visualized using goat anti-mouse IgG conjugated to Cy3 (1:200, Jackson ImmunoResearch, West Grove, PA, USA) and goat anti-rabbit IgG Alexa 488 (1:400, Molecular Probes). Images were obtained on an AX 70 Olympus microscope with a digital camera (DP70, Olympus, Tokyo, Japan) connected to a computer with Olympus DP manager software. We measured a scale bar contained obtained images and the length of Tuj-1-positive neurites along the holes of the collagen sponges. The maximum length of the neurites from each DRG explant was evaluated from all sections containing neurites.

Scanning electron microscopy (SEM) was performed. Some samples were fixed with 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd., Berks, UK) for 24 h. These samples were post-fixed with 2.0% (v/v) osmium tetroxide (TAAB Laboratories Equipment Ltd., Berks, UK) for 2 h. After dehydration in a graded series of ethanol and drying in a critical point drying apparatus (HCP-2; Hitachi) with liquid $CO_2$, samples were sputter-coated with platinum (Hitachi, Tokyo, Japan) and examined by scanning electron microscopy (S-4500; Hitachi, Tokyo, Japan).



*Spinal cord injury and cell transplantation*

For implantation, pieces of HC (1 x 1 x 3 mm) seeded with GFP-labeled BMSCs at a concentration of 3 x $10^4$ cells per scaffold or pieces of control HC (i.e., HC scaffolds without cells) were prepared and cultured with DMEM and 10% FBS for three days. Thirty-six adult female Fischer 344 rats (Sankyo Laboratory Service, Tokyo, Japan) weighing 130-160 g were used in the study. The rats were anesthetized by intraperitoneal injection of chloral hydrate (280 mg/kg; Junsei Chemical, Japan), and Eltacin (50 mg/kg; Fuji Pharma) was injected intramuscularly prior to surgery. A laminectomy was performed at the T8 and T9 level, and the dura was incised longitudinally. The exposed spinal cord was hemisected at T9, followed by removal of 3 mm of hemicord on the right side. After hemostasis was achieved, an HC scaffold (HC group: n = 18) or HC scaffold combined with BMSCs (HC/BMSC group: n = 18) was implanted between the rostral and caudal spinal cord stumps. The muscles and skin were sutured in layers. The rats were given a subcutaneous saline injection and were allowed to recover in a warm cage. They received manual

bladder expression twice a day until spontaneous urination was re-established, and they were given subcutaneous saline injections and intramuscular injections of Eltacin (50 mg/kg) every day for one week.

*Histology*

Four weeks after surgery, the rats were deeply anesthetized with chloral hydrate (400 mg/kg) and transcardially perfused with a solution of 4% PFA in 0.1 M phosphate buffer (PB). The spinal cords were post-fixed in 4% PFA in 0.1 M PB for 24 h and dehydrated in 30% sucrose (Wako Pure Chemical Ind., Osaka, Japan) in 0.1 M PB for 48 h. The 9-mm spinal cord segment centered on the implanted/injury site was dissected and embedded in 1.5% low-melting-temperature agarose (BM Equipment Co. Ltd., Tokyo, Japan) in PBS. The spinal cord segments were cut into 30-μm-thick horizontal sections using a cryostat (Leica Microsystems, Nussloch, Germany), and sections collected every 210 μm were used to assess the volume. A series of the sections was stained with Luxol fast blue and cresyl violet (LFB/CV; Wako Pure Chemical Ind., Osaka, Japan).



*Determination of tissue volume*

For histological evaluation of the tissue damage around the injury site, the total cross-sectional cord area and the area of spared myelin in the white matter were calculated using Scion Image software (Scion Corporation, Frederick, MD, USA). LFB was used to detect myelin; a foamy appearance, pale LFB color, and the invasion of inflammatory cells were all considered signs of demyelination. The tissue volume in each 9-mm cord segment was calculated using the following numerical formula:

$$\text{Estimated volume} = A1\times D + A2\times D + \cdots + AN\times D$$

(A: cross-sectional area, D: distance between sections = 210 μm, and N: total number of sections)

