Transplantation of Induced Pluripotent Stem Cell-Derived Neural Stem Cells Mediate Functional Recovery Following Thoracic Spinal Cord Injury Through Remyelination of Axons

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ABSTRACT

Neural stem cells (NSCs) from embryonic or fetal/adult tissue sources have shown considerable promise in regenerative strategies for traumatic spinal cord injury (SCI). However, there are limitations with their use related to the availability, immunogenicity, and uncertainty of the mechanisms involved. To address these issues, definitive NSCs derived from induced pluripotent stem (iPS) cells generated using a nonviral, piggyBac transposon approach, were investigated. Committed NSCs were generated from iPS cells using a free-floating neurosphere methodology previously described by our laboratory. To delineate the mechanism of action, specifically the role of exogenous myelination, NSCs derived from wildtype (wt) and nonmyelinating Shiverer (shi) iPS cell lines were used following thoracic SCI with subacute intraspinal transplantation. Behavioral, histological, and electrophysiological outcomes were analyzed to assess the effectiveness of this treatment. The wt- and shi-iPS-NSCs were validated and shown to be equivalent except in myelination capacity. Both iPS-NSC lines successfully integrated into the injured spinal cord and predominantly differentiated to oligodendrocytes, but only the wt-iPS-NSC treatment resulted in a functional benefit. The wt-iPS-dNSCs, which exhibited the capacity for remyelination, significantly improved neurobehavioral function (Basso Mouse Scale and CatWalk), histological outcomes, and electrophysiological measures of axonal function (sucrose gap analysis) compared with the nonmyelinating iPS-dNSCs and cell-free controls. In summary, we demonstrated that iPS cells can generate translationally relevant NSCs for application in SCI. Although NSCs have a diverse range of functions in the injured spinal cord, remyelination is the predominant mechanism of recovery following thoracic SCI.

SIGNIFICANCE

Gain-of-function/loss-of-function techniques were used to examine the mechanistic importance of graft-derived remyelination following thoracic spinal cord injury (SCI). The novel findings of this study include the first use of neural stem cells (NSCs) from induced pluripotent stem cells (iPSCs) derived using the clonal neurosphere expansion conditions, for the treatment of SCI, the first characterization and in vivo application of iPSCs from Shiverer mouse fibroblasts, and the first evidence of the importance of remyelination by pluripotent-sourced NSCs for SCI repair and regeneration.

INTRODUCTION

Spinal cord injury (SCI) is a devastating event that affects millions of individuals and is a significant burden on health care infrastructure [1]. The pathobiology of SCI is complex but can be broadly conceptualized as involving an initial insult or primary injury that triggers a cascade of cellular and molecular events comprising the secondary injury [2]. Secondary injury begins within minutes and persists for months following SCI. It is characterized by neural cell death, demyelination, and inflammation [3, 4]. This pathobiology of SCI provides an opportunity for cell-based interventions to attenuate or repair tissue damage in order to preserve or repair central nervous system (CNS) function. Oligodendrocytes are specifically susceptible to the components of the secondary injury including glutamate excitotoxicity, oxidative stress, and elements of the immune response [5, 6]. The demyelinated axons are vulnerable to further damage and degradation [7]. This has led our laboratory and others to develop SCI treatments that

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provide an exogenous cell source to remyelinate axons to restore function and prevent further axonal loss [8–10]. Neural stem cell (NSC) therapies have been proposed and studied for SCI. Potential mechanisms for NSC-based treatment for SCI are not limited to remyelination. Other possible mechanisms include providing a source of neurons to replace those lost during SCI [11] or creating a permissive environment for host repair by allowing neural plasticity and suppressing the secondary injury. The latter can be achieved by promoting a regenerative environment [12], providing trophic factor support [13], or modulating the inflammatory response [14]. Currently there is limited research investigating these mechanisms.

Adult neural precursor cells (aNPCs) isolated and cultured from the brain or spinal cord tissue have therapeutic potential in animal models of SCI [8, 15–17]. NSCs generated from fetal or embryonic stem cell (ESC) sources have also been used successfully following SCI [9, 18, 19]. However, concerns related to availability and/or immune compatibility could limit their translational potential. Induced pluripotent stem (iPS) cells are somatic cells that are reprogrammed to an ESC-like fate [20], and their use addresses the limited availability of tissue-derived NPCs, as well as allowing for autologous grafts that circumvent immune rejection issues [21–23].

