Prior Treatment with Anti-High Mobility Group Box-1 Antibody Boosts Human Neural Stem Cell Transplantation-Mediated Functional Recovery After Spinal Cord Injury

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Abstract
Together with residual host neurons, transplanted neural stem cell (NSC)-derived neurons play a critical role in reconstructing disrupted neural circuits after spinal cord injury (SCI). Since a large number of tracts are disrupted and the majority of host neurons die around the lesion site as the damage spreads, minimizing this spreading and preserving the lesion site are important for attaining further improvements in reconstruction. High mobility group box-1 (HMGB1) is a damage-associated molecular pattern protein that triggers sterile inflammation after tissue injury. In the ischemic and injured brain, neutralization of HMGB1 with a specific antibody reportedly stabilizes the blood-brain barrier, suppresses inflammatory cytokine expression, and improves functional recovery. Using a SCI model mouse, we here developed a combinatorial treatment for SCI: administering anti-HMGB1 antibody prior to transplantation of NSCs derived from human induced pluripotent stem cells (hiPSC-NSCs) yielded a dramatic improvement in locomotion recovery after SCI. Even anti-HMGB1 antibody treatment alone alleviated blood-spinal cord barrier disruption and edema formation, and increased the number of neurites from spared axons and the survival of host neurons, resulting in functional recovery. However, this recovery was greatly enhanced by the subsequent hiPSC-NSC transplantation, reaching an extent that has never before been reported. We also found that this improved recovery was directly associated with connections established between surviving host neurons and transplant-derived neurons. Taken together, our results highlight combinatorial treatment with anti-HMGB1 antibody and hiPSC-NSC transplantation as a promising novel therapy for SCI.

Significance Statement
Although transplantation of neural stem cells derived from human induced pluripotent stem cells (hiPSC-NSCs) represents a promising approach for the treatment of spinal cord injury (SCI), functional recovery remains far from complete. This study developed a combinatorial treatment for SCI: administering anti-high mobility group box-1 (HMGB1) antibody and then transplanting hiPSC-NSCs yielded a dramatic improvement in locomotion recovery after SCI. Results of this study found that neutralizing HMGB1, which functions as a trigger of inflammatory response to injury, alleviated blood-spinal cord barrier disruption and edema formation, and increased neurites from spared axons and the survival of host neurons. This preservation of the lesion site further enhanced hiPSC-NSC transplantation-mediated functional recovery, which was directly associated with connections established between surviving host neurons and transplant-derived neurons. SCI, but the levels of functional recovery reported to date are limited [1–4]. In addition to known factors, such as the aggressive inflammatory response after injury and the inferior

INTRODUCTION
Neural stem cells derived from human induced pluripotent stem cells (hiPSC-NSC) transplantation holds great promise for the treatment of SCI, but the levels of functional recovery reported to date are limited [1–4]. In addition to known factors, such as the aggressive inflammatory response after injury and the inferior
regenerating ability of neurons in the central nervous system (CNS), the poor preservation of damaged tissue could be one of the reasons for this limitation: in such tissue, survival of host neurons is particularly important [5] because transplant-derived neurons require intact endogenous neurons to transmit signals effectively from the brain to caudal parts through the lesion site [2, 4–7]. These considerations led us to hypothesize that preserving the lesion site should further improve functional recovery induced by transplantation after SCI.

High mobility group box-1 (HMGB1), originally identified as a nonhistone nuclear protein, has been reported to play an important role as a trigger for inflammatory responses to CNS injury [8–12]. Upon injury, HMGB1 is released from the cells in the damaged tissue into the extracellular space, where it disrupts the blood-brain barrier (BBB) by directly inducing morphological changes in pericytes and endothelial cells and the swelling of astrocyte end feet [13], and initiate innate immune responses by binding to cell membrane receptors such as advanced glycation end product (RAGE), Toll-like receptor 2 (TLR2), and TLR4 [8]. We have recently developed an inhibitory monoclonal antibody (mAb) for HMGB1 as a new and effective reagent for the treatment of various CNS disorders such as brain infarction, brain hemorrhage, and brain injury in rodent models [13–16]. These therapeutic effects of anti-HMGB1 mAb are exerted, at least in part, by the prevention of HMGB1-promoted BBB disruption and inflammatory responses; however, the effectiveness of anti-HMGB1 mAb treatment in SCI has yet to be investigated. Here, we report that administration of anti-HMGB1 mAb can help to preserve the lesion site of the spinal cord by suppressing blood-spinal cord barrier (BSCB) disruption, edema progression, and damage spread. We further reveal that preservation of the lesion site by prior treatment with anti-HMGB1 antibody boosts NSC transplantation-mediated functional recovery after injury to a degree that, to the best of our knowledge, has never before been attained. We also show that around the lesion site, anti-HMGB1 mAb treatment enhance the survival of host neurons, which can form synaptic connections with transplant-derived neurons, and that these reconstructed neural networks directly contribute to the dramatic functional recovery.

**Materials and Methods**

**Animals**

All mouse experiments were conducted in accordance with guidelines of the Kyushu University Center for Animal Resources and Development. We used female nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice (8–10 weeks old, weighing 18–22 g, Charles River, Osaka, Japan, http://www.crl.co.jp). Mice were housed under a 12-hour light-dark cycle in a specific pathogen-free facility with controlled temperature and humidity, and allowed free access to food and water. The mice were randomly divided into four groups: non-treatment group, transplantation alone group, anti-HMGB1 treatment alone group, and combination group.

