Bone marrow stromal cell sheets promote axonal regeneration and functional recovery with suppressing glial scar formation after spinal cord transection injury in rats

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Abstract

OBJECT. Transplantation of bone marrow stromal cells (BMSCs) is a theoretical potential as a therapeutic strategy in the treatment of spinal cord injury (SCI). Although a scaffold is sometimes used for retaining transplanted cells in damaged tissue, it is also known to induce redundant immunoreactions during the degradation processes. In this study, we prepared cell sheets made of BMSCs, which are transplantable without a scaffold, and investigated their effects on axonal regeneration, glial scar formation, and functional recovery in a completely transected SCI rat model.

METHODS. BMSC sheets were prepared from the bone marrow of female Fischer 344 rats using ascorbic acid, and were cryopreserved until the day of transplantation. A gelatin sponge (GS), as a control, or BMSC sheet was transplanted into a 2-mm-sized defect of the spinal cord at the T8 level. Axonal regeneration and glial scar formation were assessed 2 and 8 weeks after transplantation by immunohistochemical analyses using anti-Tuj1 and glial fibrillary acidic protein (GFAP) antibodies, respectively. Locomotor function was evaluated by the Basso, Beattie, Bresnahan (BBB) score.

RESULTS. The BMSC sheets promoted axonal regeneration at 2 weeks after transplantation, but there was no significant difference in the number of Tuj1-positive axons between the sheet- and GS-transplanted groups. At 8 weeks after transplantation,

Tuj1-positive axons elongated across the sheet and their numbers were significantly greater in the sheet group compared with the GS group. The areas of GFAP-positive glial scars in the sheet group were significantly reduced compared with those of the GS group at both time points. Finally, hind limb locomotor function was ameliorated in the sheet group at 4 and 8 weeks after transplantation.

CONCLUSIONS. The results of the present study indicate that an ascorbic acid-induced BMSC sheet is effective in the treatment of SCI and enables autologous transplantation without requiring a scaffold.

Accumulating evidence suggests that transplantation of adult bone marrow stromal cells (BMSCs) is a theoretical potential as a therapeutic strategy in the treatment of spinal cord injury (SCI). BMSCs are easily isolated and harvested *in vitro* and can be cryopreserved for a long duration.[21] BMSCs prepared from patients enable autologous transplantation. Previous studies have shown that BMSCs enhanced axonal regeneration and suppressed glial scar formation in both contused [10,16,19,28,30] and transected [33] SCI models. In the case of the transected model, a scaffold was still needed to fill the defect of the spinal cord as well as to retain the transplanted cells at the site.[33] Many kinds of scaffolds have been developed to date, including self-assembled peptide nanofibers,[15] gelatin,[7] collagen,[26] Matrigel,[18] and fibrin.[17] However, a previous report demonstrated that these biodegradable scaffolds induce inflammatory reactions during their degradation.[32] Thus, an autologous cell sheet that is transplantable without scaffolds is considered to be a useful material for the treatment of SCI.

BMSCs produce abundant extracellular matrix (ECM) proteins, which are considered suitable for tissue engineering applications.[25] Our previous study demonstrated that the addition of L-ascorbic acid phosphate (AscP) to the culture medium for BMSCs allowed the cells to disperse into a sheet form.[20] In the same study, the BMSC sheets were found to produce various growth factors such as vascular

endothelial growth factor, basic fibroblast growth factor (bFGF), platelet-derived growth factor, and insulin-like growth factor-1,[20] which have been reported to enhance axonal regeneration and to confer neuroprotection.[11,12,31] Thus, we hypothesized that transplanting a BMSC sheet into a spinal cord defect without a scaffold might enhance axonal regeneration and suppress glial scar formation. The aim of this study was to assess the usefulness of BMSC sheets in terms of axonal regeneration and the suppression of glial scar formation in a completely transected SCI model. We first examined the cell viability of BMSC sheets after recovery from cryopreservation and investigated their ECM components by immunocytochemical staining. Second, we examined whether the sheets are permissive for axonal elongation and the extension of astrocyte processes, using neurospheres co-cultured on the sheet, because previous work has shown that the permissiveness of transplants for astrocyte processes is also important to prevent glial scar formation *in vivo*. [29] Third, we investigated the number of regenerating axons and glial scar formation by immunohistochemical staining after transplantation of the BMSC sheet into a spinal cord defect. Finally, functional recovery was assessed by the Basso, Beattie, and Bresnahan locomotor rating scale (BBB scale).[5]

Methods

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Nara Medical University. Female Fischer 344 rats were used in all experiments, which were purchased from Japan SLC Inc. (Shizuoka, Japan). A total of 30 rats were used for the experiments; 18 were used for histological and motor functional analyses (8-week-old rats), 10 were used for collecting bone marrow (7-week-old rats), and two embryos (embryonic day 17 [E17]) were used for preparing neurospheres.