In this study, piggyBac transposon reprogrammed iPS cells [24] were directed to definitive NSCs (dNSCs) using neurosphere expansion methods [21, 25] for subacute intraspinal transplantation following thoracic SCI. To directly address the hypothesis that the iPS cell-derived dNSCs (iPS-dNSCs) provide functional improvement following SCI through the remyelination of axons, we compared nonmyelinating iPS cells generated from Shiverer (shi) mouse embryonic fibroblasts (MEFs) with those derived from a wildtype (wt) mouse. Shiverer mice have a mutation in the myelin basic protein (MBP) gene, causing an inability to generate compact myelin [26]; thus, the resultant shi-iPS cells and subsequent dNSCs will retain this property. Investigating the mechanism of recovery of a NSC-based treatment is difficult because of the multiple possible routes of action such as remyelination, neuronal replacement, and neurotrophin expression. The use of the loss-of-function shi-iPS-dNSCs allows this study to examine the specific role of remyelination by exogenous cells following thoracic SCI because both the wt and shi-iPS-dNSC will be capable of producing neuronal cells and neurotrophins, but only the wt-iPS-dNSC can produce compact myelin. Thus, any difference in recovery observed following SCI must be attributed to remyelination. This study is the first to generate shi-iPS cells, and use low-cell density neurosphere expansion conditions to generate iPS-dNSCs for SCI treatment, as well as the first to provide evidence of the importance of remyelination by pluripotent-sourced NSCs for SCI repair and regeneration.

**MATERIALS AND METHODS**

**Animal Information**

All experimental methodology used in this study was approved by the animal care committee of the University Health Network at the Toronto Western Research Institute and is in accordance with the policies established by the Canadian Council of Animal Care’s guide to the care and use of experimental animals. The wild-type (C57BL/6J) and Shiverer mice (C3Fe.SWV-Mbpsh/+J) were received from Jackson Laboratories (West Grove, PA, http://www.jacksonimmuno.com; Cedarlane, Burlington, ON, Canada, http://www.cedarlanelabs.com).

**iPS Cell Generation**

MEFs were prepared from CD1 and homozygous Shiverer fetuses (12.5 days post coitum) following a published protocol [27]. CD1 (p0) and homozygous Shiverer MEFs (p1) were reprogrammed by the piggyBac (PB) transposon-delivery system previously reported [24]. In brief, fibroblasts were plated on gelatinized 6-well plates at 0.75 × 10^5 cells per well. The next day, they were FuGENE HD (Roche, Indianapolis, IN, http://www.roche.com) transfected with 2 μg per well of a DNA mixture containing expression vectors for the PB transposase and PB transposons containing CAG promoter-driven reverse tetracycline transactivator, CAG-green fluorescent protein (GFP), and tetracycline-inducible promoter-driven reprogramming factors (polycistronic cMyc, Klf4, Oct4, Sox2, linked by 2A sequences). After 24 hours, the medium was supplemented with 1.5 μg/ml doxycycline (dox), and then 48 hours after transfection, the cells were cultured in mouse embryonic stem (ES) medium containing leukemia inhibitor factor (LIF) and 1.5 μg/ml dox. On days 10–14, ES-like colonies were picked on MEF feeders in 96-well plates. Clones were cultured and expanded by passing cells. The mouse iPS cell lines typically became dox-independent after day 21 of transfection.

**In Vitro iPS Cell Neutralization**

The generation of dNSCs from the iPS cell lines follows methodology previously described by our laboratory [21]. In brief, colonies of iPS cells were dissociated using TrypLE (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) to single cells and seeded in culture flasks contain serum-free media (SFM) with LIF at a cellular density of 10 cells/μl to yield primary primitive neurospheres after 7 days. To generate definitive neurospheres, the primitive neurospheres are dissociated and expanded in SFM containing fibroblast growth factor (FGF; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), heparin (Sigma-Aldrich), b27 supplement (Gibco, Grand Island, NY, http://www.invitrogen.com), and DLL4 (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com).

**RT-PCR**

Reverse transcription-polymerase chain reaction (RT-PCR) was used to examine the pluripotent iPS cells, in the vitro neutralization of the iPS cells to dNSCs, and their capacity to express neurotrophic factors. For evaluation of pluripotency, iPS cells were grown in feeder-free conditions using Matrigel-coated (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com) plates with MEF-conditioned media, and pluripotency markers were examined. For dNSC characterization, neural gene markers were examined in definitive neurospheres. Neurotrophin gene expression was examined in definitive neurospheres and following in vitro differentiation. mRNA was isolated using the RNAsesy microkit (Qiagen, Hilden, Germany, http://www.qiagen.com). A NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, http://www.thermofisher.com) was used to evaluate the concentration and purity mRNA. cdNA was synthesized using Superscript III with oligo(dT) (Invitrogen) according to instructions. RT-PCR was performed using TaqMan design primers with FAST TaqMan master mix under recommended thermocycling parameters on a 7900HT real-time PCR system. Samples were run in triplicate with a no template control. For the examination of the pluripotency and
neurotrophin gene expression, results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the relative quantity was calculated using $2^{-\Delta \Delta CT}$ [28]. For the examination of neural markers in dNSC, results were normalized to GAPDH and their iPS cell source, and gene expression levels were compared using the $2^{-\Delta \Delta CT}$ method [28] (supplemental online Table 1).

