Population suffers spinal cord injury (SCI) when a strong external force is applied to the spine and the spinal cord, often due to traffic accidents or falls. The global incidence of traumatic SCI is reported as 23 per million. SCI results in motor and sensory impairment, bladder-rectal dysfunction, and marked reduction in activities of daily living.

To date, there is no fundamental treatment for SCI. However, recent advances in regenerative medicine related to stem cell transplantations are expected to have therapeutic potential. Several clinical trials of cell transplantation therapies for SCI are currently ongoing.

Multilineage-differentiating stress-enduring (Muse) cells are recently discovered endogenous pluripotent stem cells. They exist in mesenchymal tissues, including the bone marrow and adipose and dermal tissues. Since Muse cells express the pluripotent surface marker stage-specific embryonic antigen 3 (SSEA-3), they can be harvested and cultured to be a source of regenerative medicine. It is remarkable that Muse cells do not have tumorigenicity.

OBJECTIVE Multilineage-differentiating stress-enduring (Muse) cells are pluripotent stem cells, which can be harvested from the bone marrow. After transplantation, Muse cells can migrate to an injured site of the body and exert repair effects. However, it remains unknown whether Muse cell transplantation can be an effective treatment in spinal cord injury (SCI).

METHODS The authors used a rat model of thoracic spinal cord contusion injury. For Muse cell transplantation, the clinical product CL2020 containing 300,000 Muse cells was administered intravenously 1 day after midthoracic SCI. Animals were divided into CL2020 (n = 11) and vehicle-treated (n = 15) groups. Behavioral and histological evaluations were conducted over a period of 8 weeks to see whether intravenous CL2020 administration provided therapeutic effects for SCI. The effects of human-selective diphtheria toxin on reversion of the therapeutic effects of CL2020 were also investigated.

RESULTS Hindlimb motor function significantly improved after CL2020 transplantations. Importantly, the effects were reverted by the human-selective diphtheria toxin. In immunohistochemical analyses, the cystic cavity formed after the injury was smaller in the CL2020 group. Furthermore, higher numbers of descending 5-hydroxytryptamine (5-HT) fibers were preserved distal to the injury site after CL2020 administration. Eight weeks after the injury, Muse cells in CL2020 were confirmed to differentiate most predominantly into neuronal cells in the injured spinal cord.

CONCLUSIONS Following SCI, Muse cells in CL2020 can reach the injured spinal cord after intravenous administration and differentiate into neuronal cells. Muse cells in CL2020 facilitated nerve fiber preservation and exerted therapeutic potential for severe SCI. https://thejns.org/doi/abs/10.3171/2020.7.SPINE20293

KEYWORDS Muse cell; intravenous administration; rat; spinal cord injury; cell transplantation
Importantly, when Muse cells are administered intravenously into the body, they recognize sites of injury and migrate there.15,16 Since Muse cells have the ability to self-renew and differentiate from a single cell to three germ layers, it was reported that they could differentiate into cells suitable to repair and replace damaged tissues.12 Therapeutic effects of Muse cells have been demonstrated in various experimental disease models, including acute myocardial infarction, liver damage, and chronic kidney disease.17–19 Furthermore, in recent experiments using a stroke model, Muse cells differentiated into functional neuronal cells in damaged brain, which led to functional recovery.16,20 This achievement indicated the suitability of Muse cells as donor cells for transplantation in neurological diseases.

To the authors’ knowledge, the effects of Muse cell transplantation for SCI have not yet been demonstrated. In this study, the clinical grade Muse cell–based product CL2020 was administered in an experimental SCI rat model. One day after the midthoracic SCI, CL2020 was intravenously transplanted in a rat model. Behavioral and histological evaluations followed to confirm the therapeutic effects of CL2020 against SCI.

Methods

Experimental Animals

All animal experiments were approved by the Animal Studies Ethics Committee of Tohoku University Graduate School of Medicine. All efforts were made to minimize the number of animals used and to decrease animal suffering during the experiments. In total, 36 adult female Sprague-Dawley rats (body weight 200–230 g) were used (Japan SLC, Inc.). Two or 3 rats were housed per cage and kept at a temperature of 24°C ± 0.5°C using a feedback-regulated heating pad (BWT-100, Bio Research Center). Two or 3 rats were housed per cage and kept at a temperature of 24°C with water and food ad libitum before and after surgery. Figure 1A summarizes the experimental protocols.