**SCI and Transplantation**

Mice anesthetized with a mixture of 4 mg/kg midazolam, 0.3 mg/kg medetomidine, and 5 mg/kg butorphanol received partial laminectomies and laminectomies at the ninth and tenth thoracic spinal vertebrae, respectively. The dorsal surface of the dura mater was exposed and injury was applied at the Th10 level using an SCI device (70 kdyn to induce moderate SCI [5], Infinite Horizon Impactor; Precision Systems & Instrumentation, Lexington, KY, http://www.presysin.com) as previously described [2, 6]. The muscle and skin were closed in layers. In the anti-HMGB1 mAb treatment alone group and the combination group, the mice were intraperitoneally administered anti-HMGB1 mAb (lgG2a) [14], at a dose of 8 mg/kg, at 5 minutes and 6 hours after the injury. In a series of previous studies with rat brain injury and ischemic models, a total dose of 2 mg/kg in acute-phase treatments has been suggested to be effective and used as a standard [13, 16, 17]. In chronic disease mouse models such as atherosclerosis, about 16 mg/kg (twice weekly for 8 weeks) was used [18]. As for SCI, there have been no reports using anti-HMGB1 antibody that we can refer to, but we wanted to use a somewhat lower dose than the 16 mg/kg used in the previous mouse model, since the antibody is not commercially available and thus in limited supply. Therefore, we first performed a pilot experiment with 8 mg/kg (two injections, at 5 minutes and 6 hours after SCI) and found that this dose gave a better functional recovery compared with the rat lgG2a control monoclonal antibody used at the same dose as the anti-HMGB1 antibody. Although we do not know whether 8 mg/kg is the optimal dose, we decided to use this dose having observed the beneficial effects of the antibody in the mouse SCI model. Since HMGB1 is a ready-made mediator, it should be blocked immediately after its release from cells after injury. Therefore, we injected anti-HMGB1 antibody first at 5 minutes after induction of cerebral stroke and injury in rat models [13, 14, 16]. In the following phase, because HMGB1 is thought to be released from cells around the lesion site, we then injected the antibody at 6 hours after lesion induction in the same rat models. By this two-injection procedure, we could clearly observe beneficial effects of the antibody. Based on these findings, we decided to also apply this twin-injection procedure to the present SCI model. In the non-treatment and transplantation alone groups, control IgG2a mAb [14] was injected at the same dose and time points. The mice were injected with gentamicin (10 mg/kg) intraperitoneally and underwent manual bladder evacuation in the first 3 days after injury. Seven days after injury, in the transplantation alone group, control IgG2a mAb was transplanted with hiPSC-NSCs using a glass micropipette attached to a stereotaxic injector (Narishige, Tokyo, Japan, http://www.narishige.co.jp). The tip of the micropipette was inserted into the injury epicenter in the injured spinal cord, and hiPSC-NSCs (2.5 × 10^5 μl^–1) in 2 μl of culture medium lacking growth factors were injected at a rate of 0.5 μl/minute. In the non-treatment and anti-HMGB1 treatment alone groups, medium without NSCs was injected.

**Cell Culture**

The hiPSC-NSC cell line (AF22) (a generous gift from Dr. A. Smith, Cambridge University) was maintained and used as described previously [2, 19]. AF22 cells were plated onto 0.1 mg/ml poly-L-ornithine (Sigma, St. Louis, MO, http://www.sigmaaldrich.com) and 10 μg/ml laminin (Corning, NY, USA, http://www.corning.com)-coated plates in maintenance medium, which consists of
DMEM/F12 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 2.5 mg/l insulin (Sigma) [20], 100 mg/l transferrin (Sigma), 16 mg/l putrescine (Sigma), 30 nM selenite (Sigma), 20 nM progestosterone (Sigma), 0.1 mg/ml penicillin/streptomycin (Nacalai Tesque, Kyoto, Japan, http://www.nacalai.co.jp), 1 μl/ml B27 (Gibco, Tokyo, Japan, http://www.thermofisher.com), 10 μg/ml basic fibroblast growth factor (bFGF) (PeproTech, Tokyo, Japan, http://www.peprotech.com), and 10 μg/ml epidermal growth factor (EGF) (PeproTech). The cells were passaged at a ratio of 1:3 every third or fourth day using TrypLE (Gibco). AF22 cells were infected with lentiviruses harboring luciferase and green fluorescent protein (GFP) genes connected by an internal ribosomal entry site (IRES), Efp (elongation factor promoter)-luciferase-IRES-GFP [2]. GFP-positive cells were collected using a FACSAria II CellSorter (B.D. Biosciences, San Jose, CA, http://wwwbdbiosciences.com) and used for transplantation [2].

Behavioral Analysis
We evaluated motor function of the hindlimbs for up to 14 weeks after injury. Two people, blinded to the treatment of the mice, examined motor function in an open field using the Basso Mouse Scale (BMS) locomotor rating scale [21]. Mice which showed an incomplete injury phenotype (BMS score ≥ 1) at 6 hours after injury were excluded from this analysis. Hindlimb movements of the mice were captured using a high-definition digital camcorder. We edited these movies and exported movie files using editing software. For footprint analysis [22, 23], the forelimbs and hindlimbs of the mice were dipped in red and blue dye, respectively. A narrow runway (80 cm length and 4 cm width) was lined with white paper for the animal to walk across. Paw rotation was defined as the angle between the axes of the two back paws. Measurements were taken on each side for three consecutive steps and were averaged. Mice that badly dragged their hindlimbs were excluded from these measurements. A grid walking test was performed to evaluate the ability of the mice to locomote over a wire mesh grid (2.5 × 2.5 cm² grid spaces, 35 × 35 cm² total area, 7 cm height) [24]. Only those mice that demonstrated plantar stepping in the open field (BMS score ≥ 4) were tested on the grid. Before testing, mice were trained to walk over the mesh for 3 minutes each day for 3 days. Each mouse was videotaped for 3 minutes while on the grid, and the recording was repeated three times. A mis-step was counted when a hindlimb paw protruded entirely through the grid with all toes and heel extended below the wire surface. Total moving distance was traced, and walking distances without a mis-step were calculated. For each session, the average distance without a mis-step for each mouse was measured from three trials and each value was expressed as a percentage of that in intact mice. n = 12 mice in each treated group, n = 3 mice in intact group.

Electrophysiology
Electrophysiological experiments were performed using a Neuropack S1 MEB-9402 (Nihon Kohden, Tokyo, Japan, http://www.nihonkohden.co.jp) at 12 weeks after transplantation, as previously described [2]. After mice were anesthetized (see above), their skulls were tightly fixed to a stereotaxic apparatus (Narishige). Scalping and two small craniotomies were performed with a drill over the motor cortex area, 2.1 mm posterior to the bregma, 2 mm lateral to the bregma [25]. Needle electrodes were inserted via the holes. The motor cortex was stimulated with 0.2-msec square wave pulses at a constant current of 5 mA. Recording needle electrodes were inserted into a hindlimb. A subcutaneous ground electrode was placed in the tail. Amplitudes of MEP were measured as the trough-to-peak height of the first wave from the onset. Latencies of MEP were measured as the time interval between the end of stimulation and the onset of the first wave. Indicated values are the average of ten experiments from each mouse. n = 7 mice (both hindlimbs) in the nontreatment and transplantation alone groups. n = 6 mice (both hindlimbs) in the anti-HMGBl mAb alone, combination, and intact groups.