BMSC sheets

The preparation of BMSCs from the femoral bone was performed as described previously, [2,3,23] and their application to cell sheets followed the methods described in our previous study.[20] In brief, bone marrow cells were obtained from the femoral shafts with standard culture medium: minimal essential medium (Nacalai Tesque, Kyoto, Japan) containing 15% fetal bovine serum (JRH Bioscience Inc., Lenexa, KS, USA) and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin; Nacalai Tesque). The released cells were collected in T-75 flasks (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) with the medium and maintained in a humidified atmosphere with 5%

CO_2 at 37°C. After reaching confluence, the cells were trypsinized and seeded in 6-cm dishes (60 × 20 mm; BD Falcon) at 1×10^4 cells/cm^2 with the same medium containing 0.28 mM of AscP magnesium salt n-hydrate (Wako Pure Chemical Industries, Kyoto, Japan). After 14 days in culture, the cells reached confluence and formed a sheet. The cell sheets were cryopreserved with CryoScarless medium (DMSO-Free; BioVerde, Inc., Kyoto, Japan) according to the manufacturer instructions, and maintained at –80°C until transplantation.

Neurite growth and glial extension on BMSC sheets

Neurospheres were prepared from the cortices of E17 embryos as described previously.[27] In brief, the tissues were dissociated by trituration with a fire-polished Pasteur pipette, and the cells were suspended in neurosphere culture medium: Dulbecco's modified Eagle medium/Ham's F-12 (1:1; Gibco, Carlsbad, CA, USA) containing 1% N2 supplement (Gibco), 1% penicillin/streptomycin (Nacalai Tesque), 0.2% recombinant human bFGF (PeproTech, Rocky Hill, NJ, USA), and 2% B27 supplement (Thermo Fisher, Waltham, MA, USA). Cells were seeded at 2×10^5 cells/mL in T-75 flasks containing the medium and incubated in 5% CO_2 at 37°C. After 1 week, neurospheres were collected, trypsinized, and passaged to secondary culture.

After 3.5 days, the neurospheres were collected and cryopreserved with STEM CELL-BANKER (GMP-grade; Takara Bio Inc., Shiga, Japan) in liquid nitrogen until use. For co-culture of neurospheres and a BMSC sheet, thawed neurospheres were plated on BMSC sheets attached to poly-D-lysine-coated coverslips in a 24-well plate and maintained in neurosphere culture medium for 3.5 days in 5% CO_2 at 37°C.

Cell viability assay

Cryopreserved BMSC sheets were thawed at 37°C and rinsed twice with the standard culture medium. Trypsinized cells were centrifuged at 100 ×*g* for 5 min at 4°C. The cell pellets were re-suspended with the medium and stained with trypan blue solution (Nacalai Tesque). Cell counting was performed under a microscope using a hemocytometer. Cell viability was defined as follows: [(total cell number counted – number of stained cells)/total cell number counted] × 100. The viability of thawed BMSC sheets was compared with that of fresh BMSC sheets.

SCI surgery

Cryopreserved BMSC sheets were thawed on the day of surgery as described above and maintained in the standard culture medium until transplantation. Animals

were anesthetized with 2.0% isoflurane in 2.0 L/min oxygen and subjected to laminectomy at the T8 level, leaving the dura mater intact. The spinal cord was completely transected at two points 2 mm apart with microsurgical scissors, and the fragment of the spinal cord was removed. A BMSC sheet or gelatin sponge (GS) was transplanted into the defect to fill the space between the rostral and caudal stumps of the spinal cord. After transplantation, the muscles and skin were sutured layer by layer. Food and water were provided *ad libitum*. The bladder was pressed twice a day until spontaneous voiding began.