*Immunohistochemistry*

To characterize the distribution of transplanted BMSCs in the host injured spinal cord, sections from each experimental group were stained for the marker gene GFP and/or neuronal markers. After treatment with 0.2% Triton X-100 for 5 min, the sections were blocked with 5% normal goat serum (NGS) and incubated

with rabbit polyclonal anti-GFP (1:1000, Molecular Probes), mouse monoclonal anti-SMI31 (a marker for neurofilament M+H, 1:500, COVANCE), anti-glial fibrillary acidic protein (GFAP) (a marker for astrocytes, 1:400, Sigma), or anti-myelin basic protein (MBP) (a marker for myelin, 1:1000, COVANCE) antibodies for 24 h. Primary antibodies were visualized using goat anti-mouse IgG conjugated to Cy3 (1:200, Jackson ImmunoResearch) for neuronal markers and goat anti-rabbit Alexa 488 (1:400, Molecular Probes) for GFP. Images were obtained on an AX70 Olympus microscope.

*Quantification of transplant survival in the injured spinal cord*

To quantify transplant survival in the injured spinal cord, sections selected every 210 μm in the transplantation groups were stained with anti-GFP antibody, and the GFP-positive cells were counted in all selected sections. The number of surviving cells was calculated as the sum of the GFP-positive cell counts in all selected sections in each animal.



*Estimation of the number of neurofilament-, CGRP-, and 5HT-positive axons in the injured spinal cord*

One series of mounted sections was incubated with SMI31 and rabbit polyclonal anti-GFAP (1:500, DAKO, Carpinteria, CA, USA) antibodies. Primary antibodies were visualized using goat anti-mouse IgG conjugated to Cy3 and goat anti-rabbit Alexa 647 (1:400, Molecular Probes). The implanted areas including the HC were observed in areas of the spinal cord where GFAP-positive cells were not located. The number of SMI31-positive fibers was determined by counting all labeled fibers crossing an imaginary line placed perpendicular to the center line of the implanted area. The number of regenerated fibers was calculated as the sum of the SMI31-positive fiber counts in all selected sections in each animal. Another series of mounted sections was incubated with mouse monoclonal anti-GFAP and antibodies against either calcitonin gene-related peptide (CGRP) (1:1000, Peninsula laboratories INC., San Carlos, CA, USA) or 5-HT (1:1000, ImmunoStar, Hudson, WI, USA) overnight at 4°C. Primary antibodies were visualized using goat anti-mouse IgG conjugated to Cy3 and anti-rabbit Alexa 647 (1:400, Molecular Probes) as the secondary antibody. The number of CGRP- or 5-HT-positive fibers was also determined as described

above.

*Retrograde tracing*

To assess the survival of spinal cord tracts, retrograde tracing with Fluoro-Gold (FG; Fluorochrome, Denver, CO, USA) was performed 4 weeks after transplantation (HC group: n = 6, HC/BMSC group: n = 6). A laminectomy was performed at T12, and 3 μL of 4% FG was injected bilaterally into the ventral, lateral, and dorsal columns of the white matter (total six points) using a glass pipette (Iwaki/Asahi Techno Glass, Tokyo, Japan). The brains and spinal cords were dissected 1 week after FG injection and dehydrated as described above. The cerebral cortex, brain stem, and T6-level spinal cord were cut into 30-μm-thick transverse sections, and one section every 300 μm was observed using an AX70 Olympus microscope. FG-positive neurons were counted in 2.4 mm of the cerebral cortex (eight slices), 1.5 mm of the brain stem (five slices), and 3.0 mm of the T6 spinal cord (10 slices).

*Hindlimb behavioral tests*



The Basso-Beattie-Bresnahan (BBB) locomotor rating score (4) and BBB subscores (28) were used to evaluate the recovery of hindlimb motor function. Two observers who were blind to the experimental procedures performed the BBB scoring once a week for 4 weeks. Footprint analysis was performed 4 weeks after injury. The hindpaws of the rats were inked, footprints were recorded on a paper runway with a length of 1 m and a width of 7 cm, and the limb rotation, stride length, and base of support were measured as described previously (32).

