fetal brain tissue are initiated by a rare population of $CD133^+$, $5E12^+$, $CD24^{-/lo}$, $CD34^-$ and $CD45^-$ cells; prospective isolation and propagation of these cells as neurospheres results in stable, clonogenic human CNS-stem cell neurosphere (hCNS-SCns) lines that are readily expandable and retain multilineage potential *in vitro* and *in vivo*, expressing differentiation markers for both neurons and glia[14]. Furthermore, these cells have not come into contact with animal cells and are grown in serum free conditions; concerns for embryonic cell lines[15]. Finally, hCNS-SCns transplanted into NOD-scid mouse brain survive and migrate, with no evidence of tumorigenic growth after engraftment for over 17 months[16].

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Recent studies have used a variety of rodent-derived precursor/stem cells transplanted into rodent models of spinal cord injury (SCI) or disease. These studies have generally lacked definitive identification of transplanted cells, long-term survival and engraftment data, evidence of differentiation and/or evidence of functional integration. Portions of the data presented here were presented at the Advances in Biology and Treatment of Gliomas Conference, Rome, Italy (September 24, 2005) and recently in the Proceedings of the National Academy of Sciences (PNAS)[20]. This report expands on previous work on the use of prospectively isolated human CNS-SCns as described above and demonstrates that: (1) hCNS-SCns survive, engraft and differentiate after transplantation into immunodeficient SCI mice; (2) hCNS-SCns engraftment is associated with locomotor recovery after traumatic SCI and (3) this recovery is abolished by selective ablation of human cells using diphtheria toxin (DT).

METHODS

Contusion injuries

Female NOD-scid mice (10 weeks old) were anesthetized with avertin and received a laminectomy at T9 using a surgical microscope. Mice received either a 50 kd (kilodyne) (n=38) or 60 kd (n=30) contusion SCI using an infinite horizon impactor. In the 50 kd SCI cohort three mice were excluded owing to unilateral bruising or abnormal force/displacement curves. Seven days post-SCI, the remaining 35 mice were tested using the blood–brain barrier (BBB) rating scale to ensure that the contusion injury was successful and the mice were randomized to receive hCNS-SCns (n=16) or vehicle (n=19). Before analysis, five hCNS-SCns-transplanted animals and four vehicle-injected animals were excluded using pre-hoc criteria owing to kyphosis of the spinal column (resulting from retarded muscle healing in NOD-scid mice and/or suture failure post-transplantation) that could have interfered with behavioral assessment resulting in 11 hCNS-SCns grafted and 15 vehicle control animals. In the 60 kd SCI cohort a human fibroblast (hFibroblast) cellular control group was added to the above paradigm and the injury severity increased. Four mice were excluded owing to unilateral bruising or abnormal force/displacement curves. Seven days post-SCI, the remaining 26 mice were randomized to receive hCNS-SCns, hFibroblasts or vehicle. Before analysis, two hCNS-SCns-transplanted animals and two hFibroblast-injected animals were excluded using pre-hoc criteria owing to kyphosis as above, resulting in eight hCNS-SCns, eight hFibroblast and ten vehicle controls. Behavioral and histological data (collected in the same animals) from both cohorts are included in the results. Twelve additional mice received a 50 kd SCI and hCNS-SCns and were killed to assess cell survival at hour 24 (n=4), hour 48 (n=4) or week 4 (n=4); these mice were not behaviorally assessed.

hCNS-SCns cell culture, maintenance and injection

Long-term human neurosphere cultures from fetal brain have been described previously[14]. hCNS-SCns propagated as neurospheres were shipped overnight to the University of California at Irvine (UCI); cell survival was greater than 95%. Neurospheres were passaged by harvesting the cells and enzymatically dissociating the spheres into a single cell suspension with collagenase [0.5 mg/ml in phosphate-buffered saline (PBS)/0.1% hemopoietic stem cell (HSC)] for 5–10 minutes. Neurospheres were concentrated to a density of 75,000 cells/μl in injection buffer consisting of 50% Hank's balanced salt solution (HBSS) and 50% X-*vivo* medium. In the follow-up experiment, hFibroblasts from fetal liver were grown to confluence in Iscove's MOD (1x)/10% fetal bovine serum (FBS), dissociated with trypsin, washed and concentrated to 75,000 cells/μl.

