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Role of Neuregulin-1/ErbB Signaling in Stem Cell Therapy for Spinal Cord Injury-Induced Chronic Neuropathic Pain

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Abstract

Chronic neuropathic pain is a common and debilitating consequence of spinal cord injury (SCI). In a rat contusion injury model, we observed that chronic neuropathic pain is present on day 7 after SCI and persists for the entire 56-day observation period. However, currently available pain therapies are inadequate for SCI-induced neuropathic pain. In this study, we show that spinal transplantation of mouse embryonic stem cell-derived oligodendrocyte progenitor cells (OPCs) enhances remyelination in the injured spinal cord and reduces SCI-induced chronic neuropathic pain. Moreover, we found that SCI reduces the protein level of neuregulin-1 and ErbB4 in the injured spinal cord and that OPC transplantation enhances the spinal expression of both proteins after SCI. Finally, intrathecal injection of neuregulin-1 small interfering RNA, but not the control nontarget RNA, diminishes OPC transplantation-produced remyelination and reverses the antinociceptive effect of OPC transplantation. Our findings suggest that the transplantation of embryonic stem cell-derived OPCs is an appropriate therapeutic intervention for treatment of SCI-induced chronic neuropathic pain, and that neuregulin-1/ErbB signaling plays an important role in central remyelination under pathological conditions and contributes to the alleviation of such pain.

Keywords

Spinal cord injury; Chronic neuropathic pain; Stem cell transplantation; Neuregulin-1/ErbB signaling

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Disclosure of Potential Conflicts of Interest
The authors indicate no potential conflicts of interest.

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Introduction

Spinal cord injury (SCI) is a major cause of disability in all industrialized societies. SCI can induce chronic neuropathic pain that is dependent on the nature of the lesion, neurological structures damaged, and secondary pathophysiological changes of surviving tissues [1]. Approximately two-thirds of persons who have sustained SCI experience clinically significant pain after injury, and of those, one-third have severe pain [2, 3]. The development of chronic neuropathic pain after SCI can increase with time and is often refractory to conventional treatment approaches [4]. SCI-induced chronic neuropathic pain is divided into three types: at-level pain (pain within the body segments innervated by spinal cord segments at the level of the injury), below-level pain (pain within body segments caudal to the level at which the spinal cord was injured), and above-level pain (pain within body segments rostral to the level at which the spinal cord was injured) [5].

Grafting of stem cells is a promising therapy for central nervous system (CNS) diseases, including neurodegeneration, ischemia, and SCI [6–9]. Previous studies have demonstrated that mouse or human embryonic stem (ES) cells can differentiate into oligodendrocytes and produce myelination after spinal transplantation [10–13], and that transplantation of ES cells promotes functional recovery after SCI [6]. Thus, it is possible that spinal transplantation of ES cell-derived oligodendrocyte progenitor cells (OPCs) could be used to enhance myelination in the injured spinal cord and treat SCI-induced chronic neuropathic pain.

Schwann cells in the peripheral nervous system and oligodendrocytes in the CNS produce myelin sheaths around axons. Neuregulin-1 (NRG1), a specific isoform of the growth factor [14], is expressed on the surface of axons, binds to ErbB receptor tyrosine kinases expressed by Schwann cells, and serves as a trigger molecule for myelination. Thus, the strength of NRG1/ErbB signaling determines whether Schwann cells produce myelin and how much myelin is produced. Because oligodendrocytes also respond to NRG1 [15], it is possible that this sort of NRG1/ErbB signaling mechanism exists during oligodendrocyte-mediated myelination in the CNS.

NRG1 exerts its effect by binding to either ErbB3 or ErbB4; each receptor then heterodimerizes with ErbB2, which cannot bind NRG1 directly but has an active kinase domain. Schwann cells principally express ErbB2 and ErbB3, but oligodendrocytes express all three ErbB receptors in a developmentally regulated manner [16, 17]. Transgenic mice that express a dominant-negative ErbB receptor in Schwann cells exhibit axon dysmyelination and hypersensitivity to mechanical stimuli, suggesting that the disruption of NRG1/ErbB signaling may contribute to the pathogenesis of peripheral dysmyelination and neuropathic pain [18]. Although NRG1/ErbB signaling appears to serve distinct functions in myelination of the peripheral nervous system and CNS during neural development [19], it is unclear whether this signaling is involved in remyelination under pathological conditions, such as during stem cell transplantation-produced remyelination after SCI.

In this study, we determined whether spinal transplantation of ES cell-derived OPCs can alleviate SCI-induced chronic neuropathic pain and investigated the role of NRG1/ErbB signaling in the molecular mechanism underlying the effect of OPC transplantation.