Sensory tests were also performed for mechanical and cold sensation 2 and 4 weeks after injury. The animals were placed in clear acrylic boxes (Ugo Basile Biological Research Apparatus, Comerio, Italy) and allowed to acclimate to the testing environment. The boxes were then placed on an elevated perforated plastic surface for a minimum of 30 min prior to all behavioral tests (17). A blind observer conducted the behavioral testing.

Mechanical sensitivity was measured by applying a series of calibrated von Frey filaments (0.02 - 15 g) to

the plantar aspect of the hindpaw using the up-down method (6).

For determination of cold sensitivity, using a plastic tube connected to a 1-mL syringe (Terumo Corp., Tokyo, Japan) and without touching the skin, 100 μl of acetone (Wako Pure Chemical Ind., Osaka, Japan) was applied to the plantar surface of the foot. Acetone was applied five times to each paw with an interval of at least 30 seconds, and brisk foot withdrawals in response to the acetone evaporation within 10 seconds after the application were considered to be positive. The number of positive responses was then recorded.

*Statistical analysis*

All results are expressed as the mean ± S.E.M. The data were analyzed using GraphPad Prism5 (GraphPad Software Inc., San Diego, CA, USA) statistical software. The two groups (HC scaffold alone vs. HC scaffold + BMSCs) were compared using the unpaired two-tailed Student's t-test or the Mann-Whitney U test. In the behavioral testing, the score at every time point was analyzed using a two-way ANOVA followed by Bonferroni post hoc test. $p < 0.05$ was considered statistically significant.



**Results**

*Neurites extended from dorsal root ganglia (DRGs) into honeycomb collagen sponge (HC) pores with or without bone marrow stromal cells (BMSCs)*

Efficient expression of GFP in BMSCs was observed at passage 3 (Fig. 1A). The cells were distributed in the HC pores 3 days after seeding by the low-pressure loading method (Fig. 1B). Bioactivity in vitro was analyzed using lumbar DRGs seeded over HC pieces with or without BMSCs. The DRGs extended their neurites towards and into the porous structure of the honeycomb scaffolding (Fig. 2A). Tuj-1-positive neurites were observed in the tubular HC sections after 10 days in vitro (Fig. 2B). The neurites were measured from the base of the DRG residing on the surface of the HC scaffold to their furthest discernible extension. The average maximum extension was 733.3 ± 112.4 μm in the HC pieces and 1992.7 ± 92.3 μm in the HC pieces with BMSCs. Thus, neurite growth was increased by over two fold in the HC pieces

combined with BMSCs compared to the control HC pieces (Fig. 2C). GFP-labeled BMSCs were located alongside the Tuj-1-positive neurites and on the inner walls of the HC pores (Fig. 2D and E).

*Locomotor and sensory function recovered after HC scaffold implantation with or without BMSCs*

The HC scaffold (HC group) or HC scaffold combined with BMSCs (HC/BMSC group) was implanted between the rostral and caudal spinal cord stumps. The BBB score on the injured side reached a plateau with an average score of average 11.6 ± 0.2 at 3 weeks after injury in the HC group, whereas the BBB score was 12.6 ± 0.1 in the HC/BMSC group and 11.7 ± 0.1 in the HC group at 4 weeks after injury (Fig. 3B). Hindlimb coordination was significantly improved in the HC/BMSC group at 4 weeks after injury. In the BBB subscore, the HC/BMSC group displayed a better outcome than the HC group at 4 weeks (Fig. 3C). In addition, the stride length was significantly improved in the HC/BMSC group compared to the HC group (Fig. 3D). Both the base of support and external rotation angle appeared to improve in the HC/BMSC group, but the difference did not reach statistical significance (data not shown).



After the hemisection injury, hypoesthesia or anesthesia to mechanical and cold stimuli developed in the hindpaw on the injured side. The number of animals showing a response to the 4-8 g von Frey filaments in the HC/BMSC group (n = 12) increased from two (at week 2) to seven (at week 4), whereas the respective number of responsive animals increased from four to six in the HC group (n = 12). The response to acetone was also statistically increased in the HC/BMSC group 4 weeks after injury (Fig. 3E). Thus, the HC scaffold combined with BMSCs contributed to a partial recovery of hindlimb function.