Transplantation

Nine days post-SCI, mice were anesthetized and the laminectomy site was exposed and secured in a spinal stereotaxic frame by clamping the T8 and T10 lateral vertebral processes. Polished siliconized beveled glass pipettes (i.d.=70 μm, o.d.=100–110 μm, Sutter Instruments, Novato, CA, USA) were loaded with freshly triturated cells, which were stereotaxically injected via a NanoInjector system and micropositioner (WPI Instruments, Waltham, MA, USA). Animals received four injections, bilaterally 0.75 mm from midline at both the anterior aspect of T10 and the posterior aspect of T8. Each site received either 250 nl of cells or vehicle, delivered in 50 nl puffs over 75 seconds, followed by a 2 minute delay before withdrawal of the pipette.

Behavior testing

Animals were cared for, excluded from analysis and scored for behavior by observers blinded to treatment. Functional recovery was assessed using a modified (18 point) BBB locomotor rating scale weekly for 1 month and then biweekly[17,18]. At week 16, a subset of animals was also videotaped on a horizontal ladder beam task in a series of three trials. Animals were scored for step errors (slips, drags and missed placements) over 150 rungs by two independent observers.

Human cell ablation

Following BBB assessment at week 16, mice received two i.p. injections of DT (50 μg/kg), 24 hours apart and were reassessed on the BBB 1 week later and then killed.

Perfusion and tissue collection

Mice were anesthetized and transcardially perfused with 30 ml PBS followed by 100 ml of 4% paraformaldehyde. Spinal cords were dissected and the segments corresponding to T2–T5, T6–T12 and T13–L3 were blocked. Blocks were post-fixed overnight in 4% paraformaldehyde. For immunocytochemistry (ICC), some blocks were equilibrated in 30% sucrose/PBS for 12 hours, embedded in optimal cutting temperature (OCT) and frozen at −65°C in isopentane for sectioning. For electron microscopy (EM), 1 mm thick coronal

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sections were post-fixed in 4% glutaraldehyde immediately following paraformaldehyde fixation.

ICC

Immunostaining was conducted as described previously[16,19]. SC101, a monoclonal antibody for human nuclei, SC121, a monoclonal antibody for human cytoplasm, and SC123, a monoclonal antibody specific for human glial fibrillary acidic protein (GFAP), were produced by StemCells Inc. Other primary antibodies included: β-tubulin III (Covance, Richmond, CA, USA); glutamic acid decarboxylase 67 (GAD)-67, neuronal nuclear antigen (NeuN), NG2 and Neurofilament-150 (Chemicon); pan-GFAP (DAKO); vimentin (Cell Marque, Hot Springs, AR, USA); CC-1/affinity pure rabbit anti-allophycocyanin (APC) (Oncogene); and myelin basic protein 1-188 (Biogenesis, Bournemouth, UK). Secondary antibodies in double-labeling experiments were Alexa 488 and 555 from Molecular Probes. Controls included incubations in the absence of primary/secondary antibodies and cross-reactivity tests of the paired primary antibodies versus the other secondary antibody. Confocal imaging was conducted using a BioRad Radiance 2000 system using LaserSharp 2000 software with lambda strobing to reduce bleed-through associated with simultaneous scanning.

EM

Following glutaraldehyde post-fixing, tissue was either cut at 50 μm on a vibratome and immunostained with SC121 and diaminobenzidine (DAB) or washed in 0.1 M sodium cacodylate buffer overnight, fixed with osmic acid, washed in distilled water, dehydrated through a series of alcohols, immersed in propylene oxide and embedded in Spurr resin. Semithin (1 μm) sections were stained with toluidine blue. Ultrathin sections (90 nm) were cut, transferred to a 150 mesh copper grid, stained with uranyl acetate and lead citrate and viewed on a Philips C10 transmission electron microscope.