Materials and Methods

Differentiation of Mouse ES cells

The B5 mouse green fluorescent protein (GFP)-labeled ES cells (Samuel Lunenfeld Research Institute, Canada) were differentiated via the 4–/4+ retinoic acid (RA) protocol [6, 11, 20]. The ES cells were grown in ES cell induction medium (Dulbecco’s modified

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Eagle's medium [DMEM], 10% newborn calf serum, 10% fetal bovine serum, and 10% nucleosides) for 4 days and in ES cell induction medium plus RA (all-trans-RA, 500 nM) for 4 additional days [11, 20, 21]. Induced ES cells formed floating clusters termed embryoid bodies. To produce OPCs, we partially dissociated 4–/4+ stage embryoid bodies in 0.25% trypsin with EDTA for 5 minutes at 37°C and plated them onto poly-D-lysine/laminin-coated plates. The cells were grown in modified serum-free Sato medium (DMEM supplemented with bovine serum albumin [100 μg/ml], pyruvate [1 mM], progesterone [60 ng/ml], putrescine [16 μg/ml], insulin [10 μg/ml], transferrin [100 μg/ml], sodium selenite [40 ng/ml], and *N*-acetyl cysteine [63 μg/ml]) [11] with basic fibro-blast growth factor (20 ng/ml) and platelet-derived growth factor (PDGF) (20 ng/ml). The cells were allowed to differentiate for 7–9 days at 37°C in a humidified atmosphere with 5% CO_2 and 95% air; then OPCs were partially dissociated and resuspended in Sato medium for transplantation.

Contusion SCI Model

Contusion SCI was carried out with a weight-drop device (NYU model) as reported previously [22]. Female Long-Evans rats (250 ± 25 g) were anesthetized with ketamine-medetomidine cocktail (75:0.5 mg/kg), and a dorsal laminectomy was performed. A midline incision was made, and the paravertebral muscles were dissected to expose the spinal column at the level of T8–T11. Animals were stabilized in a spinal frame by clamping T8 and T10 spinous processes. The T9 spinous process and dorsal lamini were removed. The dorsal surface of T9 spinal cord with intact dura was exposed, and a 10-g weight (2.5 mm in diameter) was dropped onto it from a height of 6.25 mm. For sham surgery, animals underwent laminectomy but no impact injury. The muscles were closed in layers and the skin was stapled. The animals were administered 0.5 ml of atipamezole (i.m.) and were kept in a 37°C incubator for recovery from anesthesia. They were hydrated with 10 ml of saline (s.c.) and 0.12 mg/kg enrofloxacin (i.m.) for 3 consecutive days. Bladders of the rats were expressed manually three times daily until reflex bladder emptying was established. All surgical interventions and postoperative animal care were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care and Use Committee of Johns Hopkins University.

Spinal Transplantation of OPCs

On day 29 after SCI, the rats were anesthetized as described above, and the laminectomy site was re-exposed. A 10-μl Hamilton syringe with a silicone-coated pulled-glass pipette (diameter: 60–70 μm) was used for cell injection [6, 11]. The glass pipette was lowered into the epicenter of the injured spinal cord (1.1 mm in depth) with a Kopf stereotaxic microinjector, and 5 μl of the ES cell suspension (2×10^5 cells per microliter) or medium was injected at a rate of 0.25 μl/min. After injection, the glass pipette was left in place for 5 minutes and then withdrawn. Animals were given 10 mg/kg cyclosporine (i.m.) 1 day before transplantation and then daily until the end of the study.

Immunofluorescence Staining

For immunofluorescence staining in vitro, cultured ES cells were collected after 18 days of differentiation. The cells were fixed for 30 minutes in 4% paraformaldehyde in phosphate buffered saline (PBS). Next, the fixed cells were treated with 0.2% Triton X-100 and 10% goat serum in PBS for 1 hour and then incubated at 4°C overnight with anti-NG2 or anti-PDGFR*α* primary antibody. The secondary Cy3-conjugated flu-orescent antibody was applied for 1 hour at room temperature. The cell nuclei were stained with Hoechst 33342. For immunofluorescence staining in vivo, animals were sacrificed by trans-cardial perfusion with 200 ml of saline followed by 400 ml of 4% paraformadehyde (in 0.1 M PBS) on day 56 after SCI. Then spinal cords were removed, postfixed for 2 hours, transferred to 20%

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sucrose at 4°C for 48 hours, and embedded in Tissue-Tek optimal cutting temperature medium. Frozen horizontal sections (20 μm) were cut with a cryostat. Selected sections were rinsed three times for 10 minutes each in PBS. After 60 minutes in 10% normal goat serum and 0.1% Triton X-100, sections were incubated at 4°C for 48 hours in the following primary antibody pairs: (a) mouse anti-GFP (1:200, Molecular Probes, Eugene, OR, http://probes.invitrogen.com) and rabbit anti-NG2 (1:200, Chemicon, Temecula, CA, http://www.chemicon.com); (b) rabbit anti-GFP (1:300, Molecular Probes) and mouse anti-APC-CC1 (1:400, Oncogene); (c) mouse anti-GFP and rabbit anti-GFAP (1:400, ImmunoStar); (d) mouse anti-GFP and rabbit anti-NeuN (1:400, Chemicon). Sections were rinsed three times for 10 minutes each and then incubated with Alexa 488- and Cy3-conjugated secondary antibodies for 1 hour to double stain different phenotypes of GFP-positive stem cells. Next, the sections were incubated in 0.5% Hoechst 33342 for 15 minutes, partially dried, and coverslipped. The slides were observed under a fluorescence microscope. Alexa 488-labeled GFP-positive cells (green) represent transplanted progenitor cells. Immunostained cells were counted with StereoInvestigator software. Negative control sections were treated the same but without exposure to primary antibodies.