Immunohistochemistry
Anesthetized mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Spinal cords were dissected and postfixed overnight in the same fixative at 4°C. The fixed tissues were cryoprotected successively in 10% and 20% sucrose in PBS overnight at 4°C, embedded in OCT compound, and sectioned sagittally at 20 μm on a cryostat. We stained tissue sections with primary antibodies to CD11b (1:200, 5C6, Serotec, Sapporo, Japan, http://www.serotec.co.jp), glial fibrillary acidic protein (GFAP) (1:500, Z0334, DAKO, Carpinteria, CA, http://www.dako.com), 5-hydroxytryptamine (5-HT) (1:200, 1338, ImmunoStar, Hudson, WI, http://immunostar.com), GFP (1:500, GFP-1010, Aves Labs, Tigard, OR, http://www.aveslabs.com), β-III-tubulin (1:500, PRB-435P, Covance, Madison, WI, http://www.covance.com), NeuN (1:500, MAB377, Millipore, Billerica, MA, http://www.millipore.com), human-specific GFAP (hgFAP, 1:500, AB-123-U-050, StemCells Science, Newark, CA, http://www.stemcellsci.com), human-specific synaptophysin (hSyn, 1:200, MAB332, Millipore), postsynaptic density 95 (PSD95, 1:200, 1350, ImmunoStar), and α-tubulin (1:500, T5168, Sigma, St. Louis, MO, http://www.sigma-aldrich.com). Sections were incubated with Alexa Fluor-conjugated secondary antibodies (1:500, Biotin, Fremont, CA, http://www.biotin.com). Nuclei were stained with Hoechst (bis-benzimide H33258 fluorochrome trihydrochloride, Nacalai Tesque). An In Situ Cell Death Detection Kit, TMR Red (Roche, Mannheim, Germany, http://www.roche.com), was used for the TUNEL assay.

Anterograde Labeling of the CST
Twelve weeks after injury, descending corticospinal tract (CST) fibers were labeled with biotinylated dextran amine (BDA; MW 10,000, 10% in saline, 2 μl per cortex; Molecular Probes, Carlsbad, CA, http://probes.invitrogen.com) [26, 27] by injection into the left and right motor cortices [25]. The skulls of anesthetized mice were tightly fixed to a stereotaxic apparatus (Narishige). Scalping and craniotomies over the motor cortex area were carried out using a micromotor system (Nakanishi, Tochigi, Japan; http://www.nsk-nakanishi.co.jp). The injection site was 2.1 mm posterior to the bregma, 2 mm lateral to the bregma, and 0.7 mm in depth [25]. For pressure injections with a 20-μm outer diameter glass capillary attached to a microsyringe (Narishige), we used 0.1-μl steps per minute until the desired volume (1 μl per injection site) was injected. Two weeks later, the animals were perfused and their spinal cords were fixed as described above. Sections (20 μm) were cut and used for immunohistochemistry. To visualize the BDA, a tyramide signal amplification fluorescence system
(Perkin Elmer, Waltham, MA, http://www.perkinelmer.com) was used.

**Image Acquisition and Quantitative Analysis**

All images were obtained using a fluorescence microscope (BZ9000, Keyence, Osaka, Japan, http://www.keyence.co.jp) and a scanning laser confocal imaging system (LSM 710 or 800, Zeiss, Jena, Germany, http://www.zeiss.com). To evaluate the lesion area, we selected five sagittal sections (20 μm thick, 180 μm apart; the section including the epicenter was defined as the third section) in each mouse, measured the GFAP-negative area around the lesion site using ImageJ (http://rsb.info.nih.gov/ij/), and estimated the tissue volume using the Cavalieri principle [28, 29]. To quantify the number of host neurons, we performed immunostaining with an anti-GFAP antibody and anti-NeuN antibody on the midsagittal sections with the largest lesion area from each spinal cord. The number of immunoreactive cells was counted in the pictures photographed at the areas of 500 μm rostral and caudal to the epicenter using a Keyence BZ9000 at ×400 magnification (0.394 mm²) and divided the area of gray matter. To quantify the proportion of transplanted cells that had differentiated, we selected representative midsagittal sections and randomly captured three regions within 1,000 μm rostral and caudal to the lesion epicenter. GFP-positive transplanted cells and nontypic marker-positive cells were counted in each section.

**Evans Blue Dye Extravasation**

The integrity of the BSCB was investigated with Evans blue dye extravasation according to previous reports [30–32]. At 1 day after spinal cord injury, 0.2 ml of 2% Evans blue dye (Sigma) solution in saline was administered intraperitoneally. Three hours later, 12-mm lengths of spinal cord centered at the injury epicenter were immediately removed from anesthetized mice after intracardiac perfusion with saline, and weighed as wet weight. The samples were homogenized in 50% trichloroacetic acid solution and centrifuged at 10,000 g for 10 minutes. Supernatants were collected, and fluorescence for Evans blue ng/g wet brain weight.

**Spinal Cord Water Content**

Three days after SCI, 12-mm lengths of spinal cord centered at the injury epicenter were immediately removed from anesthetized mice and weighed as wet weight; they were then dried at 85°C for 48 hours, and reweighed as dry weight. Percent water content was calculated as (wet weight–dry weight)/wet weight × 100 [33].

**In Vivo Imaging of Transplanted Cells**

In vivo imaging experiments were performed as described previously [34]. A Xenogen-IVIS 100 cooled CCD optical microscopic imaging system (SC BioScience, Tokyo, Japan, www.scbio.co.jp) was used for bioluminescence imaging. Mice were given an intravenous injection of D-luciferin (150 mg/kg body weight), and images were acquired at 2 minutes after administration with the field-of-view set at 10 cm. All images were analyzed using Igor (WaveMetrics, Portland, OR, http://www.wavemetrics.com) and Living Image software (Xenogen), and optical signal intensity was expressed as photon flux, in units of photons/s/cm²/steradian. Each image was displayed as a false-color photon count image superimposed on a grayscale anatomic image. To quantify the measured light, we defined regions of interest (ROIs) over the cell-implanted area and examined all values within the same ROI.

**Ablation of Transplant-Derived Cells**

Transplant-derived cell ablation experiments were performed as described previously [2, 35, 36]. Since mouse and rat cells are less sensitive to diphtheria toxin (DT) than human cells, which express human heparin-binding EGF-like growth factor as a DT receptor, we could ablate only transplanted cells by injecting DT into the hiPSC-NSC-transplanted mice. DT was purified from conditioned medium of the PW8 strain of Corynebacterium diphtheriae by diethylaminoethyl Sepharose column chromatography and diluted to an appropriate concentration with saline. Six weeks after injury, DT solution (50 μg/kg/day × 2 days) was administered to mice by intraperitoneal injection.