NOD-scid/shiverer crosses

The myelin basic protein-shiverer (MPBshi) mutation was backcrossed onto the NOD-scid background. Male shi heterozygote mice were crossed with NOD-scid females. N1 male progeny were genotyped for deletion of exons 3–7 in the MBP gene and backcrossed to another set of NOD-scid females. Backcrossing was continued for four generations. Heterozygous N4 males and N4 females were mated with one-fourth of the progeny expressing the shiverer phenotype. NOD-scid/shi mice received either intra-cerebellar grafting of hCNS-SCns at post-natal days 1–2 or a contusion injury to T9 at week 6 of age combined with cells or vehicle; survival period was 4 weeks.

RESULTS

Differentiation of hCNS-SCns following transplantation into SCI NOD-scid mice

We have previously shown that hCNS-SCns transplanted into NOD-scid mice with spinal cord contusion injuries survive and migrate extensively; critically, transplanted cells in this model do not generate large numbers of GFAP-positive astrocytes detectable with either double-labeling for human-specific nuclear (SC101) or cytoplasmic (SC121) antibodies, or with a human-specific GFAP antibody (SC123)[20]. In contrast, significant numbers of human cells assume morphologies consistent with differentiating cell populations. We have reported that cell counts using double labeling for SC121 and markers of early neuronal (β-tubulin III/Tuj-1) or oligodendroglial (NG2) lineage differentiation in confocal imaging suggest that ~26% of SC121-positive human cells exhibit colocalization with β-tubulin III neuronal staining (*Figure 1A*) and 64% of SC121-positive human cells exhibit colocalization with NG2 staining (*Figure 2A*)[20]. We present here further immunolabeling evidence of neuronal differentiation by transplanted human cells, using markers for β-tubulin III (*Figure 1A,B,B'*), GAD67 (*Figure 1C*) and NeuN (*Figure 1D,E*). Similarly, we have shown oligodendroglial differentiation of transplanted human cells using staining for NG2 (*Figure 2A*) and the terminal differentiation marker APC/CC-1 (Ref. 20). Additionally, we have shown evidence of myelination at the confocal level in the cerebellum of NOD-scid/shiverer mice (*Figure 2B–C*), which have a partial deletion in the gene for myelin basic protein (MBP), transplanted with hCNS-SCns at post-natal day 1. Here, we expand the evidence for myelination by human cells by demonstrating confocal double labeling for SC121 labeling surrounding neurofilament-positive axonal processes in the spinal cord of contusion-injured NOD-scid/shiverer mice (*Figure 2D*).

A critical question that follows from these confocal colocalization studies is whether these findings engrafted hCNS-SCns demonstrate cellular morphology at the electron microscopic level that is consistent with terminal differentiation to functional neurons and oligodendrocytes. Additionally, we have previously shown evidence for the formation of newly born neurons, extending dendrites and synaptic contacts from transplanted hCNS-SCns[20]. Additionally, we have demonstrated hCNS-SCns-mediated remyelination at the electron microscopic level in the cerebellum of NOD-scid/shiverer mice transplanted with hCNS-SCns as adults and the spinal cord of NOD-scid mice transplanted with hCNS-SCns 9 days post-SCI as adults.

Finally, a key question for these studies is whether hCNS-SCns transplantation is associated with recovery of locomotor function. A sensitive quantitative task in which to assess recovery of locomotor function is the horizontal ladder beam. Analysis of horizontal ladder beam errors showed that hCNS-SCns-grafted mice ($n=9$) exhibited significantly fewer errors than vehicle controls ($n=12$) in the 50 kd SCI study cohort ($p<0.05$) (*Figure 3*). Similarly, hCNS-SCns-grafted mice ($n=8$) from the 60 kd SCI cohort exhibited significantly fewer errors than vehicle controls ($n=10$) ($p<0.05$); hFibroblast controls ($n=8$) did not differ from vehicle controls ($n=10$) ($p<0.87$), but approached significance in comparison with hCNS-SCns-grafted mice ($p<0.17$).