*HC scaffold implantation with BMSCs did not affect the estimated tissue volume but increased nerve regeneration*

At 4 weeks, the defect was filled with the HC scaffold, which contacted both cord stumps (Fig. 4A). We estimated the total lesion volume and amount of spared myelin in 9-mm-long horizontal sections stained with LFB/CV under light microscopy. The total tissue volume and cavity volume were not significantly different between the groups (data not shown). GFP-positive cells were observed in the implantation site

of the BMSC group (Fig. 4B). The average number of GFP-positive cells was 208 (minimum number of cells = 14, maximum number of cells = 711) in each animal. Regenerated fibers were also observed as SMI31-positive fibers in the GFAP-negative implanted area (Fig. 4B). A quantitative analysis showed that more SMI31-positive fibers were present in the BMSC group than in the HC group (Fig. 4C and D).

*BMSCs increased the number of CGRP-positive fibers in the implanted site and 5-HT-positive fibers contralateral to the implanted site.*

To evaluate the growth of sensory fibers, CGRP-positive fibers were analyzed in the implanted spinal cord 4 weeks after implantation (Fig. 5A). The number of CGRP-positive fibers was higher in the HC/BMSC group than in the HC group at the implantation site, but not contralateral to the implantation site (Fig. 5B). Furthermore, to examine the growth of serotonergic fibers related to functional recovery, we quantified the 5-HT-positive fibers. Few 5-HT-positive fibers were present in the implanted area, even in the HC/BMSC group (Fig. 5C). However, more 5-HT-positive fibers were observed contralateral to the implantation site (Fig. 5D); in particular, the number of 5-HT-positive fibers was higher in the HC/BMSC group than in the HC group (Fig. 5E). The nerve fiber distribution suggested that the transplanted BMSCs enhanced and attracted the growth of sensory afferents at the implantation site and serotonergic descending fibers contralateral to the implantation site.



*HC implantation with BMSCs increased rubrospinal axonal density after injury*

The FG-labeled neurons represented the corticospinal tract, rubrospinal tract, and the short tract starting from the propriospinal neurons at the T6 segment. The red nucleus on the injured side contained a higher number of FG-positive cells in the HC/BMSC group than in the HC group (Fig. 6A and B). Thus, HC scaffold implantation with BMSCs led to axonal regeneration in at least the rubrospinal tract.

**Discussion**

We demonstrated that in vitro expanded BMSCs engrafted in a honeycomb collagen matrix enhanced

neurite elongation in vitro and directly promoted sensory and rubrospinal tract regeneration in addition to functional recovery after SCI.

Recent studies have identified axonal recovery after SCI and have thus opened a relatively new field compared to the well-established regeneration of peripheral nerves. It remains unclear whether sufficient recovery of central nervous system tissue can occur to support meaningful recovery. For functional recovery, the damaged axons must regenerate to an adequate length and also have the potential to reconnect or relay to their original distal axons. Various artificial scaffolds have been developed and studied with or without cell therapy in spinal cord injury models (23,38,48,51). We have already demonstrated that the HC matrix has the potential to aid the regrowth of axons after SCI (15). In addition to supporting regenerating axons along its entire length, the HC matrix also guides axonal regeneration through its serial tunnel structure. In the present study, the regenerated nerve fibers were clearly visible and easy to evaluate in the HC scaffold implant site. With regard to biodegradability, part of the HC scaffold remained in the host cord 4 weeks after implantation (Fig. 4A). In previous studies, HC scaffolds implanted subcutaneously were visible after 8 weeks (24), and collagen filament implants applied in the transected spinal cord partly remained after 12 weeks (64). Further observation is therefore necessary to confirm that the implanted HC scaffold degrades completely and the role, if any, that the degradation or maintenance of the scaffold in the injured cord plays in functional recovery.