Myelination Analysis

Rats were perfused with saline and paraformaldehyde as described above. Spinal tissues were dissected into 2-mm-long blocks and postfixed with 4% glutaraldehyde for 72 hours. The blocks were exposed to 1% OsO_4 for 1 hour, stained in 0.5% uranyl acetate overnight, and dehydrated in a series of alcohols (15 minutes each) followed by propylene oxide for 3 hours. After being infiltrated with a 1:1 mixture of propylene oxide and EMBed-812 (Electron Microscopy Sciences, Hatfield, PA, www.emsdiasum.com/microscopy) for 3 hours, the blocks were embedded with the embedding resin at 60°C overnight. The coronal sections were cut on a microtome at 1 μm thickness and stained with toluidine blue [23]. Next, the outlines of whole spinal cord and toluidine blue-stained white matter area were measured with Neural Lucida software. The ratio of myelinated white matter to whole spinal cord area was calculated.

Pain Behavioral Testing

Mechanical allodynia in the rats was determined by applying a series of calibrated von Frey filaments to the glabrous surface of the hindpaws. Before being tested, animals were acclimatized to the testing area for 30 minutes. The von Frey filaments (0.41–15.8 g) were applied with enough force to cause buckling of the filament, and a modification of the up-down method of Dixon [24] was used to determine the value at which paw withdrawal occurred 50% of the time [25]. This value was interpreted as paw withdrawal threshold.

Western Blotting

After SCI or SCI plus other treatment, the rats were killed by an overdose of isoflurane, and fresh spinal cord tissues from each group were harvested for Western blotting. The primary antibodies anti-NRG1 and anti-ErbB4 were used to detect the expression of NRG1 and ErbB4, respectively, in the injured spinal cord. β-Actin served as a loading control. Optical density values were measured for each band by densitometry. The optical density values were normalized to that of β-actin (a loading control).

Intrathecal Injection of NRG1 Small Interfering RNA (siRNA) with a Mini-Pump

The NRG1 siRNA for rat NRG1 (GenBank accession number: NM_031588) was designed and synthesized by Dharmacon (Thermo Scientific, Lafayette, CO, www.thermoscientific.com). The NRG1 siRNA pool includes: (a) AGUAAUGGGCACACGGAGA; (b) GGAACGAGCUGAAC CGCAA; (c)

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CCACCAAGGCUACGGGAGA; (d) UCAGAA AGCAACUCCGUAA. A scrambled nontarget RNA sequence was used as a mismatch control. A BLAST search of all the nucleotide sequences in the GenBank database revealed no substantial homology with other genes. The NRG1 siRNA and mismatch control RNA were prepared immediately before administration by mixing the RNA solution with the transfection agent i-Fect in a ratio of 1:4 (w:v) [26]. Mini-osmotic pumps (Model 2004, Alzet, Cupertino, CA, www.alset.com) implanted into the rats according to the manufacturer's instructions were used to inject either NRG1 siRNA or control nontarget RNA intrathecally for 28 days starting from day 29 after SCI. The rats were sacrificed on day 56 after SCI, and spinal cord tissues were harvested. Western blot analysis was used to confirm siRNA-induced NRG1 knockdown in the spinal cord.

Statistical Analysis

All data were expressed as means ± SEM. The expression of NRG1 and ErbB4 in the spinal cord between sham and SCI groups or between "SCI+medium" and "SCI+OPCs" groups in Figure 5 was analyzed by paired t test. Statistical analysis for other data was performed with one-way or two-way ANOVA followed by the Student-Newman-Keuls method as the post hoc test. ANOVA values and degrees of freedom were shown in Supporting Information Table S1. $p < .05$ was the minimum required to establish statistical significance.

Results

Mouse ES Cells Differentiate into OPCs In Vitro

The B5 mouse GFP-labeled ES cells were differentiated via the 4−/4+ RA protocol [6, 11, 20]. To examine OPC differentiation in culture, we harvested the cells after differentiation for 18 days in vitro and then conducted immunocytochemical analysis. Mouse ES cell-derived, GFP-labeled OPCs exhibited a bipolar morphology (Fig. 1A–1C), and more than 90% of GFP-labeled ES cells expressed OPC markers PDGFRα and NG2 (Fig. 1D–1I). Only a few cells that did not label with oligodendroglial markers were positive for glial fibrillary acidic protein (GFAP, a marker for astrocytes, Fig. 1J–1L).

Spinally Transplanted OPCs Differentiate into Oligodendrocytes

To determine whether spinally transplanted OPCs continually differentiate into oligodendrocytes that can remyelinate axons, we harvested spinal cord tissues 4 weeks after OPC transplantation and performed immunofluorescence staining. We found that approximately 10% of transplanted OPCs (GFP-positive) survived for 4 weeks in the injured spinal cord. Of those, 44.5% ± 5.1% had differentiated into mature oligodendrocytes (adenomatous polyposis coli [APC]-positive, Fig. 2). In addition, 32.8% ± 3.9% of the transplanted OPCs expressed NG2, and only a few transplanted cells (4.4% ± 3.5%) were labeled with the astrocyte marker GFAP. Transplanted cells that labeled with the neuron marker NeuN were very sparse (< 0.1%).

OPC Transplantation Inhibits SCI-Induced Chronic Neuropathic Pain

We measured paw withdrawal thresholds of rat hindpaws weekly to determine the development of below-level pain after SCI. Mechanical allodynia (decreased paw withdrawal threshold) was present on day 7 after SCI and persisted for the entire 56-day observation period in both hindpaws (Fig. 3A, 3B).