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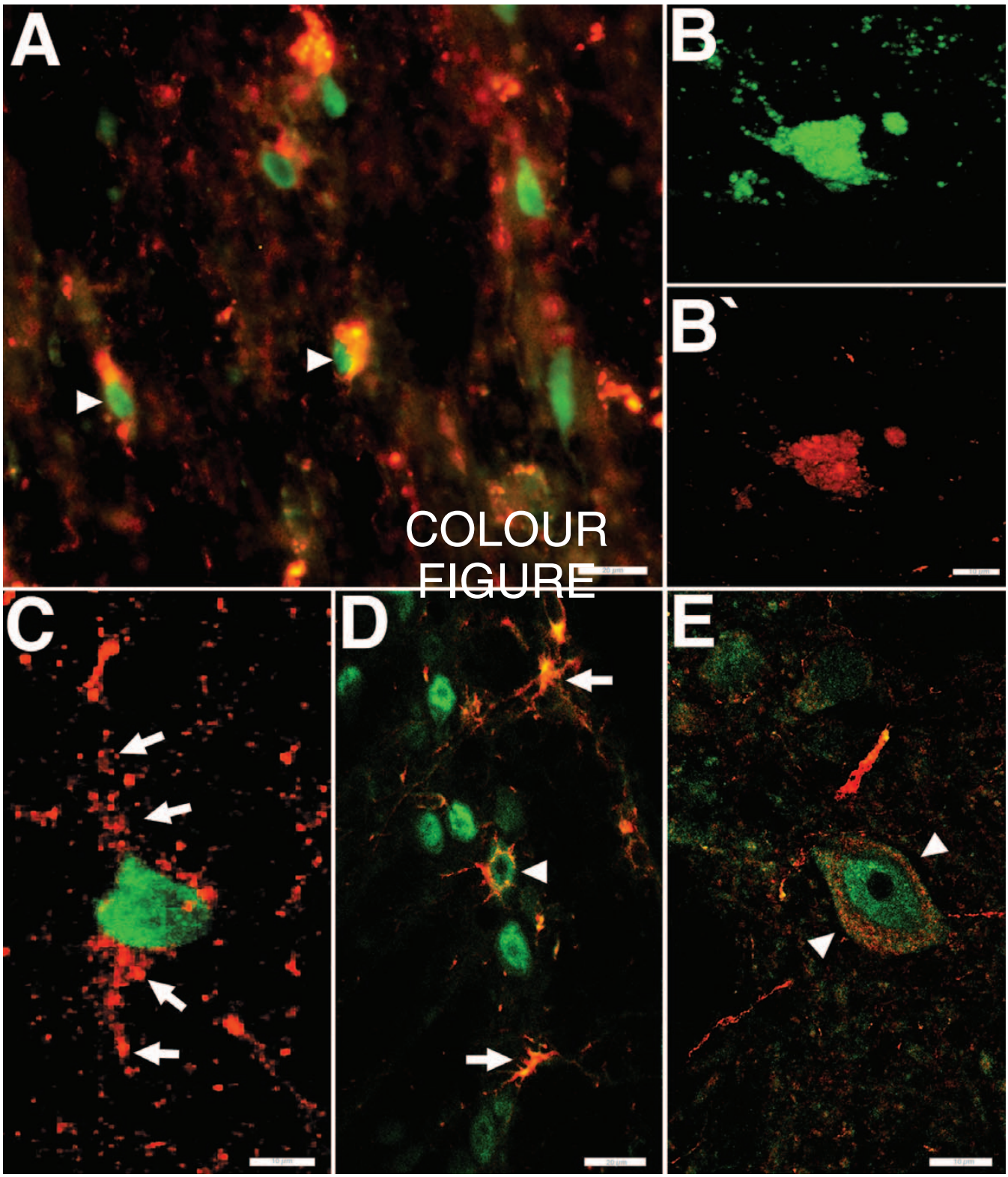

Figure 1: Transplanted hCNS-SCns are capable of neuronal differentiation. (**A**) By week 4, some human-immunopositive nuclei (SC101, green) within injured spinal cord are co-localized (arrowheads) with β-tubulin III (red). (**B**) Co-localization of a human cytoplasm positive cell (SC121, green) with (**B'**) β-tubulin (red) at week 17. (**C**) By week 17, GAD-67-immunopositive (red) processes (arrows) were occasionally co-localized with human nuclei (SC101, green), indicating a GABAergic neuron. (**D** and **E**) Human cytoplasm positive cells (SC121, red, arrows) were detected co-localized (arrowheads) with the neural marker NeuN at week 17 (green). Scale (μm): **A** and **D**=20; **B**, **C** and **E**=10. Reproduced by the kind permission of PNAS from[20]