Spinal Cord Injury

Rats were anesthetized with 2% isoflurane in 30% oxygen and 70% nitrous oxide. During surgery, the rectal temperature was maintained at 37.0°C ± 0.5°C using a feedback-regulated heating pad (BWT-100, Bio Research Center). Rats were positioned comfortably and the T9 spinous process was palpated. Skin above the T9 spinous process was shaved and cleaned in an antiseptic manner. A midline dorsal skin incision was made to perform a T9 laminectomy. After the dorsal spinal cord was widely exposed, an Infinite Horizon (IH) impactor (Precision System and Instrumentation LLC) was used to make a contusion injury to the spinal cord at T9 by the impactor (arrow). Figure is available in color online only.

One day after the injury, the Muse cell–based product CL2020 (Muse cells) Intravenous Administration

One day after SCI, 3 rats showing movements in their hindlimbs were excluded from the study. For the remaining 33 rats, either CL2020, which is a Muse cell–based product produced from human mesenchymal stem cells (MSCs) after exposing the cells to the combination stressing (Life Science Institute, Inc.) (CL2020 group, n = 12), or Dulbecco’s phosphate-buffered saline (D-PBS; Funakoshi Co., Ltd.) (control group, n = 21) was intravenously administered. Rats were assigned to groups in a blinded format. Each rat in the CL2020 group received 300,000 Muse cells in CL2020 through a tail vein. A total of 0.3 ml was slowly injected over 1 minute as determined from our previous studies.16 We made sure there was no extraneous leakage of the materials. All rats received a 0.5 mg/kg subcutaneous injection of a immunosuppressant, tacrolimus hydrate (Prograf, Astellas Pharma, Inc.), every other day for 8 weeks.

Behavioral Analysis

Hindlimb motor functions of all animals (n = 33) were evaluated using the Basso, Beattie, Bresnahan (BBB) locomotor scale before and after SCI on days 1, 5, and 7, and weekly thereafter for 8 weeks.22 BBB scores were recorded by an animal care technician who was blinded to

FIG. 1. A: Schema showing the experimental procedure of the treatment group. In the CL2020 group, rats (n = 36) received an SCI at the T9 level on day 0 (arrow). One day after the injury, the Muse cell–based product CL2020 (n = 12) or a control (n = 21) was administered intravenously (arrow). Afterward, hindlimb motor functions of the animals were evaluated weekly until 56 days after the SCI. The rats were then separated (arrow) to be evaluated for histological analyses (n = 6) and with a loss-of-function study using diphtheria toxin administration (n = 5). B: Intraoperative photograph showing how the contusion injury was made on the dorsal spinal cord at T9 by the impactor (arrow). Figure is available in color online only.
the study and animal groups. During the 8-week experiment, 1 and 6 rats died in the CL2020 and control groups, respectively. We excluded the data of the dead animals and performed further analyses. Overall, we included 11 and 15 animals in the CL2020 and control groups, respectively. BBB scores were compared between the CL2020 and control groups using multiple-measurement ANOVA followed by Bonferroni post hoc tests. All values are given as the mean ± standard deviation.

Immunohistochemical Analyses

Eight weeks after SCI, all rats except animals undergoing the loss-of-function study were anesthetized by aspirating excess isoflurane. The rats were first transcardially perfused with saline to remove blood and subsequently with 2% paraformaldehyde in 0.1 mol/L saline. The fixed spinal cords were cut in 10-mm pieces, placed with the injured portion in the center, and embedded in matrix material (Thermo Scientific Shandon M-1 embedding matrix, Thermo Fisher Scientific) to be frozen by liquid nitrogen. The prepared spinal cord was then sectioned at a 5-µm thickness with a cryostat. Slides were created from spinal cord sections of 1, 1.5, 2, 3, 4, and 5 mm in the rostral and caudal directions with the injured epicenter as 0.