**Immunocytochemistry**

Samples from undifferentiated pluripotent cells, differentiated dNSCs, and sectioned neurospheres were analyzed using standard immunocytochemical procedures. In brief, nonspecific binding was blocked by incubation with phosphate-buffered saline containing 1% bovine serum albumin, 5% normal goat serum 5% nonfat milk powder, and 0.25% Triton X-100 for 1 hour. The slides were incubated overnight at 4°C with primary antibody (supplemental online Table 2) diluted in the same blocking solution. Next, the slides were incubated for 1 hour with appropriated secondary antibody (Invitrogen) containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Glass coverslips were mounted using Mowiol (Sigma-Aldrich).

**Spinal Cord Injury**

Clip-compression spinal cord injuries at the T6 level in mice were performed similarly to previously described techniques [19]. In brief, a T5–T7 laminectomy was performed, allowing for the passage of a modified aneurism clip (FEJOTA mouse clip) at T6. The clip was applied for 30 seconds with a closing force of 6.5 g. Sham-injured mice received the laminectomy without the clip-compression injury. Buprenorphine was administered subcutaneously twice daily for 2 days following surgery.

**Intraspinal Cell Transplantation**

The NSC transplantations were performed as previously described [19]. Immune suppression using cyclosporine A (20 mg/kg) began 2 days before transplantation and continued for the duration of the experiment. The mice were randomly assigned to wt-iPS-dNSC, shi-iPS-dNSC, or cell-free groups. One week following SCI, four injections were made flanking the injury. The injections were at a rate of 0.2 μl per minute and consisted of 1 μl of media with or without 50,000 cells. Shiverer mouse spinal cords were also transplanted with dNSCs. These animals were transplanted at 6 weeks of age with 2 bilateral spinal injections at the T6 region.

**Assessment of Motor Functional Recovery**

Functional recovery was assessed using the Basso Mouse Scale (BMS) and CatWalk-assisted gait analysis. Neuropathic pain was evaluated using von Frey filaments for mechanical allodynia and tail-flick latency for thermal alldynia.

Two treatment-blinded researchers performed all behavioral data acquisition and analysis. The BMS is a global measure of hind limb function in mice [29] and was evaluated weekly, on the same day and at the same time for all 8 weeks of experimentation.

The CatWalk apparatus can measure many specific aspects of mouse hind limb movement including swing speed, stride length, and hind limb intensity [30]. Only the mice able to traverse the CatWalk platform were included in the analysis of these parameters.

Mechanical and thermal alldynia was tested weekly starting at 6 weeks post-SCI using established methods [15]. Mechanical alldynia was assessed using a 4-g von Frey filament (Semmes-Weinstein monofilaments). The filament was applied to the plantar surface of the hind paws. Paw withdrawal following stimulus was recorded as a response. Thermal alldynia was assessed by tail-flick latency time in response to a light beam using tail-flick analgesia meter (IITC Life Science, Woodland Hills, CA, http://www.iitcinc.com). The latency of the mouse to remove its tail from the heat source was recorded.

**Sucrose Gap Ex Vivo Electrophysiology**

Using a modified one-sucrose gap chamber, platinum electrodes were used to recorded compound action potentials (CAP) of the whole spinal cord segment. CAPs were evoked by a single stimulus of an 8 mA for 50 microseconds (details are given in the supplemental online data).

**Quantitative Histological Assessments**

Tissue fixation and sectioning details are given in the supplemental online data. To assess total cord and white matter area, sections were stained with hematoxylin/eosin (H&E) and Luxol Fast Blue (LFB) using standard techniques [8]. H&E/LFB-stained sections, ±2,400 μm to injury epicenter at 480-μm intervals, were analyzed using the Cavaliere Probe in Stereo Investigator (MBF Bioscience, Williston, VT, http://www.mbfbiotechnology.com). White matter was identified by the blue staining of the LFB. The injury epicenter was defined as the section with the largest cross-sectional area of lesional tissue, identified by dense fibrous tissue with inconsistent matrix.

**Immunohistochemistry**

The tissue was incubated with blocking solution (described above). The slides were incubated overnight at 4°C in primary antibody (supplemental online Table 3). The slides were incubated for 1 hour with an appropriate secondary antibody (Invitrogen) containing DAPI. Glass coverslips were mounted using Mowiol. Total GFP+ cell survival was estimated using the Stereo Investigator (MBF Bioscience) optical fractionator at 480-μm intervals through cell-engrafted tissue using a 100-μm in 300-μm counting square on a Nikon Eclipse E800. Transplantation volume was calculated by multiplying the average GFP+ area by the graft length. For lineage evaluation, nonoverlapping high magnification confocal fields were imaged throughout cell graft at intervals of 480 μm. ImageJ software was used to count GFP+ and lineage marker positive cells in each field. These data were expressed as a proportion of total GFP+ cell (individual transplantation and differentiation data are given in supplemental online Table 4).