BMSCs supply trophic support and structural support for regenerating axons in the injured spinal cord (18,19,34,35). One limitation to the use of BMSCs is that they do not survive long in the injury site after they have been implanted (1,20,34). For example, BMSCs transplanted in the subacute phase disappeared after 1 to 2 weeks in a rat contusion SCI model (20). In our study, the transplanted BMSCs survived for at least 4 weeks at the implanted site, and some cells were located along regenerated nerve fibers (Fig. 4B and C). In addition, the BMSCs clearly formed bundles along the nerve fibers into HC pores in vitro (Fig 2E). These results suggest that BMSCs have nerve guidance ability and promote nerve regeneration both

in vitro and in vivo.

Our in vivo study also demonstrated that implantation of an HC scaffold combined with BMSCs enhanced motor and sensory recovery compared to implantation of the HC scaffold alone. It should be noted that in this study, we used a two-group design (HC alone vs. HC + BMSCs) because we previously established that the HC scaffold alone provided functional benefit in this SC model (15), and the goal of the present study was thus to specifically probe the potential for BMSCs to enhance this effect, i.e., to examine the effect of adding BMSCs to the HC scaffold. We did not include a BMSC-alone group, as the lesion zone where the scaffolds are placed is a cavity with no structure for suspended cells to engraft in, and thus suspended (non-scaffolded) cells placed in the cavity would likely be immediately washed away.

We initially hypothesized that the presence of surviving BMSCs may affect functional recovery after SCI. However, the number of surviving GFP-positive cells varied among animals in the present study, and there was no obvious correlation between the number of GFP-positive cells and the functional outcome 4 weeks after implantation (data not shown). Previous reports showed that transplanted BMSCs disappeared in the early phage of SCI but provided an extracellular matrix including collagen fibrils or stimulated the production of growth factors, which resulted in the sparing of damaged tissues (18,20,34,41,42). In the present study, unexpectedly, the estimated tissue volume was not different between animals implanted with the HC scaffold engrafted with BMSCs and those implanted with the HC scaffold alone at 4 weeks after implantation. This difference between our findings and those of previous studies may be attributed to our use of an HC scaffold, which we previously showed to independently induce the regeneration of axons and functional connections, or our use of a hemisection injury model in contrast to the previous studies. Regardless of the lack of difference in tissue sparing, as mentioned above, inclusion of BMSCs in the scaffolds still enhanced functional recovery.

Our study thus suggests that the presence of sufficient surviving BMSCs in the early phase of spinal cord injury promotes eventual functional regeneration; we also consider that a longer time course of

observation in our model (i.e., longer than 4 weeks) may still reveal a correlation between the number of surviving BMSCs and functional outcome by allowing more regenerating nerve fibers to reach their target neurons or axons, as the BBB scores in the HC/BMSC group still appeared to be increasing at 4 weeks. In particular, the number of SMI31-positive fibers at the implanted site of the HC/BMSC group was more than twice as high as that in the HC-alone group, which indicates that the transplanted BMSCs facilitated the regeneration of rubrospinal axons and sensory fibers to produce functional recovery. Regarding motor function, it has been reported that functional recovery after transplantation of human adult olfactory neural progenitors is correlated with rubrospinal axon regeneration and re-innervation (61). In this study, an increase in the number or extension of regenerated rubrospinal axons toward target neurons mediated by the surviving BMSCs in the HC scaffold may have thus resulted in improvement of motor functional recovery. Our observation that BMSCs enhanced the guided regeneration of axons in vivo and neurites in vitro agrees with previous findings that transplanted BMSCs provide a guidance effect to regenerating CNS and PNS axons (2,19,36,37,57,58). However, in spinal cord repair, mesenchymal stem cells (MSCs) are also thought to migrate to the injury site, modulate inflammation by secretion of various cytokines, and produce trophic factors to induce nerve regeneration (56). In particular, BMSCs produce various trophic factors such as NGF, BDNF, GDNF, and VEGF (9,16,31,36,59,62), and the trophic factors induce different axonal responses depending on the phenotype of the receptor (22). Therefore, another possible explanation for the functional recovery observed in the present study is that trophic effects of the transplanted BMSCs mediated through secreted molecules could have affected the host spinal cord. Specifically, production of trophic factors by surviving BMSCs at the implanted site may have resulted in a higher number of CGRP-positive fibers at the implanted site and 5-HT-positive fibers at the contralateral site while contributing to the observed enhancement of axonal regeneration. Regardless of whether the effects were exerted through direct contact or through secreted molecules, in our hemi-transected SCI model, we consider that HC-engrafted BMSCs may have functioned more for a nerve guidance effect than for tissue