Assessment of locomotor function

All locomotor assessments were performed by at least two examiners blinded to the group allocations. The hind limb locomotor functions of animals in both the BMSC sheet group (sheet group) and GS group were assessed using the BBB locomotor scale[5] at 1, 2, 4, and 8 weeks after transplantation. The BBB scale (from 0 to 21 points) can be used to assess locomotor recovery, including joint movements, stepping ability, coordination, and trunk stability. A score of 21 indicates unimpaired locomotion, as observed in uninjured rats.

Immunostaining

Histological and cytological immunostainings were performed as described previously.[13,14] In brief, cultured cells plated on poly-D-lysine-coated coverslips were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 10 min. After permeabilization with 0.3% Triton X-100 in PBS (PBST), cells were blocked with 5% normal horse serum (NHS) in PBST and incubated with Alexa Fluor-546-conjugated phalloidin and the following primary antibodies: mouse anti-collagen I (1:100; Abcam, Cambridge, UK), rabbit anti-collagen IV (1:100; Progen, Heidelberg, Germany), guinea pig anti-laminin (1:200; gifted from Dr. Miyata),[22] mouse anti-Tuj1 (1:200; Covance, Princeton, NJ, USA), or rabbit anti- glial fibrillary acidic protein (GFAP; 1:1000; Abcam). They were further incubated with species-specific secondary antibodies conjugated with Alexa Fluor-488 (1:1000; Thermo Fisher) or CF-555 (1:1000; Biotium, Hayward, CA, USA). The coverslips were shielded using Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA).

For immunohistochemistry, anesthetized animals with sodium pentobarbital (100 mg/kg) were transcardially perfused with PBS followed by 4% PFA. Dissected T6–T10 spinal cord segments were post-fixed with the same fixative overnight. After

cryoprotection with 30% sucrose in PBS, 20-μm-thick sagittal sections were made with a cryostat (Leica, Wetzler, Germany) and mounted on glass slides. The sections were treated with 25 mM glycine in PBS, 0.3% PBST, and then blocked in PBST containing 5% NHS for 2 h. The sections were incubated with the following primary antibodies for 2 days at 4°C: mouse anti-Tuj1 (1:200; Covance), rabbit anti-Tuj1 (1:200; Covance), mouse anti-GAP43 (1:100; Millipore, Bedford, MA, USA), rabbit anti-GFAP (1:1000; Abcam), or guinea pig anti-laminin. The sections were incubated with species-specific secondary antibodies conjugated with Alexa Fluor-488 or CF-555 (1:1000; Biotium) for 2 h. The coverslips were shielded using Vectashield containing DAPI. All immunocytochemical and immunohistochemical images were captured using a FluoView 1000 confocal microscope (Olympus, Tokyo, Japan) with a 512 × 512-pixel array.

Quantification of axonal regeneration and glial scarring

Axonal regeneration and glial scar formation were analyzed using the morphometric software program Metamorph (Molecular Devices, Sunnyvale, CA, USA). Nine serial sections prepared at 80-μm intervals including the midline were analyzed per animal. The numbers of regenerating axons were quantified by counting

the numbers of Tuj1-positive axons with a length of at least 50 μm within the two rectangles (500 × 1000 μm^2) that were 400 μm away from the rostral and caudal stumps of the injury sites. Glial scar formation was quantified by measuring the GFAP-positive areas within the two rectangles (1250 × 500 μm^2/area) including the rostral and caudal edges of the injury sites. The numbers of axons and GFAP-positive areas obtained from nine sections were summed as the final numbers and areas, respectively.

Statistical analyses

The Kolmogorov-Smirnov test, Shapiro-Wilk test, F-test, t-test, or Welch's test was performed depending on whether the data followed a normal distribution. The t-test was performed to examine significant differences in glial scar area, and Welch's test was performed to determine significant improvements in axonal regeneration. Furthermore, the t-test and repeated-measures analysis of variance were performed to examine significant improvements in BBB scores. All data are presented as means ± SEM. Data analyses were conducted using the Statistical Package for the Social Sciences (SPSS) software package (version 23.0; SPSS Inc., Chicago, IL, USA). A probability value (p) < 0.05 was considered to denote statistical significance.