To investigate the effect of OPC transplantation on SCI pain, we injected OPCs (~ 10^6 cells) or culture medium intra-spinally on day 29 after SCI and then assessed pain behavior in the rats once a week. We found that spinal transplantation of ES cell-derived OPCs significantly

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increased paw withdrawal thresholds after SCI in both hindpaws compared to those in medium control group (Fig. 3C, 3D).

OPC Transplantation Enhances Remyelination in the Injured Spinal Cord

To determine whether transplantation of ES cell-derived OPCs affects myelination after SCI, we used toluidine staining to measure myelination in the spinal cord. We found that SCI reduced myelination in the spinal cord, and that grafting of ES cell-derived OPCs significantly enhanced myelination in different segments of the injured spinal cord (Fig. 4A–4F). Statistical analysis showed that SCI reduced the ratio of myelinated white matter area to whole spinal cord, but grafting of ES cell-derived OPCs significantly ameliorated the extent of the decrease (Fig. 4G).

SCI Downregulates Expression of NRG1 and ErbB4 in the Spinal Cord but Transplantation of OPCs Reverses These Effects

To investigate whether NRG1/ErbB signaling is involved in the pathogenesis of SCI-induced chronic neuropathic pain, we used Western blot analysis to determine whether spinal expression of NRG1 and ErbB4 is altered after SCI. We found that SCI markedly reduced the protein levels of NRG1 and ErbB4 in the spinal cord compared to those in the sham group (Fig. 5A, 5B). Next, we wanted to assess whether the analgesic effect of OPC transplantation after SCI is mediated through NRG1/ErbB signaling; therefore, we performed a similar analysis after transplantation of OPCs into the mildly contused spinal cord. We found that OPC transplantation increased spinal NRG1 and ErbB4 expression, counteracting the reduction produced by SCI (Fig. 5C, 5D).

Knockdown of NRG1 Reduces Remyelination and Inhibits the Analgesic Effect of OPC Transplantation on SCI-Induced Chronic Neuropathic Pain

To further investigate the role of NRG1/ErbB signaling in transplanted OPC-mediated remyelination and analgesia, we used NRG1 siRNA to knock down the expression of NRG1 in the spinal cord. Intrathecal injection of the NRG1 siRNA significantly and specifically decreased the expression of spinal NRG1 but had no effect on the expression of spinal ErbB4 (Fig. 6A, 6B). We further found that intrathecal injection of the NRG1 siRNA not only diminished OPC transplantation-induced remyelination (Fig. 6C) but also blocked the antinociceptive effect of OPC transplantation (Fig. 6D, 6E). However, the control nontarget RNA did not alter the OPC effect (Fig. 6). The differences between the SCI group and the “SCI–OPCs+NRG siRNA” group as well as between the “SCI–OPCs+vehicle” group and the “SCI+OPCs+nontarget RNA” group were not significant (Fig. 6C–6E).

Discussion

Demyelination (loss of myelin) and dysmyelination (abnormal myelination) are important contributors to behavioral deficits associated with SCI. Remyelination of the injured spinal cord is one of the key elements for functional recovery from SCI. Oligodendrocyte injury and death play substantial roles in the demyelination and dysmyelination that follow traumatic and ischemic SCI [27, 28]. Thus, stem cell-based oligodendrocyte replacement therapy has become a realistic approach to promote the remyelination process and functional recovery after SCI. In this study, we have demonstrated that spinally transplanted ES cells with oligodendrocyte differentiation can be developed as a therapy for SCI-induced chronic neuropathic pain. Using our culture protocol, more than 90% of ES cells can be differentiated into OPCs before transplantation. In the contusion SCI model, spinally transplanted OPCs can survive, further differentiate into mature oligodendrocytes, and enhance remyelination in the injured spinal cord, thereby inhibiting SCI-induced chronic

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neuropathic pain. Our data suggest that oligodendrocyte-mediated remyelination may contribute to the mechanism by which OPC transplantation alleviates SCI pain.

NRG1/ErbB signaling is necessary for normal myelination and sensory function [15, 29]. This signaling has been shown to regulate morphology and gene expression of myelinating Schwann cells during the process of myelination [18]. In the CNS, NRG1/ErbB signaling has been implicated in a broad range of processes, including neuronal migration, axonal path-finding, and synaptic plasticity [30, 31]. In vitro studies have shown that this signaling affects oligodendrocyte specification, differentiation, myelination, and survival [16, 32]. Given that oligodendrocytes also respond to NRG1 [15], we hypothesized that altered expression of NRG1 and ErbB4 in the injured spinal cord may not only be involved in the pathogenesis of SCI-induced demyelination and consequent chronic neuropathic pain but may also contribute to the OPC transplantation-produced analgesic effect. In our contusion SCI model, we found that SCI decreased the expression of spinal NRG1 and ErbB4 but OPC transplantation reversed the effect of SCI on the expression of the two proteins in the injured spinal cord, indicating that NRG1/ErbB signaling may be involved in the pathogenesis of SCI and contribute to the mechanism underlying the effect of transplanted OPCs on remyelination and SCI-induced neuropathic pain. However, a previous study [19] reported that oligodendrocytes have evolved an NRG1/ErbB-independent mechanism of myelination control during neural development. Thus, it is possible that the role of NRG1/ErbB signaling differs during physiological development and under pathological conditions. By using siRNA knockdown strategy, we further demonstrate that downregulation of NRG1 reduces OPC transplantation-produced remyelination in the injured spinal cord and inhibits the analgesic effect of OPC transplantation after SCI. Our findings suggest that NRG1/ErbB signaling may play an important role in the development of SCI-induced chronic neuropathic pain and the analgesic effect produced by OPC transplantation.