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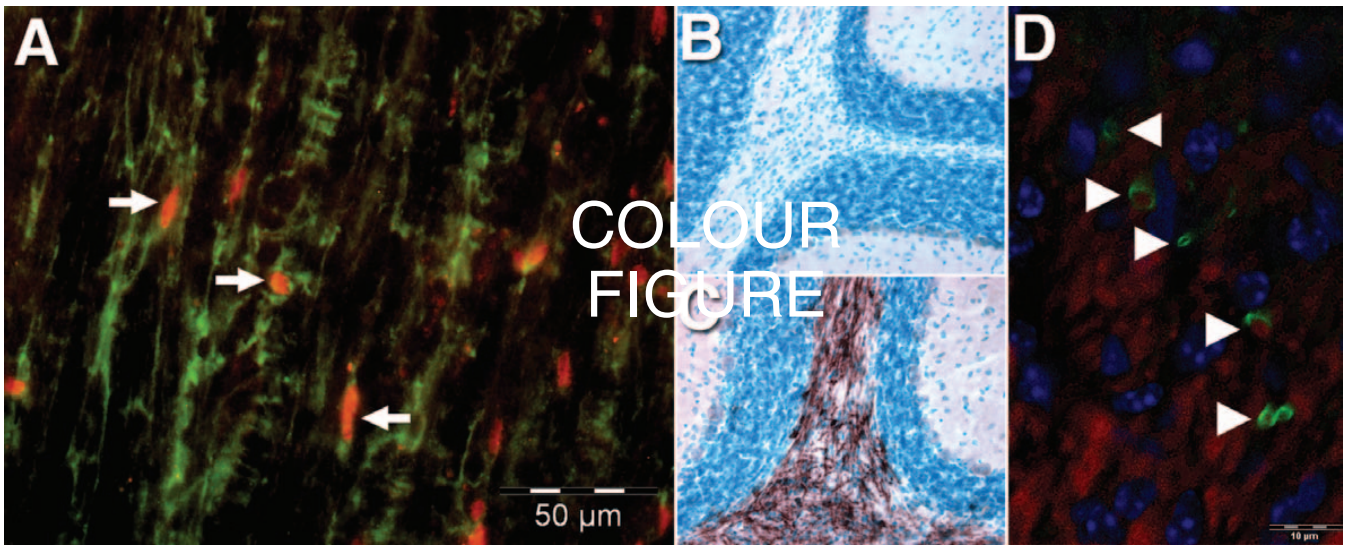

Figure 2: Transplanted hCNS-SCns are capable of oligodendrocyte differentiation**.** (**A**) By week 4, some human-immunopositive nuclei (SC101, red) within injured spinal cord are co-localized (arrowheads) with NG2 (green). Arrows denote double-labeled cells, suggesting that some hCNS-SCns have differentiated along an oligoprogenitor pathway. (**B**) Demonstration of the lack of myelin formation (anti-MBP antibody) in the cerebellum a shiverer mouse that did not receive an hCNS-SCns graft. (**C**) In the cerebellum of a shiverer littermate, hCNS-SCns have differentiated into mature oligodendrocytes capable of forming myelin (brown immunoreactivity within the white matter. (**D**) In the injured shiverer spinal cord, grafted hCNS-SCns produced normal MBP (green) that surrounds (arrowheads) neurofilament-positive mouse axons (red), the remaining axons are not ensheathed with normal myelin. Scale (μm): **A**=50; **D**=10

Similar results were observed in open-field BBB testing[20].