**Ablation of Local Neurons**

Endogenous neuron ablation experiments were performed as described previously [6]. Twelve weeks after injury, we stereotaxically injected the excitotoxic glutamate agonist N-methyl-D-aspartic acid (NMDA) (1 μl of 10 mM in saline per site) into three sites of the spinal cord gray matter at 1 mm rostral, the epicenter, and 1 mm caudal to the lesion site (tenth thoracic level).

**Statistical Analysis**

All data are presented as mean ± standard error of the mean (SEM). An unpaired two-tailed Student’s t test was used for single comparisons, and analysis of variance (ANOVA) followed by the Tukey-Kramer test was used for multiple comparisons. Repeated measures two-way ANOVA followed by the Bonferroni post hoc test was used for the analysis of BMS scores, BDA-labeled positive fibers, and survival of transplanted cells at each point. p < .05 was considered significant.

**Results**

**Combination Therapy Involving Anti-HMGB1 mAb and hiPSC-NSC Transplantation Dramatically Enhances Functional Recovery**

In this study, we first sought to examine whether the tissue-protective anti-HMGB1 mAb has any beneficial effect on NSC transplantation-mediated functional recovery after SCI. We intraperitoneally injected anti-HMGB1 or control mAb into SCI model mice at 5 minutes and 6 hours after injury (Fig. 1A). At 7 days post injury, we injected hiPSC-NSCs or control medium into the epicenter of the injured spinal cord (Fig. 1A). Hindlimb locomotor recovery was evaluated using the Basso Mouse Scale (BMS) open-field motor score [21] (Fig. 1B and Supporting Information Video 1–4). In accordance with previous reports [2, 4], NSC transplantation alone indeed improved functional recovery compared with non-treatment, but the extent of recovery was relatively small. In contrast, the functional recovery in two groups that were treated with anti-HMGB1 mAb, either alone or combinatorially with hiPSC-NSC transplantation, was significantly higher than that in the transplantation alone group, at 6 weeks post SCI. Interestingly,
although the recovery of the mAb alone group then reached a plateau and became not significantly different from the transplantation alone group from 8 weeks after SCI, locomotor function in the combinatorially treated group continued to improve until at least 12 weeks. Furthermore, all three treated mouse groups exhibited less toe dragging compared with non-treatment mice in a footprint analysis [22, 23] at 12 weeks after SCI (Fig. 1C). Since paw positioning is a reliable index for evaluating motor function of mice that can step to support their weight (BMS score ≥ 4), we measured the angle of paw rotation, defined as the angle between the axes of the two hind paws. Because non-treatment mice badly dragged their hindlimbs (Fig. 1C), we could not quantify paw rotation and excluded this group. Quantitative analysis showed that the angle was smallest in the combination group, indicating that combinatorial treatment improved functional recovery most highly among all groups (Fig. 1D). To further evaluate fine motor control including coordination and foot- placing accuracy, which require a degree of sensory feedback, we performed a grid walking test [24] for mice in the three treated groups but not in the non-treatment group, which was excluded for the same reason as mentioned above. Combinatorially treated mice displayed significantly better functional recovery in this test compared with mice that received either treatment alone (Fig. 1E). Next, to clarify the conduc- tion capability of descending pathways from the motor cortex to the hindlimb motor neurons, we monitored motor-evoked potentials (MEPs) at 12 weeks after SCI (Fig. 2A). The amplitudes of MEP (tough-to-peak height of the first wave from the onset) showed no significant difference among the four groups, although there was a trend toward higher amplitudes in the three treated groups, especially in the combination group, compared with the non-treatment group (Fig. 2A, 2B). Furthermore, the latencies of MEP responses (the time interval between the end of stimulation and the onset of the first wave) in the anti-HMGB1 mAb-treated and combination- treated groups were significantly shorter than that in the non- treatment group (Fig. 2A, 2C). Taken together, these observa- tions indicate that the administration of anti-HMGB1 mAb during the acute phase of SCI is beneficial, and further enhances NSC transplantation-mediated functional recovery after SCI.

**Effects of Anti-HMGB1 mAb in the Acute Phase of SCI**

Injury to the CNS disrupts the BBB and increases the extravasa- tion of fluid and proteins into the affected tissue, causing edema and damage spread [37]. To investigate whether anti-

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**Figure 1.** Combinatorial treatment of hiPSC-NSC transplantation with prior anti-HMGB1 mAb administration dramatically improves locomotion recovery after SCI. (A): Summary of anti-HMGB1 mAb administration and hiPSC-NSC transplantation protocol. (B): Time course of functional recovery of hindlimbs after SCI. n = 16 mice in non-treatment and transplantation alone group. n = 12 mice in anti-HMGB1 mAb alone group. n = 14 mice in combination group. *, p < 0.05; **, p < 0.01; †††, p < 0.001 versus non-treatment mice; †, p < 0.05; ††, p < 0.01; ††††, p < 0.001 versus mice treated with transplantation alone; ‡, p < 0.05; ‡‡, p < 0.01; ‡‡‡‡, p < 0.001 versus mice treated with anti-HMGB1 mAb alone (repeated measures ANOVA). (C): Representative pictures of footprint analysis of mice in the four groups at 12 weeks post SCI. Forepaws and hind paws are indicated by red and blue footprints, respectively. Toe dragging is indicated by arrowheads. Dotted lines show axis lines of hind paws. (D): Quantification of paw rotation in footprint analy- sis. n = 10 mice per indicated group. †††, p < 0.001 versus combination-treated mice (one-way ANOVA with Tukey-Kramer test). (E): Normalized walking distances without mis-step in a grid walking test of the three treatment groups at 12 weeks after SCI. n = 12 mice per group. †††, p < 0.001 versus combination-treated mice (one-way ANOVA with Tukey-Kramer test). Data represent mean ± SEM for (B, D, E). Abbreviations: hiPSC-NSC, neural stem cell derived from human induced pluripotent stem cells; HMGB1, high mobility group box-1; mAb, monoclonal antibody; SCI, spinal cord injury; i.p., intraperitoneal injection; BMS, Basso Mouse Scale.
Anti-HMGB1 mAb Increases Neurites from Spared Axons and Survival of Host Neurons

Given that anti-HMGB1 mAb treatment has beneficial effects on the preservation of lesion tissue in the acute phase of SCI, we next attempted to determine how this protective effect of the antibody in the acute phase results in the pathology of the injured spinal cord in the chronic phase. To this end, we checked the extent of damage spread by measuring the area that harbored CD11b-positive inflammatory cells and were surrounded by GFAP-positive reactive astrocytes 12 weeks after injury [42]. Immunostaining analysis demonstrated that the lesion area in anti-HMGB1 mAb-treated mouse groups irrespective of NSC transplantation was significantly smaller than that in untreated mice (Fig. 4A, 4B).