In this study, we use mouse ES cell-derived OPCs to treat SCI-induced chronic neuropathic pain. To develop a potential stem cell therapy for SCI patients, we are currently using human ES cells to produce OPCs for spinal transplantation. It is important to demonstrate that human sources of OPCs can also reduce pain and promote functional recovery after SCI. Further studies will be needed to fine-tune stem cell differentiation before transplantation and optimize stem cell transplantation strategies, thereby providing insights into clinical application of a powerful stem cell therapy for SCI patients.

Conclusion

Our data illustrate that SCI inhibits spinal NRG1/ErbB signaling, reduces myelination in the injured spinal cord, and induces chronic neuropathic pain. Our study also shows that spinal transplantation of mouse ES cell-derived OPCs counteracts the effect of SCI on NRG1 expression, enhances remyelination in the injured spinal cord, and alleviates SCI pain. By knocking down NRG1 using an siRNA strategy, we further revealed that NRG1/ErbB signaling contributes to the effects of transplanted OPCs on remyelination and SCI pain. These findings provide new insight into the molecular mechanism by which stem cell transplantation can treat SCI-induced chronic neuropathic pain.

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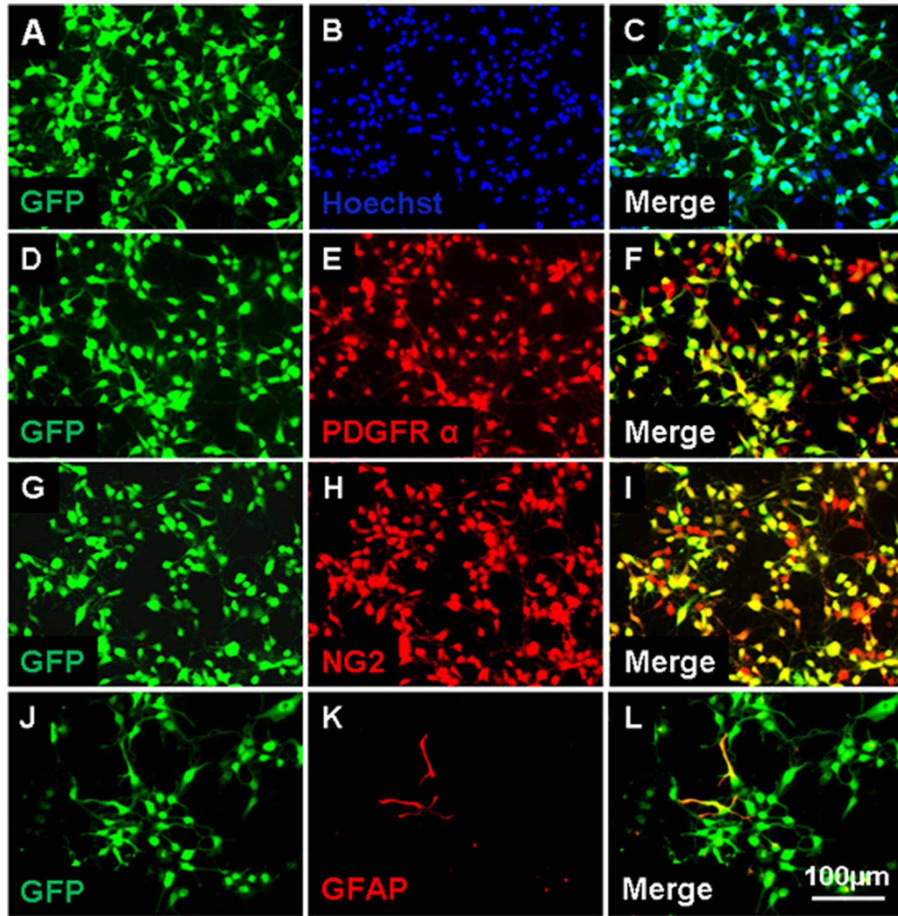

Figure 1.

Mouse embryonic stem (ES) cells (B5 cell line, GFP-labeled) differentiate into oligodendrocyte progenitor cells (OPCs) in vitro. After differentiation for 18 days in culture, mouse ES cell-derived, GFP-labeled OPCs exhibited a bipolar morphology **(A–C)**. Immunofluorescent staining with OPC markers (PDGFRα and NG2) showed that most GFP-labeled OPCs expressed PDGFRα **(D–F)** and NG2 **(G–I)**. Only a few cells expressed GFAP **(J–L)**, a marker for astrocytes. Nuclei were counterstained with Hoechst 33342 (Hoechst). The data shown are representative of three independent experiments. Scale bar = 100 μm. Abbreviations: GFP, green fluorescent protein; GFAP, glial fibrillary acidic protein; NG2, chondroitin sulfate proteoglycan; PDGFRα, platelet derived growth factor receptor α.