**Transmission Electron Microscopy**

Fixed spinal cords were embedded in 2% agarose for vibratome sectioning at a thickness of 50 μm. Immunoperoxidase staining with photooxidation of GFP was performed following adopted methods [31]. In brief, tissue was incubated overnight at 4°C in anti-GFP antibody followed by a 2-hour incubation of horseradish peroxidase rabbit secondary antibody at room temperature. Incubation with 0.05% 3,3′-diaminobenzidine (DAB) with 0.1% H2O2 was performed under a light microscope to visualize the DAB.
reaction product as well as using an X-Cite 120Q series fluorescence lamp (Luman Dynamics) with appropriate settings for GFP excitations to photobleach samples. Next, the tissue was embedded in Epon-Araldite epoxy resin [32]. After complete polymerization, the samples were sectioned using an Ultracut E microtome (Reichert; Labequip Ltd., Markham, ON, Canada, http://www.labequip.com) at 70-nm thickness and collected on copper mesh. The sections are counterstained using saturated uranyl acetate followed by Reynold’s lead citrate. A Hitachi H7000 transmission electron microscope at an accelerating voltage of 75 kV was used to image samples (details are given in the supplemental online data).

Experimental Design and Data Analysis

All animals were randomized into injury and treatment. All data were collected and quantified in a blinded fashion. All data are presented as means ± SEM. A Student’s unpaired t test, one-way or two-way analysis of variance was performed with post hoc Fisher’s least significant difference for pairwise comparison of subgroups where applicable. Results were considered to be statistically significant if p < .05. Statistics were performed using Statplus:Mac (Microsoft).

RESULTS

In Vitro Validation of iPS Cell Lines and Their Neuralization

In order to address the role of myelination in iPS-dNSC treatment for SCI, a cell line capable of myelination needed to be generated alongside a nonmyelinating cell line (supplemental online Fig. 1A). Pluripotency gene markers were evaluated using RT-PCR to compare the wt-iPS and shi-iPS cell lines with mES(R1) cells [27]. There were similar expression levels between cell lines for these genes (supplemental online Fig. 2A). To support the RT-PCR, immunocytochemical labeling of pluripotent cell colonies showed ubiquitous expression of OCT4 by all GFP+ ES and iPS cells (supplemental online Fig. 2B). The in vitro neuralization of the wt-iPS cells and shi-iPS cells was confirmed prior to in vivo experiments (supplemental online Fig. 1A). It is critically important to demonstrate equivalent neuralization for both iPS cell lines prior to their in vivo application to ensure that any differences observed in SCI treatment can be attributed to remyelination and not intrinsic cell line variation. To neutralize the iPS cells, protocols routinely used in our laboratory were implemented [21]. Phase-contrast microscopy showed qualitative similarities between wt- and shi-iPS cell colonies (Fig. 1A, left), LIF-dependent primitive neurospheres (Fig. 1A, center), and FGF-dependent/LIF-independent definitive neurospheres (Fig. 1A, right). RT-PCR analysis of dNSCs from wt- and shi-iPS cell lines showed equivalent expression levels of neural genes (Fig. 1B). Expression of the NSC marker, NESTIN, was confirmed using immunocytochemical labeling of cryopreserved and sectioned definitive neurospheres (Fig. 1C). Lastly, in vitro differentiation of the wt-iPS-dNSC and shi-iPS-dNSC was evaluated following differentiation conditions. Immunolabeling for astrocytes, neurons, and oligodendrocytes demonstrate the capacity for dNSCs from both iPS cell lines to differentiate to neural cells (Fig. 1D). Although the in vitro environment appears to favor astrocytic differentiation, this assay was only intended to demonstrate...
the ability of the iPS-dNSCs to yield neural cells. Because of the vastly different instructive niches present in the in vitro and in vivo environments, different differentiation profiles are often shown with the same cell lines [8, 33, 34].

**Neurotrophic Factor Expression Capacity of iPS-dNSC**

Neurotrophins have been shown to convey benefit following neurotrauma; cell therapies that focus on delivery of these factors can improve functional outcome [35]. We examined a series of neurotrophic gene markers in mRNA isolated from dNSCs and following in vitro differentiation conditions. Gene profiles from wt- and shi-iPS-dNSCs were similar to each other, as both dNSCs and differentiated cells. It was also noted that many key neurotrophins, such as Bdnf, Pdgfa, and Vegfa, were upregulated upon differentiation (Fig. 1E).

**In Vivo Validation of iPS-dNSCs in the Dysmyelinated Shiverer Mouse Spinal Cord**

Prior to their application in our SCI models, the in vivo usage of the dNSCs was examined in the Shiverer mouse model (supplemental online Fig. 1B). This model allows for easy assessment of the integration and myelinating ability of the exogenous cells. Six weeks following transplantation, GFP+ iPS-dNSCs were identified in the spinal tissue. Both iPS-dNSC lines survived and integrated into the Shiverer spinal tissue (supplemental online Fig. 3A). Oligodendrocyte, neuron, and astrocyte immunohistochemical markers colabeled with GFP+ from wt- and shi-iPS-dNSCs. Upon quantification of the neural cell markers in GFP+ cells, it was observed that differentiation was predominantly toward an oligodendroglial fate (supplemental online Fig. 3B).