Although we found that the lesion area of anti-HMGB1 mAb-treated mice after SCI was smaller than that in untreated mice, this finding does not explain why the antibody-treated mice exhibited improved functional recovery. Because signal transmission from the rostral to the caudal part of the injured site should be enhanced when functional recovery improves [43], we next investigated the regeneration and/or retention of neurites of supraspinal tracts in the injured spinal cord at 14 weeks after injury. We first examined regeneration of the CST, which controls finer details of locomotion [43]. By injecting the anterograde tracer BDA into the motor cortices of mice, we labeled CST axons and evaluated their regeneration in the four groups [26, 27] (Supporting Information Fig. S1A). By enumerating BDA-labeled fibers at positions from rostral (−5 mm) to caudal (+3 mm) around the lesion site, we found almost no CST axons below the lesion sites in all treatment groups of mice even though they clearly showed functional recovery after SCI (Supporting Information Fig. S1A, S1B). Therefore, we next focused on 5-HT-positive serotonergic fibers (Fig. 4C). We found no significant difference in the number of sprouted 5-HT-positive serotonergic fibers in the areas beyond 1 mm caudal to the lesion sites between intact and anti-HMGB1 mAb treatment groups, regardless of hiPSC-NSC transplantation (Fig. 4D). Although we cannot currently distinguish whether increased numbers of 5-HT serotonergic fibers in anti-HMGB1 mAb treatment groups were attributable to the retention of pre-existing fibers or to newly sprouted fibers after injury, these data suggest that anti-HMGB1 mAb treatment led to an increase in 5-HT-positive fibers in the spinal cord after injury, resulting in functional recovery as was observed in previous studies [44–46].

Figure 2. MEP assessment to evaluate the effect of treatments on spinal cord conduction capability. (A): Representative MEP waves of intact mice and SCI mice that received the indicated treatment, recorded at 12 weeks after injury. Intervals between the horizontal dashed lines: amplitude; intervals between the short vertical lines: latency. (B, C): MEP amplitude and latency. n = 7 mice in non-treatment and transplantation alone group. n = 6 mice in anti-HMGB1 mAb alone, combination, and intact groups. *, p < .05; ***, p < .001 versus non-treatment mice (one-way ANOVA with Tukey-Kramer test). Data represent mean ± SEM for both panels. Abbreviations: HMGB1, high mobility group box-1; mAb, monoclonal antibody; MEPS, motor-evoked potentials; SCI, spinal cord injury.

HMGB1 mAb administration can attenuate these pathologies in the acute phase of SCI, we evaluated BSCB permeability at 1 day after SCI by Evans blue assay (Fig. 3A). Because Evans blue dye has a strong affinity for albumin, its leakage indicates extravasation of serum albumin from capillary vessels in the CNS. The amount of Evans blue dye extravasation in SCI mice with control mAb was over twice as much as that in intact mice (Fig. 3A, left), whereas extravasation in SCI mice treated with anti-HMGB1 mAb was significantly attenuated, indicating that anti-HMGB1 mAb treatment protected the BSCB from SCI-caused disruption, consistent with previous findings for brain injury [16]. Moreover, to assess edema, which increases and reaches a plateau by 3 days after SCI [33, 38, 39], we measured the water content of the injured spinal cord 3 days after SCI (Fig. 3B). The water content of anti-HMGB1 mAb-treated spinal cord was significantly lower than that of untreated spinal cord, suggesting that anti-HMGB1 mAb treatment suppresses swelling of the injured spinal cord. It is generally known that following BBB or BSCB disruption, inflammatory responses initiated by infiltrated immune cells, oxidative stress, and glutamate-mediated excitotoxicity induce secondary damage, leading to neuronal apoptosis [30, 40, 41]. Thus, we next examined the effect of anti-HMGB1 mAb treatment on apoptotic cell death at 1 day after SCI by TUNEL staining (Fig. 3C). TUNEL-positive cells were observed around the lesion area but their numbers were significantly lower in the anti-HMGB1 mAb-treated group than in the control mAb-treated group (Fig. 3D). Thus, these findings indicate that administration of anti-HMGB1 mAb decreased BSCB disruption, probably resulting in the reduction of edema progression and of apoptosis of host neurons.

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In addition to supraspinal tracts, it has been shown that host neurons also play critical roles in the functional recovery after SCI, since they can form synaptic connections with endogenous and transplant-derived neurons for the reconstruction of neural circuits [2, 6]. In light of these facts, we checked survival of host neurons by counting their number within the regions approximately 500 µm rostral and caudal to the lesion epicenter, and observed, regardless of hiPSC-NSC transplantation, that the surviving host neuron numbers in anti-HMGB1 mAb treatment groups were significantly higher than those in anti-HMGB1 mAb-untreated groups (Fig. 4E, 4F).

Anti-HMGB1 mAb Treatment Does Not Affect Survival, Migration, or Differentiation of Transplanted Cells

We have shown that anti-HMGB1 mAb treatment improves functional recovery after SCI. However, the antibody treatment with hiPSC-NSC transplantation achieved much greater improvement compared with the antibody treatment alone, at and beyond 6 weeks after SCI (Fig. 1B), suggesting the importance of transplant-derived cells for the enhanced functional recovery. Therefore, we next examined the behavior of transplanted cells with or without prior treatment of anti-HMGB1 mAb. As assessed by a bioluminescence imaging system that detects luciferase photon signals emitted only from living cells [2, 34], the survival rates of engrafted cells were the same in the transplantation alone and combination groups (Fig. 5A, 5B). In addition, immunohistochemical analyses demonstrated that irrespective of the treatment with anti-HMGB1 mAb, transplanted cells were located around the lesion epicenter and a high proportion of them differentiated into β-III-tubulin-positive neurons; conversely, their differentiation into GFAP-positive astrocytes was relatively low, as was observed in our previous study [2] (Fig. 5C–5E). Thus, we concluded that anti-HMGB1 mAb treatment does not affect survival, migration, or differentiation of transplanted cells.