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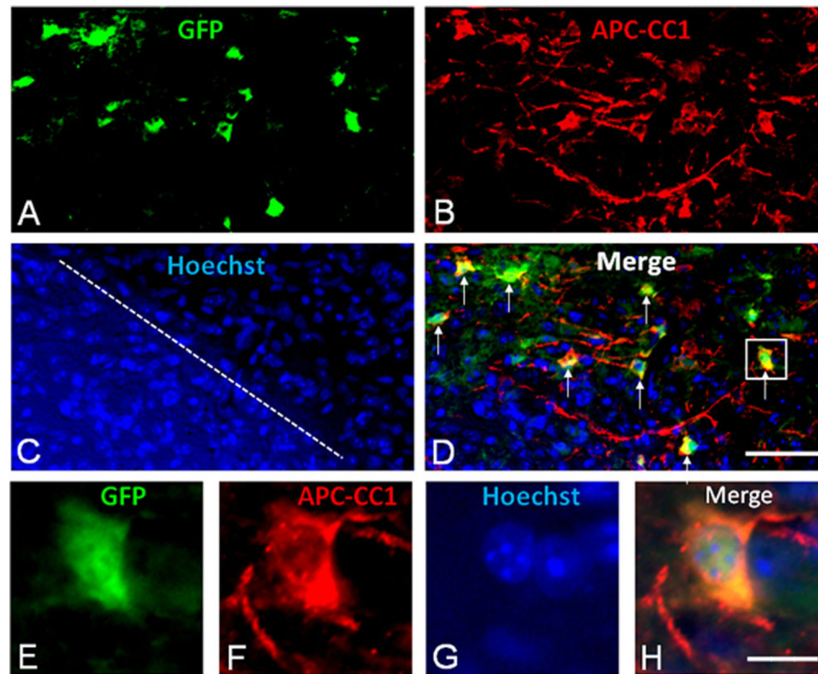

Figure 2.
Spinally transplanted embryonic stem (ES) cell-derived oligodendrocyte progenitor cells (OPCs) are further differentiated into mature oligodendrocytes. Immunofluorescence staining showed that 44.5% of transplanted GFP-positive cells were labeled with APC-CC1, a marker for mature oligodendrocytes. **(A):** GFP-positive cells. **(B):** APC-CC1-positive cells. **(C):** Nuclei stained with Hoechst 33342. The dashed line represents the border between the ES graft (upper right) and the host tissue (lower left). **(D):** Merged image of the photomicrographs in (A), (B), and (C). The arrows indicate cells double-labeled for GFP and APC-CC1. The boxed area in (D) is shown at higher magnification in (E), (F), (G), and (H). Scale bar = 50 μm (D) is for (A), (B), (C), and (D). Scale bar = 5 μm (H) is for (E), (F), (G), and (H). Abbreviations: APC, adenomatous polyposis coli; GFP, green fluorescent protein.

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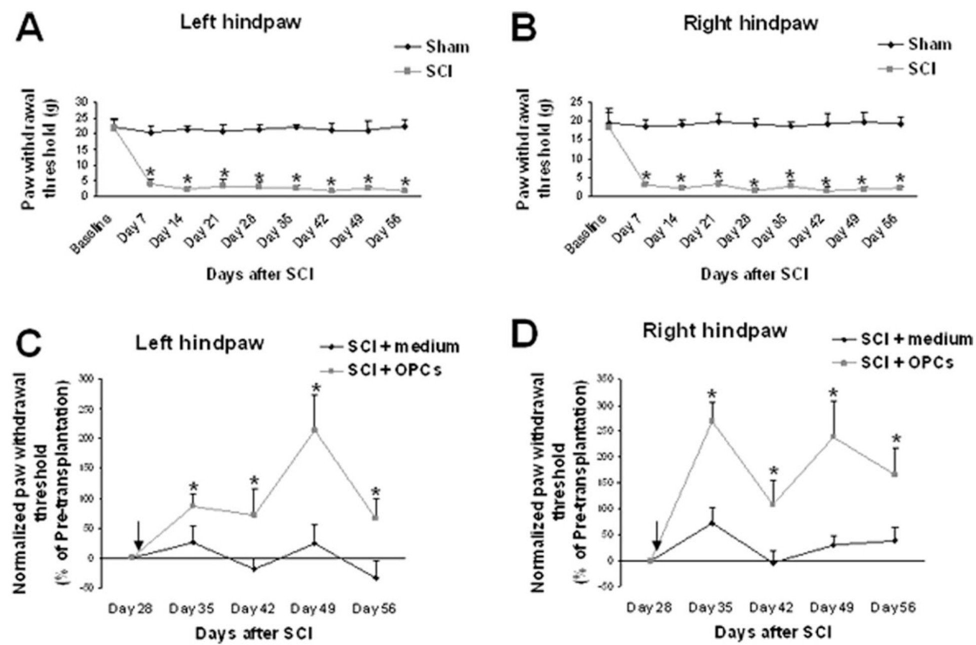

Figure 3.