Results

Cell viability of cryopreserved BMSC sheets

The cell viability of the BMSC sheets after cryopreservation was 92.88 ± 1.32% (n = 7), which was considered to represent good recovery after freezing. We therefore used cryopreserved BMSC sheets for all transplantation experiments.

The BMSC sheets provided neural cells with a permissive environment for elongation *in vitro*

BMSC sheets prepared using AscP were easily detached from the culture dishes with a cell scraper (Fig. 1A) and could be lifted up with forceps (Fig. 1B). Immunohistochemical results showed that BMSC sheets expressed collagen I and laminin (Fig. 1C and 1E), but not collagen IV (Fig. 1D). Immunostaining of neurospheres cultured on a BMSC sheet revealed that both Tuj1-positive neurites and GFAP-positive astrocyte processes could elongate on the sheet (Fig. 1F). These results indicated that the BMSC sheets do not inhibit the elongation of axons and astrocyte processes.

BMSC sheets promoted axonal regeneration

A spinal cord defect (Fig. 2A) was filled with a folded BMSC sheet (Fig. 2B) or GS (Fig. 2C). Two weeks after transplantation, double labeling of Tuj1 and GAP43 (a marker for growing axons) was performed. The results showed that the majority of Tuj1-positive axons at the sheet-transplanted site overlapped with GAP43-positive axons (Fig. 3A), indicating that almost all axons at the injured site were regenerating axons.

Double labeling of Tuj1 and GFAP was performed at 2 and 8 weeks after transplantation. More Tuj1-positive axons beyond the GFAP-positive glial scar were observed in sheet-transplanted rats compared with GS-transplanted rats at 2 weeks after transplantation (Fig. 3B). At 8 weeks after transplantation, elongated Tuj1-positive axons crossed the transplanted site in the sheet-transplanted animals, but not in the GS-transplanted animals (Fig. 3C). The number of Tuj1-positive axons (green boxes in Fig. 3D) was significantly greater in the sheet group compared with the GS group at 8 weeks after transplantation (Fig. 3E; BMSC, 429 ± 97.17; GS, 118 ± 19.84; $p = 0.047$, $n = 4$ per group). However, there was no significant difference ($p = 0.124$) between the sheet and GS groups at 2 weeks after transplantation (Fig. 3E; BMSC, 64.75 ± 25.00; GS, 12.25 ± 5.79, $n = 4$ per group). These results indicate that BMSC sheets promote axonal regeneration after SCI.

BMSC sheets attenuated glial scar formation and affected the shape of the glial scar

GFAP immunoreactivity in the rostral and caudal spinal cord stumps appeared weaker in the sheet-transplanted rats compared with those of the GS-transplanted rats at 2 and 8 weeks after transplantation (Fig. 4A and 4B). Furthermore, GFAP immunoreactivity at 8 weeks after transplantation showed an irregular and straggly shape lengthwise in the rostro-caudal direction in sheet-transplanted animals, whereas it showed a tightly packed shape with the spinal cords walled off from the transplant at the rostral and caudal stumps in GS-transplanted animals (Fig. 4C). GFAP-positive areas (black-outlined boxes in Fig. 4D) in the sheet group were significantly reduced compared with those of the GS group at both time points (Fig. 4E, 2 weeks: BMSC, 799 429 ± 71 588 μm^2; GS, 159 0592 ± 190 938 μm^2; $p = 0.008$, $n = 4$; 8 weeks: BMSC, 403 005 ± 95 128 μm^2; GS: 130 5847 ± 186 634 μm^2; $p = 0.005$, $n = 4$ per group). These results indicated that the BMSC sheets attenuated glial scar formation after SCI with affecting the shape of reactive astrocytes.

BMSC sheets improved locomotor function compared to GS

The BBB scores were measured at 0, 1, 2, 4, and 8 weeks after transplantation (Fig. 5). Significant differences in BBB scores between the sheet and GS groups were observed at 4 weeks (sheet: 5.13 ± 0.32; GS: 2.75 ± 0.48; $p < 0.05$, $n = 6$) and 8 weeks (sheet: 5.25 ± 0.14; GS: 3.00 ± 0.29; $p < 0.05$ vs. GS, $n = 6$) after transplantation. These results indicated that the BMSC sheet improved locomotor function compared with GS transplantation.