Transplant-Derived Neurons in the Spinal Cord Form Synaptic Connections with Endogenous Neurons Around the Lesion Site

Since previous studies indicated that signals from the brain to the caudal part of the spinal cord are transmitted in a relay manner through transplant-derived neurons after SCI [2, 3, 6, 7], we then sought to assess whether hiPSC-NSC-derived neurons also form synapses with host neurons in the present experimental setting. Immunoreactive signals of human-specific hSyp (presynaptic marker) were comparable on GFP-
Figure 4. Anti-HMGB1 mAb increases neurites from spared axons and survival of host neurons. (A): Representative sagittal sections of injured spinal cord in four groups at 12 weeks after SCI. Sections were stained with anti-CD11b (green) and -GFAP antibody (red). The epicenter of the SCI is indicated as (*). Scale bar, 500 μm. (B): Quantitative analysis of area surrounded by GFAP-positive cells. n = 6 mice per group. **, p < .01; ****, p < .001 versus non-treatment mice (one-way ANOVA with Tukey-Kramer test). (C): Representative pictures of 5-HT-positive fibers (red) at 3 mm caudal to the lesion site at 14 weeks after SCI. Hoechst (blue) shows nuclear staining. Scale bar, 50 μm. (D): Quantification of labeled 5-HT-positive fiber numbers. The x-axis indicates specific locations along the rostro-caudal axis of the spinal cord, and the y-axis shows the ratio of the mean fiber number at the indicated sites to that at 5 mm rostral to the lesion site. n = 3 mice per group. *, p < .05; **, p < .01; ****, p < .001 versus intact mice (repeated measures ANOVA). Data represent mean ± SEM. (E): Representative pictures of surviving host neurons that were positive for NeuN (red) and negative for GFP (green) at 500 μm rostral to the lesion epicenter. Scale bar, 50 μm. (F): Quantification of the number of surviving host neurons at 500 μm rostral and caudal to the lesion epicenter. Data represent mean ± SEM for (B, D, F). Abbreviations: HMGB1, high mobility group box-1; mAb, monoclonal antibody; SCI, spinal cord injury; 5-HT, 5-hydroxytryptamine; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein (GFP); hiPSC-NSC, neural stem cell derived from human induced pluripotent stem cell.
negative and β-III-tubulin-positive host neurons around lesion sites of both the transplantation alone and the combination group of mice (Fig. 6). We also confirmed the colocalization of hSyp and GFP signals (Supporting Information Fig. S2A).

Moreover, adjacent immunoreactive signals of PSD95 (a postsynaptic marker) and hSyp were observed, suggesting that functional synaptic connections were formed (Supporting Information Fig. S2B). Although we have not examined

Figure 5. Anti-HMGB1 mAb treatment does not affect survival, migration, or differentiation of transplanted cells. (A): Survival of transplanted cells was checked at the indicated times using a bioluminescence imaging system. (B): Time course of the survival of transplanted cells in transplantation alone (n = 8) and combination groups (n = 10). Optical signal intensity was measured using the bioluminescence imaging system. Quantification of photon intensity revealed that there was no significant difference between the two groups. p = .9283 (one-way ANOVA with Tukey-Kramer test). (C): Representative pictures of the distribution of transplant-derived cells that were positive for GFP (green). The epicenter of the SCI is indicated as (*). Scale bar, 500 μm. (D): Representative pictures of engrafted cells at 12 weeks after SCI. Spinal cords were stained with anti-GFP (green), anti-β-III-tubulin (red), anti-human-specific GFAP (hGFAP) (blue) antibodies and Hoechst (gray). Scale bar, 10 μm. Each lower panel shows a higher-magnification view of the boxed area in the corresponding upper image. (E): Quantitative analysis of β-III-tubulin-positive neurons and hGFAP-positive astrocytes, respectively. n = 4 mice per group. (Student’s t test). Data represent mean ± SEM for (B, E). Abbreviations: HMGB1, high mobility group box-1; mAb, monoclonal antibody; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; SCI, spinal cord injury.
whether transplant-derived neurons form synaptic connections with supraspinal tracts, this is an important challenge for future study.

Neuronal Circuits Composed of Transplant-Derived Neurons and Host Neurons Around the Lesion Site Are Critical for Dramatic Functional Recovery

Finally, we performed ablation experiments to evaluate the contribution of both host and transplant-derived neurons to functional recovery after SCI [2, 6]. First, to ablate transplanted cells, combinatorially treated mice were injected intraperitoneally with DT at 6 weeks post SCI (Fig. 7A), when the extent of functional improvements from anti-HMGB1 mAb treatment alone and combinatorial therapy begin to become distinct (Fig. 1B). Successful ablation was confirmed using both an in vivo imaging system (at 7 and 12 weeks) and immunohistochemistry (at 12 weeks) after SCI (Supporting Information Fig. S2A, S2B). After ablation, the BMS score of mice in the DT-treated combination group declined to a level similar to those of both the transplantation and the anti-HMGB1 mAb treatment alone groups (Fig. 7B). This reversed functional recovery was also observed in footprint analysis and the grid walking test (Fig. 7C–7E). To examine whether the reversed functional recovery resulted from inflammation induced by the ablation of cells, we assessed CD11b signals in the ablated regions (Supporting Information Fig. S2C, S2D). Three days after the first injection of DT, when residual GFP-positive cells could still be observed, we saw no increase in CD11b signals in the DT-injected spinal cord compared with signals without the injection (Supporting Information Fig. S2D), suggesting that substantial inflammation was not induced even by the ablation of cells with DT. Therefore, the reversed functional recovery in the DT-treated combination group suggested that transplanted cells contribute directly to functional recovery. Additionally, since hiPSC-NSCs require several months to become mature and fully functional neurons [4, 47], the ablated transplant-derived cells at 6 weeks after SCI were assumed to be immature neurons which would later become fully functional and play an important role in enabling more complicated neuronal networks to execute finer details of locomotion [47].

Second, to ablate endogenous neurons around the lesion area that had been rescued by administration of anti-HMGB1 mAb, NMDA was then injected into the injury epicenter of the spinal cord in the DT-treated combination group of mice at 12 weeks after SCI (Fig. 7A, 7F). After ablation of these cells, the BMS score dropped to below that of even the nontreatment group (Fig. 7G), indicating that remaining endogenous neurons around the lesion site also have an important role. Collectively, these data indicate that the reconstructed neuronal networks are composed of both surviving host neurons around the lesion site and transplant-derived neurons, yielding a dramatic improvement in motor functional recovery after SCI.

Discussion

Although many pharmacological interventions for SCI have been investigated, none of the agents examined in these studies are currently recommended for use. For example, methylprednisolone, one of the most frequently used drugs for the treatment of SCI, is no longer recommended for this purpose because its efficiency is controversial and it can have harmful side effects, inducing complications such as wound infection, gastrointestinal hemorrhage, sepsis, pulmonary embolism, and severe pneumonia [48]. Moreover, a recent study has reported that transient hyperglycemia induced by methylprednisolone administration impairs functional improvement after SCI [49]. Therefore, new effective reagents for SCI have become desirable.