OPC transplantation inhibits SCI-induced mechanical allodynia. **(A, B):** The rats subjected to SCI showed chronic mechanical allodynia as evidenced by significant reductions in paw withdrawal threshold ($n = 12$, *, $p < .01$ vs. baseline). **(C, D):** Post-transplantation paw withdrawal thresholds were normalized to the pretransplantation threshold recorded on day 28 after SCI. Note that spinal transplantation of mouse embryonic stem cell-derived OPCs increased paw withdrawal threshold compared to that of culture medium-treated rats ($n = 10$; *, $p < .05$ vs. "SCI+medium" group). Arrow (↓) indicates spinal transplantation on day 29 after SCI. Abbreviations: OPC, oligodendrocyte progenitor cells; SCI, spinal cord injury.

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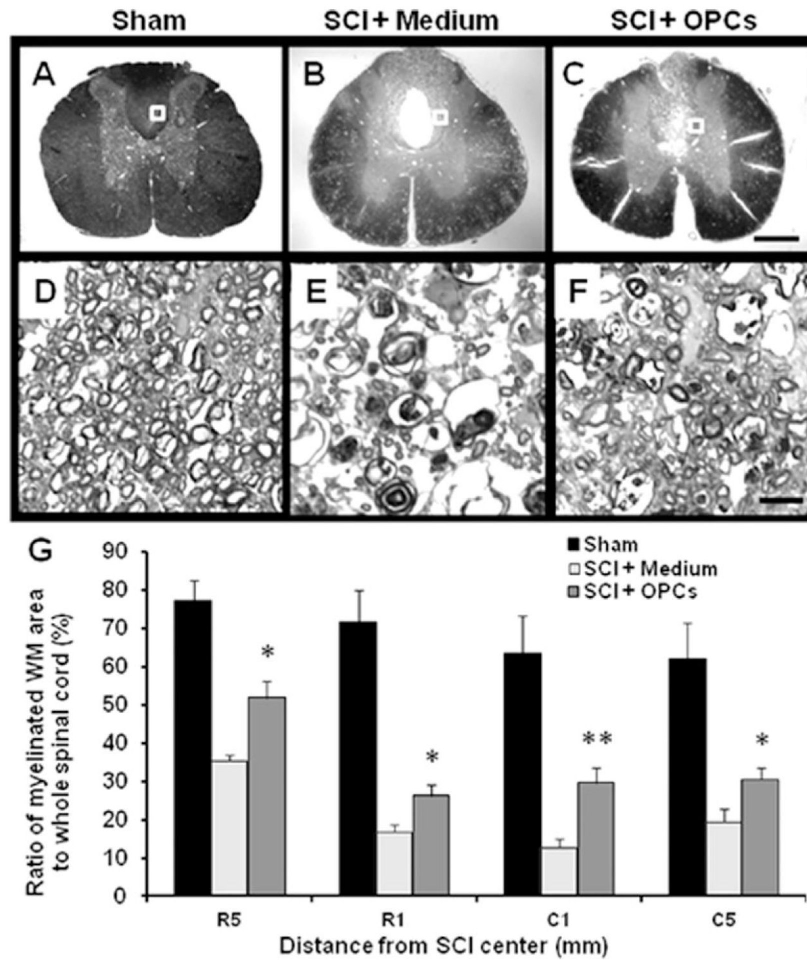

Figure 4.

OPC transplantation enhances remyelination in the injured spinal cord. **(A–C):** Transverse spinal sections (5 mm rostral from injury epicenter; 1 μm thick) from different groups were stained with toluidine blue for myelinated WM. **(D–F):** Higher magnification images of the indicated square areas in (A–C), respectively. **(G):** SCI reduced the ratio of myelinated WM area to whole spinal cord, but grafting of embryonic stem cell-derived OPCs significantly ameliorated the extent of the decrease ($n = 8$). Scale bar = 0.5 mm (C) is for (A), (B), and (C); scale bar = in 10 μm (F) is for (D), (E), and (F). *, $p < .05$; **, $p < .01$ versus "SCI +medium" group. Abbreviations: C caudal; OPC, oligodendrocyte progenitor cell; SCI, spinal cord injury; R, rostral; WM, white matter.

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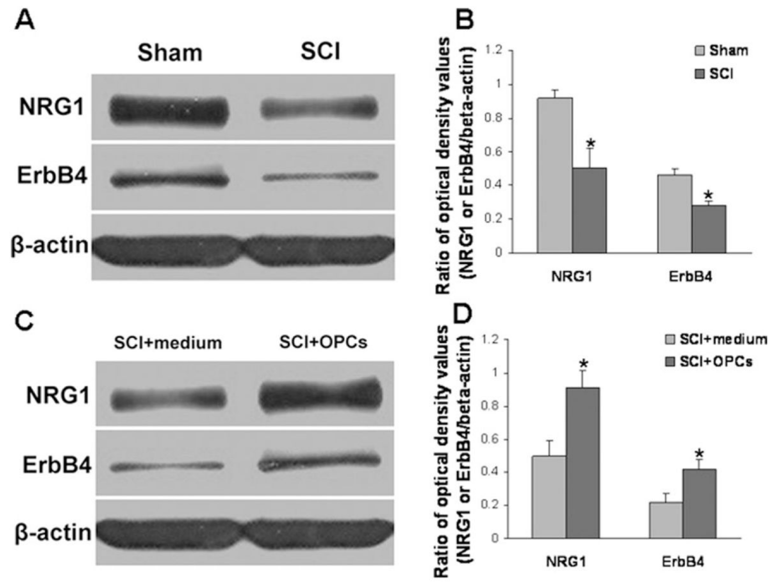

Figure 5.