In the wt-iPS-dNSC transplanted spinal cords, MBP colocalizes with GFP as long tubes in the longitudinal sections (Fig. 2A) and as circles in the transverse sections (Fig. 2B). The shi-iPS-dNSCs have a mutation in the MBP gene and are not expected to be capable of myelination. This was confirmed with a lack of MBP-positive labeling in GFP+ areas engrailed shi-iPS-dNSCs (Fig. 2C). Transmission electron microscopy (EM) was used to examine the nature of the remyelination. The myelination profile of the Shiverer spinal cord is characterized by fewer layers of noncompacted myelin (Fig. 2D, left). With wt-iPS-dNSC transplantation thick, multilayer, and compacted myelin was observed (Fig. 2D, center) as compared with the shi-iPS-dNSC transplanted tissue that remains similar to the naive Shiverer spinal tissue (Fig. 2D, right).

**Transplanted iPS-dNSCs Survived, Integrated, and Differentiated in the Injured Spinal Cord**

Following the validation of the iPS-dNSCs, these cells were used in our model of SCI with engraftment at 7 days postinjury (supplemental online Fig. 1C). Spinal tissue was isolated 8 weeks post-SCI for immunohistochemical analysis. The tissue was examined for the presence and distribution of GFP+ cells. Both the wt-iPS-dNSC and shi-iPS-dNSC integrated in the spinal tissue following SCI in an indistinguishable fashion (Fig. 3A), with similar cell numbers (Fig. 3B), graft length (Fig. 3C), and transplantation volume (Fig. 3D) observed.

The neural fates of the transplanted cells were identified. Positive labeling for neural cells was seen in both transplantation groups. Oligodendroglial progenitors and oligodendrocytes were labeled with Olig2 and APC (Fig. 3E, 3F), whereas astrocytes and neurons were labeled with glial fibrillary acidic protein (GFAP) (Fig. 3G) and NeuN (Fig. 3H), respectively. The proportion of GFP+ cells labeled for each neural marker was counted (Fig. 3I) with iPS-dNSCs primarily differentiating to an oligodendrocytic fate with 56.9% ± 0.4% of wt-iPS-dNSC and 54.4% ± 0.2% of shi-iPS-dNSC positive OLIG2, along with 24.8% ± 0.4% of wt-iPS-dNSC and 23.3% ± 0.1% of shi-iPS-dNSC positive for the mature oligodendrocyte marker CC-1. Astrocytes were present in both wt-iPS-dNSC and shi-iPS-dNSC transplanted groups at 4.0% ± 0.5% and 5.3% ± 0.4%, respectively. Neurons were present in both wt-iPS-dNSC and shi-iPS-dNSC transplanted groups with 16.0% ± 0.4% and 17.1% ± 0.2%, respectively. Total neural cell differentiation was calculated by adding the proportions of OLIG2 with GFAP+ and NeuN-positive cells. Approximately 80% of the transplanted cells displayed a neural marker, leaving 20% of cells undefined. This is comparable to our previous results with ES-dNSCs [19, 33] and aNPCs [8, 36] in SCI. Although a subpopulation of cells was unidentified by our markers, there was no sign of neoplastic cell growth in any of the transplanted animals.
Behavioral and Functional Outcomes Improved With wt-iPS-dNSC Treatment

The BMS quantifies hind limb locomotor function in mice. There was a significant improvement in locomotor function with wt-iPS-dNSC compared with both the shi-iPS-dNSC and media control groups (wt vs. shi, \( p = .00008 \); wt vs. media control, \( p = .00001 \)) observed over the experimental period (Fig. 4A). At 8 weeks post-SCI, the wt-iPS-dNSC reached a BMS score of 5.0 ± 0.7, whereas shi-iPS-dNSC and media control animals had scores of 3.3 ± 0.4 and 2.6 ± 0.7, respectively. The score of 5 on the BMS, achieved by the wt-iPS-dNSC group, represents the first point in the scale that takes into consideration coordination and is a key milestone in the locomotor recovery of the injured animals. Sham-injured animals did not display a motor deficit and received the maximum BMS score of 9 during all evaluations postsurgery.

CatWalk analysis began at 3 weeks post-SCI because some locomotor function is required. A subset of animals from all groups successfully traversed the CatWalk platform at 3 weeks, although no statistically significant differences in gait parameters were observed (data not shown). At 8 weeks postinjury, 100% (8 of 8) of wt-iPS-dNSC mice traversed the platform, whereas 75% (6 of 8) of shi-iPS-dNSC mice and 83% (5 of 6) of media control mice completed the task. Statistically significant differences between the wt-iPS-dNSC compared with shi-iPS-dNSC and media control groups were seen in hind limb intensity (wt vs. shi, \( p = .01531 \); wt vs. media control, \( p = .01224 \); Fig. 4B, left), swing speed (wt vs. shi, \( p = .01307 \); wt vs. media control, \( p = .0228 \); Fig. 4B, center),
and stride length \((wt \text{ vs. } shi, p = .00929; wt \text{ vs. media control}, p = .0227; \text{Fig. 4B, right})\) 8 weeks post-SCI. To demonstrate the effects of iPS-dNSCs on action potential conduction in the injured spinal cord, CAPs were recorded using the sucrose gap technique in isolated spinal cord segments ex vivo (Fig. 5A, 5B). The amplitude \((wt \text{ vs. } shi, p = .00395; wt \text{ vs. media control}, p = .00971; \text{Fig. 5C})\) of CAPs was significantly increased in \(wt\)-iPS-dNSC-implanted mice compared with \(shi\)-iPS-dNSC- and media-injected mice.