Cystic Cavity Measurement

Klüver-Barrera staining was used for morphological evaluation. Areas of cystic cavity in the spinal cord were measured using ImageJ (W. J. Rasband, National Institutes of Health, https://imagej.nih.gov/ij/). Cystic cavity volumes were calculated using the cavity areas on the slides of 1, 1.5, 2, 3, 4, and 5 mm in the rostral and caudal directions with the injured epicenter as 0. In the CL2020 and control groups, the cavity volumes were calculated and compared using the Mann-Whitney U-test.

Quantification of Preserved 5-Hydroxytryptamine Fibers

To evaluate preserved 5-hydroxytryptamine (5-HT) fibers in the spinal cord, fluorescent staining of anti–5-HT antibody (ab66047, Abcam) was employed. In a section perpendicular to the rostral-caudal axis of the spinal cord and 3 mm caudal from the epicenter of the injury, the number of 5-HT–immunolabeled axons was quantified with ImageJ. Differences between the CL2020 and control groups were compared with the Mann-Whitney U-test.

Muse Cell Identification in the Injured Spinal Cord

To detect human Muse cells in CL2020 homing into the injured spinal cord and evaluate their differentiation into neural lineages, immunostaining with antibodies against human mitochondria (hMit) (1:50; ab3398, Abcam), microtubule-associated protein-2 (MAP-2) (1:500; ab5392, Abcam), glial fibrillary acidic protein (GFAP) (1:500; 130330, Invitrogen), and glutathione S-transferase pi (GST-pi) (1:200; MBL312, Medical & Biological Laboratories) was performed. The samples were incubated with each secondary antibody conjugated with Alexa 488 anti-mouse, Alexa 488 anti-rabbit, Alexa 568 anti-chicken, and Alexa 488 anti-rat antibodies and enclosed with a mounting agent (Prolong diamond antifade mountant with DAPI: P36962, Thermo Fisher Scientific). The samples were inspected under a laser confocal microscope (C2si, Nikon) or a color microscope camera (DFC7000 T, Leica).

Diphtheria Toxin Administration

Eight weeks after SCI, loss-of-function studies were performed using diphtheria toxin from Corynebacterium diphtheriae (Sigma-Aldrich Co. LLC). Human cells are 100,000 times more sensitive to the toxin than rodent cells. Therefore, diphtheria toxin has been used as a tool for targeted ablation of human cells in rodent models. Five rats from both the CL2020 and control groups received intraperitoneal injections of diphtheria toxin (50 µg/kg) twice at 24-hour intervals. Hindlimb motor functions were assessed 5 days after diphtheria toxin administration. Paired t-tests were used to evaluate BBB scores before and after the diphtheria toxin administration.

Statistical Analysis

BBB scores were compared between CL2020 groups and control groups using multiple-measurement ANOVA followed by Bonferroni post hoc tests. The cavity volumes and the number of 5-HT fibers were compared using the Mann-Whitney U-test. Paired t-tests were used to evaluate BBB scores before and after diphtheria toxin administration.

Results

Intravenous CL2020 Improves Hindlimb Locomotor Function After SCI

Rats received CL2020 1 day after SCI. CL2020-treated rats showed greater improvement in BBB scores than control rats (2–8 weeks post-SCI in Fig. 2). Eight weeks after the injury, the average BBB scores were 9.7 ± 3.3 and 6.2 ± 3.7 in the CL2020 and control groups, respectively.
CL2020 Prevented Spinal Cord Damage and Contributed to Structural Preservation

The cystic cavity volume in rats treated with CL2020 was significantly smaller than that in the control group (p < 0.05; Fig. 3). In addition, higher numbers of 5-HT–positive axons were preserved in the caudal spinal cord in the CL2020 group animals (p < 0.01; Fig. 4). These results suggest that Muse cells in CL2020 exerted neuroprotective activity in the injured spinal cords. Histological findings were compatible with the functional improvement exhibited by the CL2020-treated animals.