Identifying an appropriate cellular control for neural stem cell transplantation experiments is a difficult task. Although we selected fibroblasts as comparison cell population for transplantation, these cells are likely to supply trophic factor support or other positive effects on the host environment in and of themselves. In fact, fibroblasts engineered to augment this capacity and transplanted into the injury cavity in rat SCI models attract sprouting host fibers into the graft; however, these fibers do not continue past the graft in significant numbers to re-establish connections with denervated targets[21,22]. Accordingly, it is not surprising to observe a trend towards improvement of locomotor recovery in our paradigm in animals transplanted with hFibroblast cells. Nonetheless, comparison of hCNS-SCns and hFibroblast transplantation in this model may assist in beginning to dissect the mechanisms of recovery involved, as discussed below.

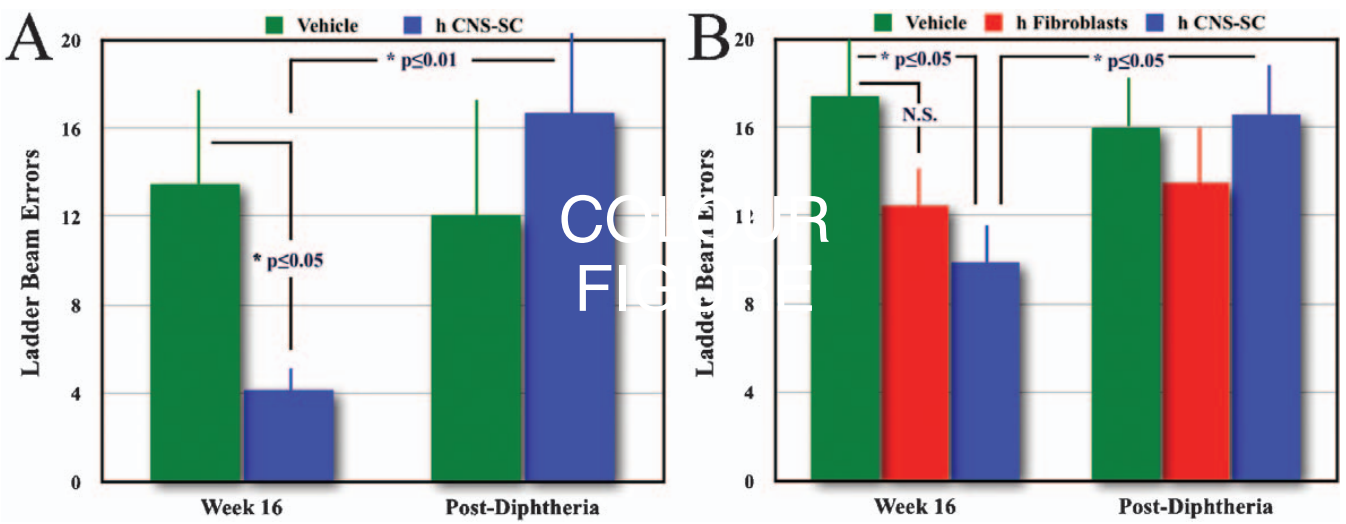

Figure 3: Locomotor recovery induced by hCNS-SCns is lost following ablation of human cells with diphtheria toxin. (**A**) Errors on a ladder beam task are significantly decreased in 50 kd SCI hCNS-SCns-grafted mice compared with vehicle controls ($p \leqslant 0.05$) and in (**B**) 60 kd SCI hCNS-SCns-grafted mice compared with vehicle controls ($p \leqslant 0.05$). No significant differences were observed between either vehicle versus hFibroblasts ($p \leqslant 0.87$) or hFibroblasts versus hCNS-SCns, although there was a trend toward the latter ($p \leqslant 0.17$). Ladder beam improvements in hCNS-SCns-transplanted mice were reversed 1 week after treatment with DT, suggesting the presence of hCNS-SCns is required to maintain functional recovery

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To investigate whether locomotor recovery was dependent on survival of transplanted human cell populations, NOD-scid mice were treated with DT after behavioral recovery was stabilized and locomotor performance was reassessed on the BBB and ladder beam 1 week later. Murine cells are 100,000-fold less sensitive to DT than human cells[23] and DT has been used extensively as a tool for targeted cell ablation in transgenic rodent models. These models either conditionally drive DT A chain expression in specific subpopulations of cells or express the human DT receptor under a promoter that is specific to a selective cell population. DT-targeted cells die via apoptosis with little or no effects on non-targeted cells and neighboring tissues[24–26].