The possible risk of increased neuropathic pain with cell-based treatment is a potential concern [16]. To examine this, mechanical and thermal allodynia was assessed. There was no difference at any examined time point between experimental groups in responses to von Frey filament application to the plantar surface of hind paw (supplemental online Fig. 4A) or latency times for tail removal from a focal heat source (supplemental online Fig. 4B). These data indicate that the application of these cell-based therapies does not exacerbate neuropathic pain in our model of SCI.

### iPS-dNSC Transplantation Affects Spinal Cord Histological Parameters Following SCI

Histological differences between the \(wt\)-iPS-dNSC and \(shi\)-iPS-dNSC with media injections were evaluated using H&E/LFB staining. Representative stained sections for each group at the injury epicenter and 1,440 \(\mu\)m caudal and rostral to the epicenter demonstrate the key histological difference in spinal cord parameters following SCI (Fig. 6A). The injury epicenter was determined as the tissue section with the greatest damage noted by dense networks of lesion tissue and clusters of small basophilic cells. The total cord area quantification showed that both iPS-dNSC lines preserved overall tissue compared with the cell-free control, in which a decrease in cross-sectional area with a compressed atrophic shape was seen \((wt \text{ vs. media}, p = .00001; shi \text{ vs. media control}, p = .00002; \text{Fig. 6B})\). Luxol Fast Blue staining identifies the white matter region of the spinal cord by dying the lipoprotein-rich myelin sheath and, as such, can be used a surrogate marker of myelination status. Quantification of LFB staining showed that a significantly greater amount of white matter was present throughout the SCI tissue with \(wt\)-iPS-dNSC treatment compared with either \(shi\)-iPS-dNSC or controls \((wt \text{ vs. } shi, p < .00001; wt \text{ vs. media control}, p < .00001; \text{Fig. 6C})\). It should be noted that \(shi\)-iPS-dNSC treatment showed significantly more white matter compared with media-only control \((shi \text{ vs. media}, p = .00015; \text{Fig. 6C})\), likely a result of the overall preserved tissue observed in this group. Furthermore, there was no evidence of tumor formation in any mice receiving either iPS-dNSC treatment during histological analysis, supporting the finding reported above in the immunohistochemical analysis.

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**Figure 4.** Improved outcomes in locomotor testing following SCI with \(wt\)-iPS-dNSC transplantation compared with \(shi\)-iPS-dNSC and media controls were observed. (A): Following SCI, mice that received transplantation of \(wt\)-iPS-dNSCs performed significantly better than \(shi\)-iPS-dNSC and cell-free media controls in open field analysis of hind limb locomotor function, evaluated using the BMS. (B): For CatWalk analysis of gait, the animals must display some stepping ability. At 8 weeks postinjury, 100\% (8 of 8) of \(wt\)-iPS-dNSC mice could traverse the platform, whereas 75\% (6 of 8) of \(shi\)-iPS-dNSC mice and 83\% (5 of 6) of media control mice completed the task. \(wt\)-iPS-dNSC-treated mice performed significantly better than \(shi\)-iPS-dNSC and media control animals when evaluating hind limb intensity (left), stride length (center), and swing speed (right) among those animals able to complete the CatWalk trial. The data represent means ± SEM. *, \(p < .05\) two-way analysis of variance (ANOVA) with Fisher’s least significant difference (LSD) post hoc analysis for BMS comparison and one-way ANOVA with Fisher’s LSD post hoc analysis for CatWalk comparison \((wt, n = 8; shi, n = 8; \text{media, } n = 6)\). Abbreviations: BMS, Basso Mouse Scale; dNSC, derived neural stem cell; iPS, induced pluripotent stem; Max, maximum; SCI, spinal cord injury.
wt-iPS-dNSC Engrafted Tissue Displayed Evidence of Exogenous Myelination

To further examine the nature of exogenous myelination by wt-iPS-dNSCs, additional experimental outputs were analyzed. Because shi-iPS-dNSCs were shown not to myelinate in the Shiverer spinal cord, these additional measures were not evaluated for that group. To assess whether oligodendrocytes derived from wt-iPS-dNSCs contributed to myelin formation in injured spinal cords, transplanted spinal cord tissue sections were stained for MBP, NF200, and DAPI. Exogenous GFP labeling was colocalized with MBP associated with axons (Fig. 7A). EM showed compacted multilayer myelin along with peroxidase deposition, indicating an exogenous origin (Fig. 7B). Lastly, contribution to nodal architecture was examined by immune labeling for the paranodal proteins CASPR and Kv1.2. The localization of the proteins is disrupted as a result of demyelination during SCI (supplemental online Fig. 5). The wt-iPS-dNSCs are shown to localize with the nodal proteins and display proper nodal channel distribution (Fig. 7C, 7D).