preservation, particularly considering that the HC/BMSC group showed increased axonal regeneration without any difference in spared tissue volume relative to the HC-alone group.

BMSCs have been shown to differentiate into various phenotypes in vitro (5,47,63). BMSCs have also been suggested to differentiate into neural phenotypes upon implantation in host neural tissues, although such neural differentiation remains controversial (5,10,19,27,37). In the present study, we did not observe neuronal or glial differentiation of the surviving BMSCs in the host spinal cord at 4 weeks after implantation (data not shown). Furthermore, few surviving BMSCs expressed CD90 (data not shown), therefore suggesting that most of the surviving BMSCs did not maintain their undifferentiated, stem-like state. However, a subset of the surviving BMSCs expressed fibronectin (data not shown), which is a mesenchymal marker that has been shown to promote nerve growth (25) and to be expressed by undifferentiated donor BMSCs associating with axons in a contusion spinal cord injury model (19). It therefore appears that in the host tissue, the transplanted BMSCs do not undergo neural differentiation; rather, their therapeutic effects appear to be mediated either by donor cells that lose their undifferentiated, stem-like status to take on a phenotype with therapeutic effects on endogenous neural tissue components or by a sub-population of donor cells that remains undifferentiated, providing a niche to enhance the proliferation, differentiation, and/or therapeutic effects of endogenous cells in the injured spinal cord (40).



MSCs can be derived from not only bone marrow but also adipose tissue (ASCs) or umbilical cord blood (UCB-MSCs). Both BMSCs and ASCs can be obtained from autologous tissues of SCI patients, thus avoiding immunological complications and ethical concerns (49). In recent comparative studies, transplanted ASCs were shown to modulate the SCI environment with increase of the BDNF expression to a higher extent than BMSCs (8,65). In contrast to ASCs and BMSCs, UCB-MSCs are derived from perinatal tissues and are currently under initial evaluation for CNS repair (50,56). UCB-MSCs were shown to have higher nerve regeneration and anti-inflammation activity than other types of MSCs in a comparative study in a canine SCI model (44). Although ASCs and UCB-MSCs thus offer some

advantages, BMSCs may be the closest to clinical application for spinal cord injury because MSCs were originally obtained from the bone marrow, and numerous basic and clinical reports of applications of BMSCs for CNS repair have thus already been reviewed (26,54,60). In particular, culture techniques for BMSCs have already been optimized, and autologous BMSC transplantation has been confirmed as safe in SCI patients in previous clinical trials, thus establishing BMSCs as potentially neuroregenerative somatic stem cells without tumorigenicity. Further in vivo studies under the same experimental conditions are needed to determine which type of MSC promotes the greatest extent of functional recovery after SCI.

In summary, we have shown that administration of a HC scaffold engrafted with BMSCs significantly enhanced functional recovery and axonal regeneration compared to treatment with the HC scaffold alone when evaluated at 4 weeks after SCI. Our in vitro and in vivo data also support that the BMSCs surviving in the lesion zone may exert their therapeutic effect by stimulating and guiding nerve fiber regeneration. A therapy that combines HC scaffolds with BMSCs for transplantation may therefore be a clinically effective approach to fill gaps or cavities in the injured spinal cord.



**Figure legends**

Figure 1. In vitro expanded BMSCs after lentiviral infection and engraftment of BMSCs in the honeycomb collagen sponge. BMSCs were expanded and infected at P2 by GFP-gene-encoding lentiviruses. The cells were cultured and fixed at P3. GFP (green in A) was efficiently expressed in the BMSCs. The BMSCs were introduced into the honeycomb collagen sponge (HC) by a low-pressure loading method and cultured for 5 days. The GFP-labeled cells (green in B) were attached to the wall of HC pores (arrows). Scale bar, 50 μm (A and B).