Spinal expression of NRG1 and ErbB4 is downregulated after SCI, and OPC transplantation counteracts the effect of SCI on NRG1 and ErbB4 expression. **(A, B):** Compared to expression levels in the sham group, SCI markedly reduced the protein level of NRG1 and ErbB4 in the injured spinal cord ($n = 6$, *, $p < .01$ vs. the sham group). **(C, D):** Spinal transplantation of mouse embryonic stem cell-derived OPCs increased the expression of spinal NRG1 and ErbB4 after SCI, counteracting the SCI-induced reduction ($n = 6$, *, $p < .01$ vs. “SCI+medium” group). β-Actin served as a loading control. Abbreviations: NRG1, Neuregulin-1; OPC, oligodendrocyte progenitor cell; SCI, spinal cord injury.

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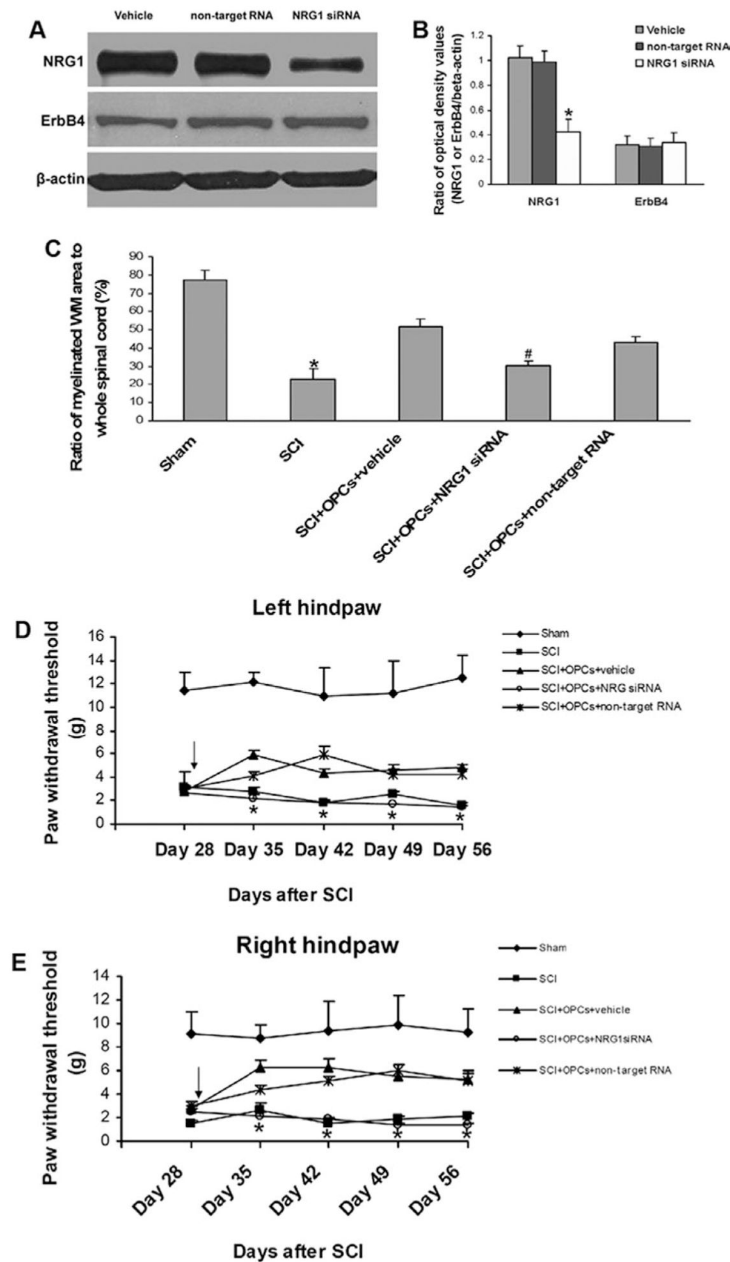

Figure 6.

Intrathecal injection of NRG1 siRNA knocks down NRG1 expression in the spinal cord and inhibits the effect of OPC transplantation on remyelination and SCI pain. **(A, B):** Spinal NRG1 expression was significantly decreased after intrathecal injection of NRG1 siRNA but not after intrathecal injection of control nontarget RNA ($n = 8$, *, $p < .05$ vs. the vehicle group). NRG1 siRNA did not affect the expression of ErbB4, indicating that it was specific for NRG1. **(C–E):** The knockdown of spinal NRG1 not only diminished OPC transplantation-induced remyelination (C, $n = 8$, *, $p < .05$ vs. the sham group; $^{\#}$, $p < .05$ vs. the "SCI+OPCs+vehicle" group) but also blocked the antinociceptive effect of OPC transplantation (D, E, $n = 8$, *, $p < .05$ vs. the "SCI+OPCs+vehicle" group). Note that the differences between the SCI group and the "SCI+OPCs+NRG siRNA" group as well as between the "SCI+OPCs+vehicle" group and the "SCI+OPCs+nontarget RNA" group were

not significant. The arrow (↓) in (D) and (E) indicates spinal transplantation on day 29 after SCI. Abbreviations: NRG1, Neuregulin-1; OPC, oligodendrocyte progenitor cell; siRNA, small interfering RNA; SCI, spinal cord injury; WM, white matter.

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