DISCUSSION

Key results of this study include the successful use of iPS cell-derived NSCs to achieve functional recovery following SCI, while demonstrating clear evidence that graft-derived remyelination is the principal mechanism mediating this recovery based on loss of function experiments comparing shi- with wt-iPS-dNSCs.

The transplanted cells survive and integrate into the injured spinal tissue without signs of tumor formation. Our laboratory has previous experience examining the neoplastic growth of aberrant cells following pluripotent stem cell-derived NSC spinal transplantation [19]. The integration pattern of the wt- and shi-iPS-dNSCs is consistent with previous findings using aNPCs, as well ES-dNSCs, and no qualitative signs of teratoma formation were displayed [8, 19]. However, an 8-week experimental time frame may be too brief to fully determine tumorigenicity of a cell-based therapy for SCI, and extended investigation into safety will be required for any cell treatment prior to clinical applications.

Our data strongly suggest that remyelination is the key process by which NSCs restore function in thoracic SCI. This result is supported by the pathology of SCI, particularly the selective vulnerability of oligodendrocytes and white matter loss during the propagation of the secondary injury [5, 6]. The thoracic region of the spinal cord, as opposed to the cervical or lumbar segments, is more suitable for cell-based treatment that focuses on a myelination strategy because of the prevalence of white matter injury and a lack of correlation between functional deficit and neuronal loss [8, 37]. Furthermore, demyelinated axons persist for a considerable time following SCI in both animal models [38] and human patients [39], creating an extensive therapeutic window, provided inflammation and scarring is addressed. Our laboratory and others have demonstrated that NSCs, from adult tissue or pluripotent sources, transplanted following SCI differentiate to oligodendrocytes, exogenously myelinate and provide functional improvements [8–10, 19]. iPS-NSCs, pre-evaluated for safety,
from mouse and human sources have been studied for SCI. Tsuji et al. [40] demonstrated that NSCs from mouse iPS cells improved locomotor function, preserved white matter and exogenous myelination, and enhanced serotonergic innervation of the dorsal cord in immune-deficient NOD/SCID mice with lower thoracic level contusion injury. Nori et al. [41] showed that NSCs from human iPS cells confer a benefit, also in the immune-compromised contusion model. Both remyelination and axonal growth were affected by NSC treatment compared with controls [41]. Although these studies allude to remyelination as a key mechanism of repair by pluripotent cell-derived NSCs, the predominant difference is the source of NSCs used. This study is the first to show that remyelination is a key mechanism of repair by pluripotent cell-derived NSCs. The use of IPS-NSCs allows for a near limitless supply and the possibility of patient-specific autologous grafts, potentially giving IPS-NSCs a clinical advantage. Our SCI models use a modified aneurysm clip to deliver injury, compared with the impactor contusion model used by Yasuda et al. Both are valid models of SCI, but only the clip-compression model combines the contusion injury associated with the closing of the clip with a sustained compression injury, creating a clinically relevant SCI model that recapitulates many aspects of human SCI pathology [43, 44]. Furthermore, we used pharmacological immune suppression beginning 5 days following SCI in an immune-competent mouse, as opposed to genetically modified immune-deficient NOD/SCID mice. Crucial elements of SCI progression may require a functional immune system. Although this may also

Figure 6. Both wt- and shi-iPS-dNSC groups had preserved spinal cord tissue, whereas the wt-iPS-dNSC group had significantly more white matter than controls. (A): Representative histological images with hematoxylin and eosin/Luxol Fast Blue-stained spinal cord cross-sections for each group at the injury epicenter and 1,440 μm rostral and caudal to the SCI epicenter. (B): Quantification of total cord area showed that wt-iPS-dNSC and shi-iPS-dNSC treatment preserved cord size following SCI compared media transplantation control. (C): Histological evaluation of white matter showed a significantly greater area in wt-iPS-dNSC treatment group compared with both shi-iPS-dNSC and media-treated animals. Significantly greater white matter preservation was observed in shi-iPS-dNSC group compared with media-only controls. The data represent means ± SEM. *p < .05 two-way analysis of variance with Fisher’s least significant difference post hoc for area analysis (n = 6). Abbreviations: dNSC, derived neural stem cell; iPS, induced pluripotent stem.
be true with pharmacologically suppressed animals, this condition more closely resembles clinical cases, particularly those in which autologous transplantation is not possible. Our current study is a logical extension and builds on the important work of Yasuda et al. by expanding the role of remyelination to pluripotent sourced cells in a different, clinically translatable model of SCI.