In this study, we focused on the function of HMGB1, which is known to trigger secondary damage after SCI [11, 12]. After SCI, HMGB1 translocates from the nucleus to the cytoplasm and its expression increases earlier than that of proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and interleukin-6. We demonstrated that anti-HMGB1 mAb administration after SCI prevented BSCB disruption, minimized CD11b-positive cell packed area surrounded by GFAP-positive cells, and increased neurites from spared
axons and survival of host neurons (Fig. 4A–4F). Furthermore, it improved functional recovery after SCI to a level equivalent to hiPSC-NSC transplantation alone (Fig. 1B). In addition to treatment involving drug administration, cell transplantation therapy holds great promise for the treatment of SCI. Mechanisms by which transplanted NSCs promote

![Figure 7](image-url)
functional recovery after SCI have been suggested, including neuroprotection, promotion of axonal regeneration, modification of inhibitory properties of reactive astrocytes [50], immunomodulation, and replacement of lost neural cells [51]. In this context, we and others have previously shown that rebuilding neuronal networks composed of both surviving host neurons and transplant-derived neurons is important for hindlimb functional recovery after SCI [2, 5, 6]. These findings imply that the surviving host neurons are critical when transplant-derived neurons contribute to the functional recovery. It has indeed been demonstrated that when endogenous neurons in the region to be injured were pre-ablated, NSC transplantation failed to induce functional recovery after moderate SCI [4]. In the present study, we demonstrated that anti-HMGBl antibody administration preserved the lesion site, resulting in increased numbers of surviving host neurons (Fig. 4E, 4F), and these neurons formed synapses with each other (Fig. 6 and Supporting Information Fig. S3A, S3B). Considering these two points, this antibody treatment can increase the abundance of synaptic connections between transplant-derived and host neurons; treatment with anti-HMGBl increases the number of surviving host neurons, leading to an increased likelihood that transplant-derived neurons will find their targets.

We have established that the newly reconstructed local neuronal networks, composed of surviving host neurons around the lesion site and transplant-derived neurons, are important for hindlimb functional recovery. We also found that re-extension of CST axons does not occur even when mice are combinatorially treated Supplemental online Fig. 1, but that the numbers of 5-HT-positive neurites from spared axons are increased in response to anti-HMGBl antibody treatment after SCI (Fig. 4C, 4D). However, in the present study, we did not examine the interaction between these neurons and these host axonal systems. In this context, using a monosynaptic tracing system with modified rabies virus, Adler et al. have recently shown that transplant-derived neurons in the injured spinal cord receive numerous synaptic inputs that normally innervate the spinal cord, including cortical, brainstem, spinal cord, and dorsal root ganglia inputs, among which the connections from spinal interneurons were the most abundant [52]. Therefore, further investigation is warranted to precisely understand how the combinatorial treatment dramatically improves functional recovery after SCI.

Considering clinical application, it is known that the injured human spinal cord has a large lesion size and forms a cavity around the lesion area [53]; therefore, the preservation of tissue encompassing endogenous neurons seems particularly important. However, anti-HMGBl treatment may overcome this obstacle, as has been shown in our animal models. Although applying our findings to the treatment of human patients will require further investigation, we believe that the combinatorial treatment with anti-HMGBl mAb and hiPSC-NSC transplantation represents a novel and highly promising therapeutic strategy for the treatment of SCI.

**CONCLUSION**

In summary, we have revealed that treatment with anti-HMGBl mAb alleviated BSCB disruption and edema formation, and increased sprouting neurites from spared axons and survival of host neurons, resulting in improvement of functional recovery equivalent to the level achieved by hiPSC-NSC transplantation alone. We have also demonstrated that preservation of the lesion tissue, including host neurons, by prior treatment with anti-HMGBl mAb dramatically enhances the functional recovery achieved by hiPSC-NSC transplantation after SCI.

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**Figure 7.** Host neurons and transplant-derived neurons both contribute directly to the functional recovery of hindlimb movement after SCI. (A): Summary of ablation experiment protocol against transplanted cells. (B): Time course of the changes in BMS scores in SCI model mice. The hindlimb function of mice that had undergone combinatorial treatment deteriorated after DT administration (magenta line). n = 16 mice in transplantation alone group. n = 12 mice in anti-HMGBl mAb alone group. n = 14 mice in DT-untreated combination group. n = 11 mice in DT-treated combination group. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus mice treated with transplantation alone (repeated measures ANOVA). (C): Representative pictures of footprint analysis of combinatorially treated mice with or without DT administration at 12 weeks after SCI. Dotted lines show the axis lines of hind paws. (D): Quantification of paw rotation in footprint analysis. n = 10 mice in the DT-untreated combination group. n = 8 mice in the DT-treated combination group. *, p < 0.05 versus combination-treated mice [Student’s t test]. (E): Normalized walking distance without mis-step in a grid walking test at 12 weeks after SCI. n = 12 mice in the DT-untreated combination group. n = 10 mice in the DT-treated combination group. **, p < 0.01 compared with mice in the DT-untreated combination group (Student’s t test). (F): Summary of ablation experiment protocol against remaining host neurons. Mice in the DT-treated combination group were divided into two groups and treated with or without NMDA 12 weeks after injury. NMDA was injected into the injury epicenter to ablate host neurons around the lesion site. (G): Time course of functional recovery of hindlimbs after SCI. n = 8 mice in non-treatment group. n = 4 mice in DT-treated combination group with or without NMDA injection. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus non-treatment mice; †††, p < 0.001 versus DT-treated combination without NMDA injection group (repeated measures ANOVA). Data represent mean ± SEM for (B, D, E, G). Abbreviations: BMS, Basso Mouse Scale; DT, diphtheria toxin; HMGBl, high mobility group box-1; mAb, monoclonal antibody; NMDA, N-methyl-D-aspartic acid. SCI, spinal cord injury; i.p., intraperitoneal injection.