Mice that received cell transplants and vehicle retained an extensive number of hCNS-SCns or hFibroblasts, whereas grafted mice that received DT had few or no remaining human cells (*Figure 4*). Vehicle-treated hCNS-SCns-transplanted mice were unaffected or improved slightly whereas DT-treated hCNS-SCns-transplanted mice showed significant decrements on the horizontal ladder beam (*Figure 3*) as well as the BBB[20]. Comparable results were observed in both the 50 and 60 kd SCI cohorts.

Critically, no statistical trends or significant differences in behavioral performance were observed after DT treatment in mice transplanted with hFibroblasts as a cellular control. This result suggests that a general toxic effect of human cell ablation on host locomotor function is possibly. Additionally, although hFibroblast-transplanted mice did exhibit locomotor recovery scores on the horizontal ladder beam and BBB that were intermediate to vehicle controls and hCNS-SCns-transplanted mice, the mechanism of any potential partial improvements in animals that received hFibroblasts may be different from that underlying recovery in animals that received hCNS-SCns.

DISCUSSION

Hypothetically, cell-based therapeutics for SCI could affect histological and/or functional outcome in at least three ways: (1) differentiation and functional integration of new cells into spared spinal cord circuitry, e.g. forming new oligodendrocytes and/or neurons; (2) increasing host neuronal, oligodendroglial or axonal survival, or decreasing host glial scaring and (3) increasing host-mediated regeneration or remyelination. Critically, one must consider that it may be quite unlikely that a cell therapy strategy will exert a single effect on the host environment and therefore that multiple mechanisms may contribute to any observed recovery of function in cell transplantation paradigms.

We report that transplanted hCNS-SCns can remyelinate axons, in naive NOD-scid/shi CNS and both injured NOD-scid/shi and NOD-scid spinal cord. These data are the first to demonstrate remyelination by engrafted human neural stem cells in a traumatic SCI model. Similarly, these are the first data supporting the potential for transplanted human stem cells to form synapses with the host in the injured spinal cord. Critically, however, whether sufficient myelination to affect conduction velocity or functional bridge circuits resulting from human-mouse synaptic connections are established by hCNS-SCns in our paradigm to account for the locomotor recovery observed is not clear and electrophysiological evidence for functional circuit integration remains necessary. Finally, it is important to note that we selected the NOD-scid mouse in order to assess the optimal potential of transplanted human cells in an environment where long term engraftment and the potential of these cells to differentiate and integrate with the host are optimized because of minimal xenotransplantation barriers[20]. It is highly likely that the transplantation model chosen could and would affect the fate of transplanted human cells, as well as observed effects on behavioral outcome. It is possible that the NOD-scid mouse provides a view of cell transplantation potential comparable with a human-human allograft; however, studies to assess the capacity of these cells in an immune environment that more closely simulates a human-human allograft would be of particular interest.

The observed reversal of locomotor improvement in DT-treated, hCNS-SCns-engrafted, SCI NOD-scid mice suggests that hCNS-SCns survival in the host plays a role in the maintenance of improved performance, but the potential for non-specific effects of DT-mediated cell ablation on behavioral performance non-specifically cannot be entirely ruled out. Accordingly, further studies to identify additional or alternate mechanisms of recovery (e.g. effects on host lesion volume/tissue sparing, host regeneration, host glial scarring, etc.) are in progress. However, taken together, these data suggest that hCNS-SCns differentiation to myelinating oligodendrocytes and neurons with EM criteria for human-host synaptic connections could be one mechanism for sustained locomotor recovery in this model.

Together, these findings suggest that hCNS-SCns could have potential benefits for multiple CNS diseases and injuries. However, clearly additional animal studies are necessary to evaluate the mechanism(s) of recovery and possible therapeutic potential of these cells.