Discussion

Several studies have demonstrated that transplantation of BMSCs (non-sheet form) was effective for axonal regeneration and enhanced hind limb motor function in both contusive[4,8,24] and transected[33] SCI rat models. The present study demonstrates that the sheet form of BMSCs is also effective for promoting axonal regeneration and functional improvement in a transected SCI model. Transplanted BMSCs do not tend to differentiate into neural cells in the host's central nervous system.[6,9] Thus, the observed regenerating axons from the rostral to caudal stumps are considered to be the hosts' neurons.

Recent evidence has shown that the inhibition of axonal regeneration is mainly due to

the formation of a glial scar, which is a type of secondary damage following SCI.[34] The present study also showed that BMSC sheets suppress glial scar formation in the same manner as observed for the non-sheet form of BMSCs.[1,10,24,30] Considered together with the result of the co-culture experiment of the sheet and neurospheres, BMSC sheets seem to provide a permissive environment for injured axons. Recent evidence suggests that a glial scar formed by GFAP-positive astrocytes does not always inhibit axonal regeneration, but rather the shape of the scar is a more important factor for its inhibitory effect.[29] The same study described that the penetration of astrocyte processes into a transplant is an indicator of a permissive environment for axonal regeneration. Our results showed that GFAP-positive processes were elongating toward the sheet-transplanted site, especially at 8 weeks after transplantation. In contrast, these features were not observed in the GS-transplanted site, where tightly packed GFAP-positive astrocytes walled off the spinal cord from the transplant. These results demonstrate that BMSC sheets not only suppress glial scar formation but also likely provide a good environment for the regeneration of injured axons by affecting the morphology of reactive astrocytes.

Cell sheet technology has attracted broad attention in tissue engineering because transplanted cell sheets can maintain their biological activity and prevent the spilling out

of transplanted cells without scaffolds. Most cases of SCI in clinical settings result from contusion, and the chronic phase involves the formation of cavities and a glial scar. Although the present study used a transection model of SCI, we suppose that BMSC sheets would also be clinically useful for filling these cavities. Another possible usage of BMSC sheets is to fill the defect formed after surgical resection of the glial scar, because the scar can be the wall inhibiting regenerating axons even after cell transplantation. Finally, the present study showed that co-culture of neurospheres and BMSC sheets is possible, which highlights the potential for the co-culture of BMSC sheets and neural stem cells derived from induced pluripotent stem cells as a great step towards clinical application. Further studies are needed before clinical trials of BMSC sheets can be conducted, such as experiments using non-human primates.

Conclusions

The present study indicates the potential therapeutic effects of BMSC sheets for SCI, which would enable autologous transplantation without requiring scaffolds.

Conflict of interest

The authors report no conflict of interest concerning the materials or methods

used in this study or the findings specified in this paper.

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Figure legends

Figure 1. BMSC sheets are permissive for axonal growth and astrocyte extension. (**A** and **B**) The appearance of a BMSC sheet. The sheet could be scraped and picked up with forceps. (**C–E**) Double labeling of ECM proteins, collagen I (**C**), collagen IV (**D**), laminin (**E**), and actin with phalloidin in BMSC sheets. The sheets were immunoreactive for collagen I and laminin, but not for collagen IV. (**F**) Neurospheres were cultured on a BMSC sheet for 3.5 days. Immunocytochemistry using Tuj1 and GFAP revealed extending Tuj1-positive neurites and processes of GFAP-positive astrocytes. All scale bars are 100 μm.

Figure 2. A T8 laminectomy was performed and the spinal cord was transected at the same level with a 2-mm defect (**A**, arrow). A BMSC sheet (**B**) or GS (**C**) was transplanted into the defect in contact with both spinal stumps (arrowheads).