CL2020 Engrafts and Differentiates Into Neuronal Cells in the Injured Spinal Cord

Eight weeks after CL2020 administration, double immunostaining with hMit and DAPI identified hMit-positive cells in the injured spinal cord. In particular, the transplanted human cells localized simultaneously to the corticospinal tract perilesional areas rostral and caudal to the lesion. In triple staining, hMit-positive cells expressed MAP-2 (Fig. 5). Positivity for MAP-2 was found in 58.3% ± 1.2% of hMit-positive cells, GFAP in 15.7% ± 3.8%, and GST-pi in 21.1% ± 3.8%. These results suggest that intravenously administered CL2020 homed into the injured spinal cord and predominantly engrafted as neuronal marker–positive cells.

Deterioration of Hindlimb Function After Diphtheria Toxin Administration

A loss-of-function study was performed using diphtheria toxin to selectively cancel functional improvements that CL2020 provided in a rodent SCI model. Five days after the diphtheria toxin administration, BBB scores in the CL2020 group (n = 5) significantly decreased (p < 0.01; Fig. 6). During the same period, there were no significant differences in the BBB scores of the control group even after diphtheria toxin was given.

Discussion

The present study demonstrated that the Muse cell–based product CL2020 migrated into the injured spinal cord when intravenously delivered 1 day after SCI. This result suggests that the intravenous administration of CL2020 may be a feasible option for cell-based therapy. Further, Muse cells engrafted into neuronal lineage cells in the injured spinal cord and were associated with improvements in hindlimb motor function over an 8-week period. When diphtheria toxin selectively ablated engrafted Muse cell functions, BBB scores of the spinally injured animals in the CL2020 group significantly deteriorated. These results support the observation that Muse cells directly contributed to functional recovery in hindlimb functions after SCI. Histological evidence also supported these findings.
Namely, Muse cell–treated rats had smaller cystic cavities and higher numbers or 5-HT fibers preserved distal to the injury.

**Muse Cells Can Migrate Into the Injured Spinal Cord**

In this experimental model, we found hMit-positive cells in the injured spinal cord 8 weeks after intravenous CL2020 administration. This result suggests that Muse cells recognized the spinal cord as a damaged site and migrated there. In previous studies, after intravenous administration of CL2020, such homing has been confirmed in the brain, the heart, the liver, and the kidney in various experimental models. Recently, using a lacunar infarction model, our group observed Muse cell migration into the damaged brain when CL2020 was administered in subacute (9 days after the onset) and chronic (30 days after the onset) phases. These results indicated that intravenous CL2020 administration may be a reasonable approach to deliver Muse cells to the injured spinal cord.

Mechanisms underlying the migration and homing of Muse cells after intravenous administration may be related to sphingosine-1-phosphate (S1P). When cell membranes are injured, S1P is generated and serves as a signal of acute inflammation. Muse cells express S1P receptor 2 and therefore can sense the trigger and migrate where the injury occurred.

**Muse Cells Differentiated Into Neuronal Lineage Cells**

Muse cells express pluripotency markers such as Sox2, Oct3/4, and Nanog. After homing, Muse cells spontaneously differentiate into cells that comprise the damaged tissues for replacement and repair. When Muse cells were transplanted into an acute myocardial infarction model, they spontaneously differentiated into cells posi-
Muse Cells Contributed to Functional Recovery

In this study, animals with Muse cells transplanted through CL2020 administration showed significant functional improvements. To determine whether intravenous CL2020 administration directly related to the recovery, we selectively ablated transplanted human Muse cells by administering diphtheria toxin, as previously reported. Importantly, in our SCI model, BBB scores of CL2020-treated animals significantly dropped 5 days after DT administration. In the control group, there was no change of BBB scores after DT administration (**p < 0.01). ns = not significant.

Although distinct mechanisms remain to be established, in the present study we were able to determine that Muse cells in CL2020 acted as interneurons and contributed to the recovery.