Cell-based therapies designed to replace neuronal loss could have therapeutic utility in SCIs. This may be more relevant in the cervical or lumbar SCI, in which neuronal loss is more associated with functional deficits and interneurons of the central pattern generator (CPGs) are located. Many of these types of treatments have been developed without convincing evidence of functional recovery (reviewed by Bareyre [45]). Researchers have shown that neuronal restricted cells are not well tolerated in the injured spinal cord [46, 47]. Neuronal restricted cells cotransplanted with glial restricted cells have improved survival, integration, and outcome measures [48, 49]. However, delineation of the mechanism of action of transplanted neuronal and glial cells remains to be explored.

Replacing lost cells following SCI using an exogenous source is not the only mechanism through which transplanted NSCs could be eliciting a benefit. Other possible mechanisms exist and include creating a permissive environment for endogenous repair and regeneration, modifying the inflammatory response, and protecting the vascular integrity of the blood-spinal cord barrier. The contribution of each of these mechanisms is poorly understood. Cell-based treatments for SCI that involve non-neural cells pose a compelling argument that direct cell replacement is not required for a beneficial effect following SCI. For example, bone marrow stromal cells have been used following SCI and produced functional improvement; the transplanted cells could be acting as a substrate for axonal growth and/or secreting neurotrophins to alter the SCI environment [50, 51]. However, Cummings et al. [18] demonstrated an improvement with hCNS-NSCs following mouse thoracic SCI that was lost upon ablation of the transplanted cells. These data suggest that the integration and function of the exogenous cells supersede their neuroprotective actions. We showed that the NSCs from both iPS cell sources expressed neurotrophic genes at similar levels. It was noted that key neurotrophins such Bdgf [52], Pdgfα [53], and Vegfα [54], which have been implicated in aspects of remyelination and angiogenesis, are up-regulated. This could suggest a trophic support mechanism, but because no difference was seen between wt- and shi-iPS-dNSCs, we can state that, in this situation, remyelination predominated over trophic support.

The nonmyelinating Shiverer iPS-dNSCs did not cause an improvement in locomotor function but did have a positive effect on the spinal cord following injury. Both wt- and shi-iPS-dNSCs preserved total spinal tissue as compared with the compressed atrophied tissue of the cell-free control. Although the wt-iPS-dNSC group had a greater amount of white matter compared with the other groups, the shi-iPS-dNSC-implanted animals did have

Figure 7. wt-induced pluripotent stem (iPS)-derived neural stem cells (dNSCs) display evidence of exogenous remyelination and contribute to proper nodal architecture. (A): Immunofluorescence images from longitudinal spinal cord sections show GFP+ wt-iPS-dNSC expressing MBP that associates with NF200+ axons. Scale bars = 15 μm (upper) and 3 μm (lower). (B): Transmission electron microscopy of spinal tissue shows exogenous, multilayered myelination of host axons. Arrows denote peroxidase reaction product. Scale bars = 2 μm and 100 nm, respectively. (C, D): wt-iPS-dNSCs are shown to contribute to normal nodal architecture as seen by GFP colabeling CASPR and Kv1.2. Scale bars = 15 μm. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; MBP, myelin basic protein.
significantly more white matter than the cell-free controls. This indicates that elements of neuroprotection are involved in the benefits of dNSC transplantation but are insufficient to improve functional outcome. These data are in accordance with the abovementioned study by Yasuda et al. [34], in which similar spinal cord histology results and functional outcomes were observed. Furthermore, this is supported by a study that examined gray matter injury at thoracic and lumbar levels. Equivalent gray matter excitotoxic insults had drastic effects in the lumbar cord and only a slight deficit in the thoracic cord [37]. This confirmed the importance of white matter preservation/regeneration in the thoracic cord. Because the effect of white matter injury in the thoracic spinal cord overshadows the effect of gray matter insult, it is not surprising that overall preservation of neural tissue with limited white matter sparing failed to elicit a functional improvement in our study.

A key milestone in recovery following SCI is coordinated stepping. We analyzed coordination in open field testing, as well as in the CatWalk analysis of gait. By 8 weeks post-SCI, the wt-iPS-dNSC-treated group displayed considerable coordination, whereas the shi-iPS-dNSC and cell-free controls demonstrated only modest improvement. Hind limb coordination is often associated with the CPG, made up of interneurons in the lumbar cord that typically play a role in hind limb coordination. However, in our study, coordination was only improved significantly in the wt-iPS-dNSC-treated group, which suggests that other mechanisms are involved in hind limb coordination.

CONCLUSION

This study provides compelling data that iPS-dNSCs act to restore function following SCI predominantly by remyelination rather than other proposed mechanisms. This does not preclude value in designing therapies that involve replacing lost neurons or promoting endogenous repair, because successful treatment of SCI will likely require combinatorial strategies that are targeted to the nature and level of injury.

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AUTHOR CONTRIBUTIONS

R.P.S.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; R.A.M., L.L., C.S., and M.M.: collection and assembly of data, data analysis and interpretation; A.N.: conception and design, data analysis and interpretation, final approval of manuscript; M.G.F.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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