Figure 3. The transplantation of BMSC sheets promoted axonal regeneration across the BMSC sheet-transplanted site. All sections were sliced in the coronal plane. The majority of Tuj1-positive axons at the sheet-transplanted site overlapped with

GAP43-positive axons at 2 weeks after transplantation (**A**, arrow). Many more Tuj1-positive axons beyond the GFAP-positive glial scar were observed at the sheet-transplanted site compared with the GS-transplanted site at 2 and 8 weeks after transplantation (**B**, **C**, the upper and lower figures are the rostral and caudal sides, respectively). At 8 weeks after transplantation, elongated Tuj1-positive axons across the transplanted site were observed only in the sheet-transplanted animals (arrow), but not in the GS-transplanted animals (arrowhead) (**C**). The number of axons with a length of at least 50 μm was counted within the two rectangles 400 μm away from the rostral or caudal stump of the injury site (**D**, counting areas are within the green boxes). The numbers of axons obtained from nine sections at 80-μm intervals were summed (**E**, sheet; $n = 4$, GS; $n = 4$, *Welch's test $p < 0.05$). All scale bars are 200 μm.

Figure 4. BMSC sheets inhibited glial scar formation and resulted in an irregular glial scar shape. GFAP immunoreactivity in the rostral and caudal stumps of the spinal cord appeared to be weaker in the sheet-transplanted animals compared with the GS-transplanted animals at 2 and 8 weeks after transplantation (**A**, **B**, the upper and lower figures are the rostral and caudal sides, respectively). At 8 weeks after transplantation, high-magnification images of GFAP immunoreactivity showed irregular

shapes lengthwise in the rostro-caudal direction in the sheet-transplanted animals, and sharp shapes at the rostral and caudal stumps in the GS-transplanted animals (**C**). GFAP-positive areas within the two rectangles including the rostral or caudal edge of the injury site are shown (**D**, the calculated area is in the black-lined boxes). GFAP-positive areas obtained from nine sections at 80-μm intervals were summed (**E**, all groups, $n = 4$, **t-test $p < 0.01$ vs. GS). All scale bars are 200 μm.

Figure 5. The hind limb locomotor assessment. The graph shows BBB scores in sheet-transplanted and GS-transplanted animals at 1, 2, 4, and 8 weeks after transplantation. Data are presented as the mean ± SEM. Sheet group, $n = 6$; GS group, $n = 6$. *$p < 0.05$ vs. GS.