Figure 2. Scanning electron microscopy (SEM) micrographs of DRGs on HC pieces and neurites

extending from a DRG cultured with BMSCs. The DRG explant was attached to the honeycomb collagen scaffold and extended neurites towards and into the porous structure (arrows in A). Tuj-1-positive neurites were observed to enter the HC piece (B). The neurites were longer in the HC piece combined with BMSCs than in the HC piece without BMSCs (C). GFP-labeled BMSCs were distributed into the HC pores (green in D and E), and some of the cells were localized alongside the Tuj-1-positive neurites (arrows in D). Higher magnification view of the BMSCs showed extending processes along the neurites (E). Scale bar: 300 μm (A), 1 mm (B), 50 μm (D), and 20 μm (E). *** $p < 0.0001$, Mann-Whitney-U-test (C)

Figure 3. Locomotor and sensory recovery after HC implantation with and without BMSCs

The BBB score at every time point was not significantly different between the HC and HC/BMSC groups by two-way ANOVA followed by Bonferroni post hoc test (A). In the comparison study of the BBB score at the final time point, there was a significant difference (B). The HC/BMSC group presented a better outcome in the BBB subscore than the HC group at 4 weeks following SCI (C). The relative value for the stride length (injured side/contralateral side) in the footprint analysis was significantly improved in the HC/BMSC group (D). Cold sensation in response to acetone was observed in both groups at 4 weeks. There was a significant difference between the responses at 2 and 4 weeks in the HC/BMSC group (E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, unpaired two-tailed Student's t-test (B, D and E), two-way ANOVA followed by Bonferroni post hoc test (C and E).

Figure 4. HC scaffold implantation into the hemisected spinal cord and nerve regeneration in the HC scaffold with or without BMSCs.

The implanted HC scaffold partially remained between the cord stumps 4 weeks after injury, but the staining intensity of LFB was maintained in the cord adjacent to the HC implant (A). The immunohistological study showed that GFP-positive cells (green in B) had survived in the

implantation site of the HC/BMSC group. The regenerated nerve fibers are shown as SMI31-positive fibers (arrows in B) in the GFAP-negative area. The number of SMI31-positive fibers was determined by counting all labeled fibers crossing imaginary lines placed perpendicular to the center line of the implanted site (arrows in C). A higher number of SMI31-positive fibers was observed in the HC/BMSC group than in the HC group (D). Scale bar, 1 mm (A), 100 μm (B), 200 μm (C). *** $p < 0.001$, Mann-Whitney-U-test (D).

Figure 5. Quantification of regenerated fibers expressing CGRP or 5-HT in the HC with or without BMSCs.

CGRP-positive fibers (arrows in A) were observed in HC scaffolds with GFP-positive cells (green in A). A quantitative analysis showed that the number of CGRP-positive fibers in the HC/BMSC group was significantly higher than that in the HC group at the implanted site (B). However, there was no significant difference contralateral to the implantation site (B). Few 5-HT-positive fibers (arrows in C) were observed in HC scaffolds with GFP-positive cells (green in C) at the implanted site. However, a quantitative analysis contralateral to the implanted site showed that more 5-HT-positive fibers (arrows in D) were present in the HC/BMSC group than in the HC group (E). Scale bar, 100 μm (A and C), 200 μm (D). ** $p < 0.01$, Mann-Whitney-U-test (B and E).



Figure 6. Quantification of retrograde neuronal tracing 4 weeks after implantation

FG-labeled neurons were quantified in sections of the cortex, red nucleus, and T6 cord segment in both the implanted and contralateral sides (A). A higher number of FG-labeled neurons in the HC/BMSC group relative to the HC group was observed only in the red nucleus. In a representative section of a red nucleus in the HC/BMSC group, the FG-labeled neurons were present at both the implanted and contralateral side (B). Scale bar, 300 μm. * $p < 0.05$, Mann-Whitney-U-test (A).

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