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Figure 4: Photomicrographs of diphtheria ablation of human cells. After behavioral assessment at week 16, mice received two i.p. injections of DT (50 μg/kg), 24 hours apart or injection of PBS. One week later, mice were killed and immunostained for SC121 (brown) and counterstained with methyl green. DT administration ablated the majority of human cells, whether hCNS-SCns or hFibroblasts, yet no evidence of toxicity to surrounding host tissues was apparent. Scale bar for low-power photos (left column) is 100 and 50 μm for high-power photos (right column). Reproduced by the kind permission of PNAS from[20]

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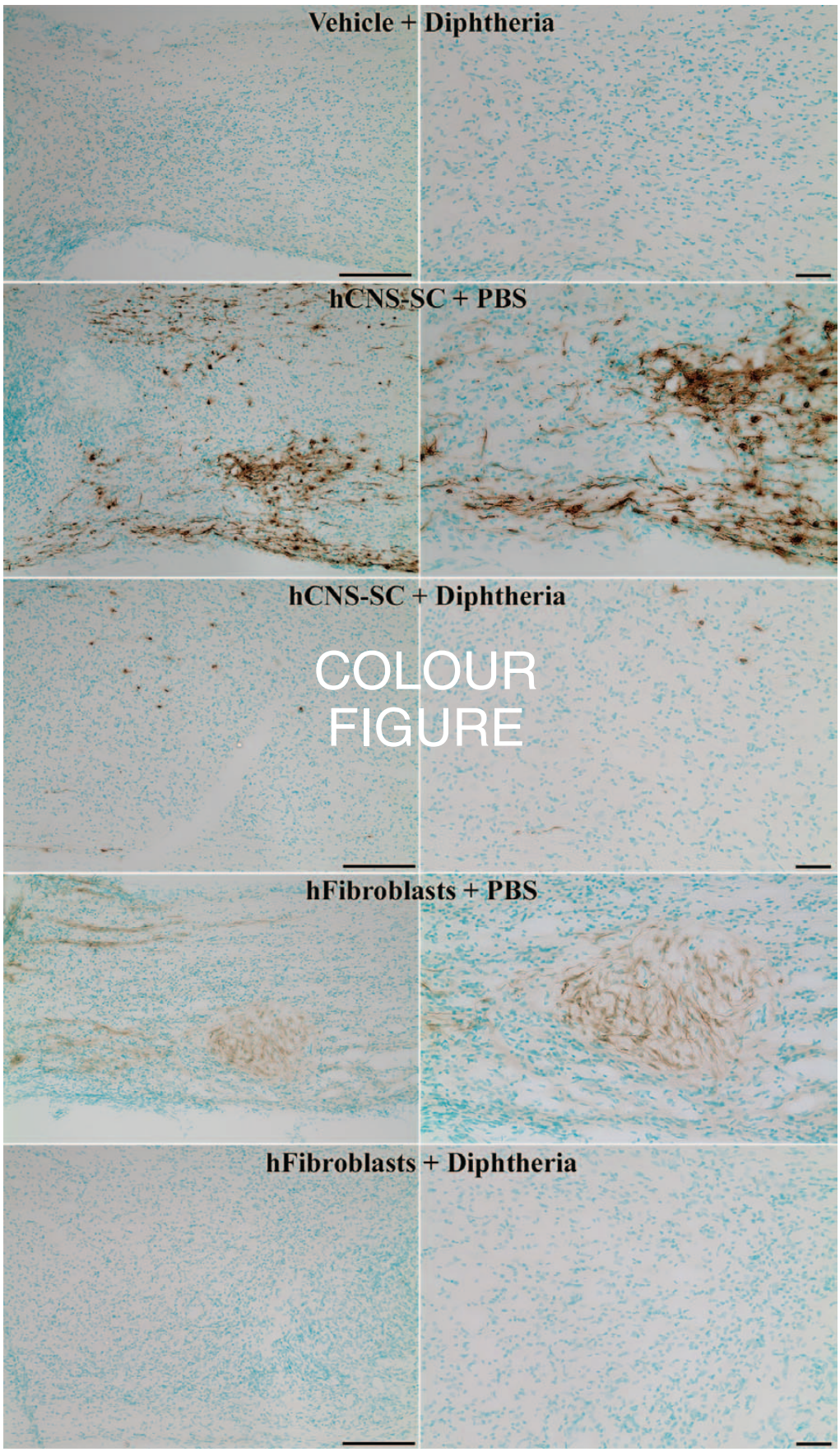

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