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Figure 1

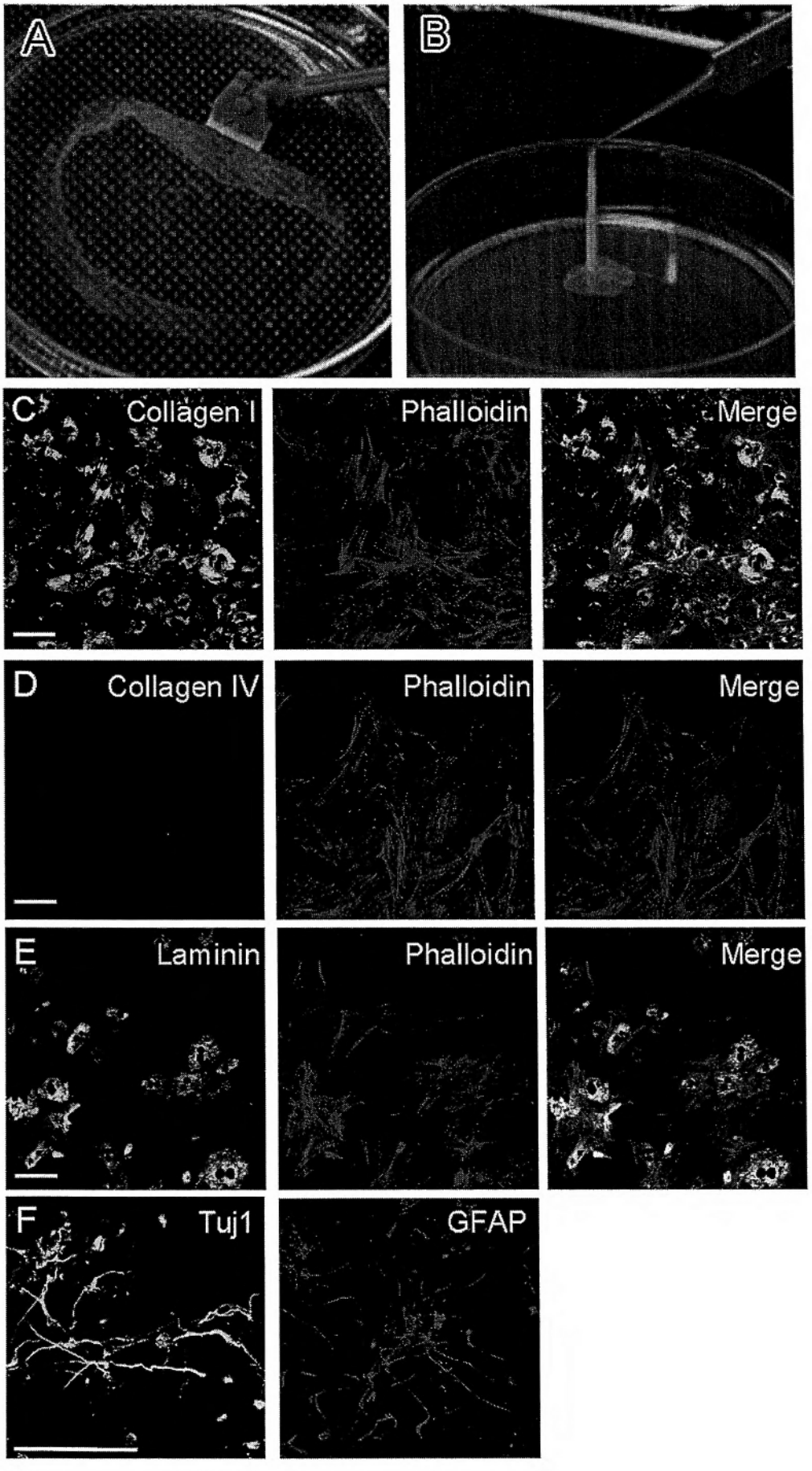

A
B
C
Collagen I
Phalloidin
Merge
D
Collagen IV
Phalloidin
Merge
E
Laminin
Phalloidin
Merge
F
Tuj1
GFAP