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antibody ameliorates brain infarction induced by transient ischemia in rats. FASEB J 2007; 21:3904–3916.
15 Haruma J, Teshigawara K, Hishikawa T et al. Anti-high mobility group box-1 (HMGB1) antibody attenuates delayed cerebral vasospasm and brain injury after subarachnoid hemorrhage in rats. Sci Rep 2016; 6:37755.
16 Okuma Y, Liu K, Wake H et al. Anti-high mobility group box-1 antibody therapy for traumatic brain injury. Ann Neurol 2012; 72: 373–384.
17 Nakamura Y, Moroika N, Abe H et al. Neuropathic pain in rats with a partial sciatic nerve ligation is alleviated by intravenous injection of monoclonal antibody to high mobility group box-1. PLoS One 2013; 8: e37640.
18 Kanellopis P, Agrotis A, Kyaw TS et al. High-mobility group box protein 1 neutralization reduces development of diet-induced atherosclerosis in apolipoprotein e-deficient mice. Arterioscler Thromb Vasc Biol 2011; 31: 313–319.
19 Falk A, Koch P, Kesavan J et al. Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons. PLoS One 2012; 7:e29597.
20 Rhee YH, Choi M, Lee HS et al. Insulin concentration is critical in culturing human neural stem cells and neurons. Cell Death Dis 2013;4:e766.
21 Basso DM, Fisher LC, Anderson AJ et al. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 2006;23:635–659.
22 Kunkel-Bagden E, Dai HN, Bregman BS. Mapping of the motor cortex of the white matter in adult paraplegic rats. Exp Neurol 1993;119:153–164.
23 Cheng H, Almström S, Giménez-Lloret L et al. Gait analysis of adult paraplegic rats after spinal cord repair. Exp Neurol 1997;148: 544–557.
24 Ma M, Basso DM, Walters P et al. Behavioral and histological outcomes following graded spinal cord contusion injury in the C57Bl/6 mouse. Exp Neurol 2001;169: 239–254.
25 Pronchik IV, Lenkov DN. Functional mapping of the motor cortex of the mouse by a microstimulation method. Neurosci Behav Physiol 1998;28:80–85.
26 Hata K, Fujitani M, Yasuda A et al. RGMa inhibition promotes axonal growth and recovery after spinal cord injury. J Cell Biol 2006;173:47–58.
27 Kaneko S, Iwanami A, Nakamura M et al. A selective Sema3A inhibitor enhances regenerative responses and functional recovery of the injured spinal cord. Nat Med 2006;12:1380–1389.
28 Akbas H, Sahin B, Erolgu L et al. Estimation of breast prostheses volume by the Cavallieri principle using magnetic resonance images. Aesthetic Plast Surg 2004;28:275–280.
29 Aleksic D, Aksic M, Divac N et al. Throminominal water promotes axonal sprouting but does not reduce gial scar formation in a mouse model of spinal cord injury. Neural Regen Res 2014;9:2174–2181.
30 Lee JY, Kim HS, Choi HY et al. Flutextine inhibits matrix metalloprotease activation and prevents disruption of blood-spinal cord barrier after spinal cord injury. Brain 2012; 135:2375–2389.
31 Lee JY, Choi HY, Ahn HJ et al. Matrix metalloprotease-3 promotes early blood-spinal cord barrier disruption and hemorrhage and impairs long-term neurological recovery after spinal cord injury. Am J Pathol 2014;184:2985–3000.
32 Wang HL, Lai TW. Optimization of Evans blue quantitation in limited rat tissue samples. Sci Rep 2014;4:6588.
33 Kimura A, Hsu M, Seldin M et al. Protective role of aquaporin-4 water channels after contusion spinal cord injury. Ann Neurol 2010;67:794–801.
34 Okada S, Ishi K, Yamane J et al. In vivo imaging of engrafted neural stem cells: its application in evaluating the optimal timing of transplantation for spinal cord injury. FASEB J 2005;19:1839–1841.
35 Furukawa N, Saito M, Hakoshima T et al. A diphtheria toxin receptor deficient in epi- dermal growth factor-like biological activity. J Biochem 2006;140:831–841.
36 Saito M, Iwawaki T, Taya C et al. Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. Nat Biotechnol 2001;19:746–750.
37 Laird MD, Shields JS, Sukumari-Ramesh S et al. High mobility group box protein-1 promotes cerebral edema after traumatic brain injury via activation of toll-like receptor 4. Glia 2014;62:26–38.
38 Fan ZK, Lv G, Wang YF et al. The protective effect of salvinorin A on blood-spinal cord barrier after compression spinal cord injury in rats. J Mol Neurosci 2013;51: 986–993.
39 Cohen DM, Patel CB, Ahbola-Vajjula P et al. Blood-spinal cord barrier permeability in experimental spinal cord injury: Dynamic contrast-enhanced MRI. NMR Biomed 2009; 22:332–341.
40 Wang D, Liu K, Wake H et al. Anti-high mobility group box-1 (HMGB1) antibody inhibits hemorrhage-induced brain injury and improved neurological deficits in rats. Sci Rep 2017;7:46243.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.
41 Nagoshi N, Okano H. iPSC-derived neural precursor cells: Potential for cell transplantation therapy in spinal cord injury. Cell Mol Life Sci 2017;75:899–1000.

42 Okada S, Nakamura M, Katoh H et al. Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. Nat Med 2006;12:829–834.

43 Liu X, Wang X, Li W et al. A sensitized IGF1 treatment restores corticospinal axon-dependent functions. Neuron 2017;95:817–833 e814.

44 Nothias JM, Mitsui T, Shumsky JS et al. Combined effects of neurotrophin secreting transplants, exercise, and serotonergic drug challenge improve function in spinal rats. Neurorehabil Neural Repair 2005;19:296–312.

45 Kim D, Murray M, Simansky KJ. The serotonergic 5-HT(2C) agonist m-chlorophenylpiperazine increases weight-supported locomotion without development of tolerance in rats with spinal transections. Exp Neurol 2001;169:496–500.

46 Ghosh M, Pearse DD. The role of the serotonergic system in locomotor recovery after spinal cord injury. Front Neural Circuits 2014;8:151.

47 Lu P, Ceto S, Wang Y et al. Prolonged human neural stem cell maturation supports recovery in injured rodent CNS. J Clin Invest 2017;127:3287–3299.

48 Rogers WK, Todd M. Acute spinal cord injury. Best Pract Res Clin Anaesthesiol 2016;30:27–39.

49 Kobayakawa K, Kumamaru H, Saiwai H et al. Acute hyperglycemia impairs functional improvement after spinal cord injury in mice and humans. Sci Transl Med 2014;6:256ra137.

50 Lukovic D, Valdes-Sanchez L, Sanchez-Vera I et al. Brief report: Astrogliosis promotes functional recovery of completely transected spinal cord following transplantation of hESC-derived oligodendrocyte and motoneuron progenitors. Stem Cells 2014;32:594–599.

51 Volarevic V, Erceg S, Bhattacharya SS et al. Stem cell-based therapy for spinal cord injury. Cell Transplant 2013;22:1309–1323.

52 Adler AF, Lee-Kubli C, Kumamaru H et al. Comprehensive monosynaptic rabies virus mapping of host connectivity with neural progenitor grafts after spinal cord injury. Stem Cell Reports 2017;8:1525–1533.

53 Norenberg MD, Smith J, Marcillo A. The pathology of human spinal cord injury: Defining the problems. J Neurotrauma 2004;21:429–440.

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