After diphtheria toxin administration, the BBB scores of CL2020-treated animals declined significantly but not exclusively. Behavioral recovery was also evident as early as 2 weeks after the administration of CL2020. These results indicate that in addition to neuronal network reconstruction, Muse cells in CL2020 exerted other beneficial effects for SCI. Having considered that Muse cells are distinct stem cells in mesenchymal cell populations, we can suggest possible mechanisms of CL2020 related to those of MSCs. For SCI, MSC transplantation can promote functional recovery. Postulated beneficial effects of MSCs included trophic support, neuroprotection, and providing environments to accommodate axonal sprouting. Histological evaluations in the current study confirmed that CL2020-treated animals had smaller cystic cavities. This finding suggests a neuroprotective function of Muse cells in SCI. In addition, more 5-HT–positive fibers were identified distal to the injury in rats after CL2020 transplantation compared with fiber numbers in the control rats. The 5-HT systems are descending brainstem tracts. Following MSC transplantation in SCI rats, 5-HT–positive nerve fibers were identified along the engrafted MSC bundles and considered to reflect functional recovery.

Timing of CL2020 Administration

In the current protocol, CL2020 was intravenously administered 1 day after SCI. We determined the timing based on a previous report in which SCI rats demonstrated the greatest functional recovery when they received MSCs 1 day after the injury. Immediately after SCI, a series of pathological and reactive alterations take place in the damaged spinal cord. Ischemia and necrosis make the injured spinal cord a hostile environment for the survival of transplanted cells. Matsushita et al. reported that the blood–spinal cord barrier disruption occurred soon and peaked at 2 weeks after the injury. If the Muse cells can pass through the blood–spinal cord barrier and reach the injured site, Muse cell transplantation administered during the subacute phase might exert beneficial effects. Although the appropriate timing for cell transplantation therapy remains controversial, if we apply the CL2020 product in future clinical trials for SCI, it is worthwhile that we now have evidence of the effects of Muse cells administered acutely in CL2020 1 day after SCI.

Study Limitations

The results of this study suggest that intravenously administered Muse cells migrated into the injured spinal cord and contributed to functional recovery. However, the distinct mechanisms by which the cells reached the injured spinal cord and contributed to recovery remain to be elucidated. Recently, we reported surrogate imaging results using Muse cells tagged with a luminescent protein, Nano-Lantern. In vivo live imaging can possibly disclose distribution of the Muse cells and indicate the best timing for the cell transplantation therapy in future experimental studies.
Conclusions
Intravenous administration of the human-derived product CL2020 containing Muse cells successfully led to significant functional recovery from SCI. One day after the injury, engrafted Muse cells in CL2020 migrated and homed in on the spinal cord. The cells then differentiated into neuronal cells and contributed to the restoration of descending spinal tracts. CL2020 may offer a feasible treatment option in a future clinical trial for SCI patients.

Acknowledgments
We would like to thank Enago for the English-language review. We appreciate Dr. Haruo Kanno, Dr. Michiharu Matsuda, Ms. Natsumi Konno, and Ms. Marisa Ota for their invaluable scientific input and technical support for this project.

References
Kajitani et al.


Disclosures
This study was partially supported by the MEXT/AMED Translational Research Network Program (J190000613), a JSPS Grant-in-Aid for Scientific Research (C), the HIROMI Medical Research Foundation, the Ichiro Kanehara Foundation, the SENSHIN Medical Research Foundation, the Okinaka Memorial Institute for Medical Research, and co-research expenses with Life Science Institute Inc. (LSII; Tokyo, Japan). Dr. Takatsugu Abe, Prof. Kuniyasu Niizuma, and Prof. Teiji Tominaga are parties to a joint research agreement with LSII. Prof. Niizuma has a contract of a clinical trial agreement with LSII. Prof. Tominaga received consulting fees from LSII.

Author Contributions
Conception and design: Endo, Niizuma, Tominaga. Acquisition of data: Endo, Kajitani, Iwabuchi, Takahashi, Abe. Analysis and interpretation of data: Endo, Kajitani, Inoue, Abe, Niizuma. Drafting the article: Endo, Kajitani. Critically revising the article: Iwabuchi, Inoue, Abe, Niizuma, Tominaga. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Endo. Statistical analysis: Kajitani, Iwabuchi, Takahashi, Abe, Niizuma. Administrative/technical/material support: Kajitani, Niizuma, Tominaga. Study supervision: Niizuma, Tominaga.

Correspondence
Toshiki Endo: Tohoku University Graduate School of Medicine, Sendai, Japan. endo@nsg.med.tohoku.ac.jp.