Figure 2

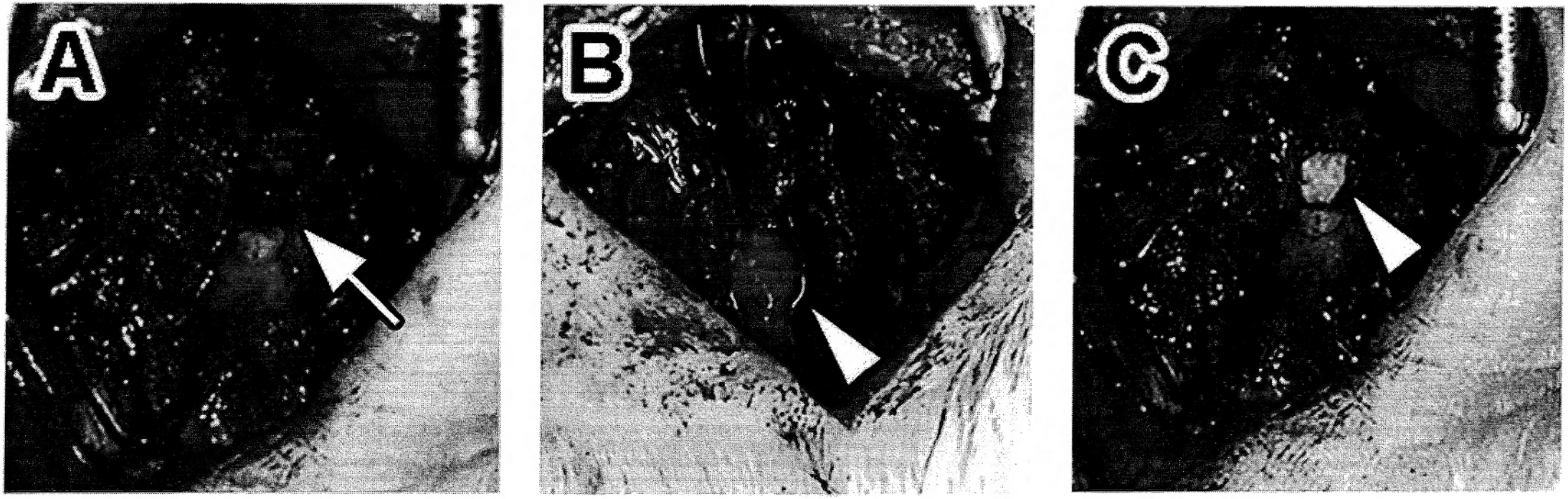
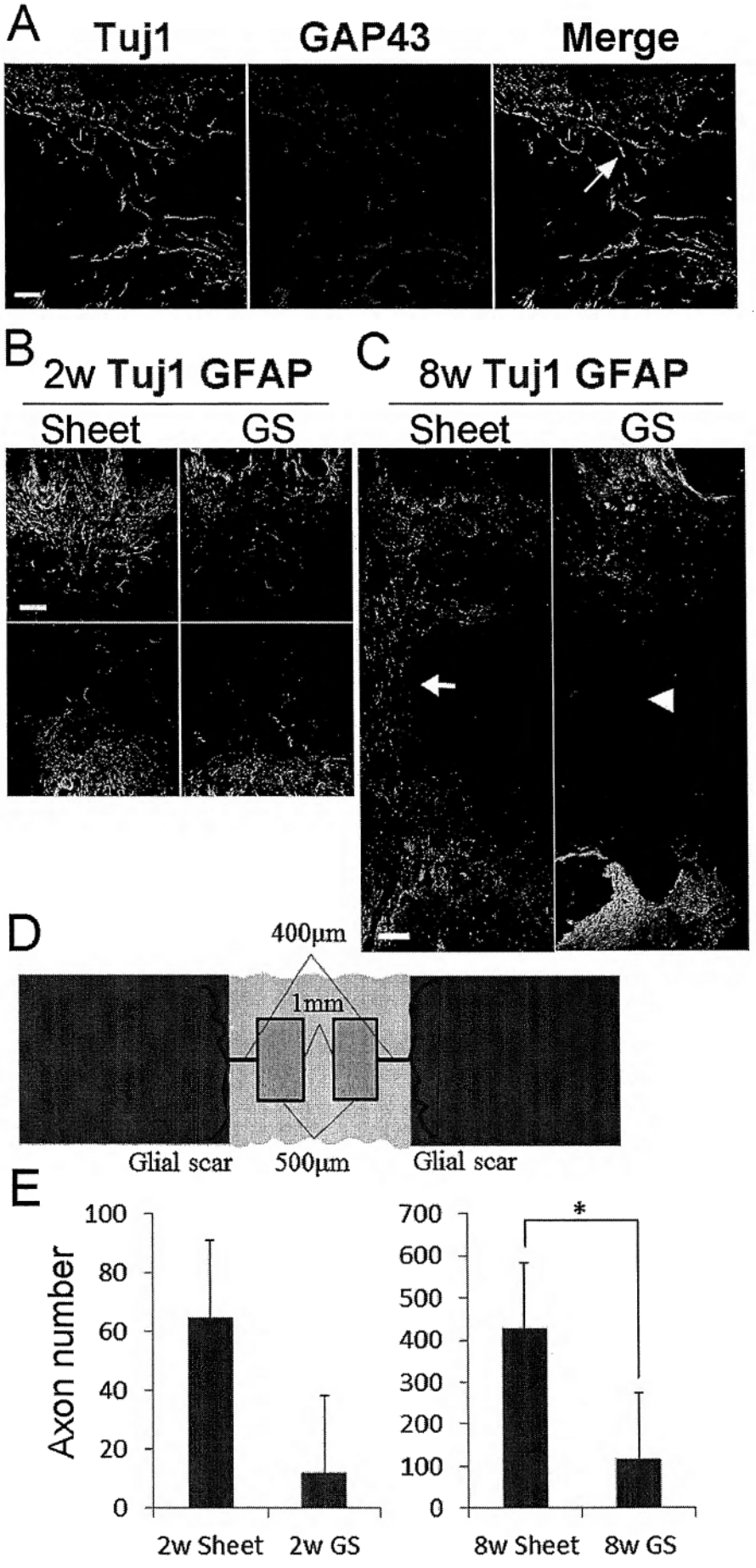


Figure 3


A
Tuj1
GAP43
Merge
B
2w Tuj1 GFAP
Sheet
GS
C
8w Tuj1 GFAP
Sheet
GS
D
400μm
1mm
Glial scar
500μm
Glial scar
E
Axon number
100
80
60
40
20
0
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2w GS
700
600
500
400
300
200
100
0
*
8w Sheet
8w GS

Figure 4

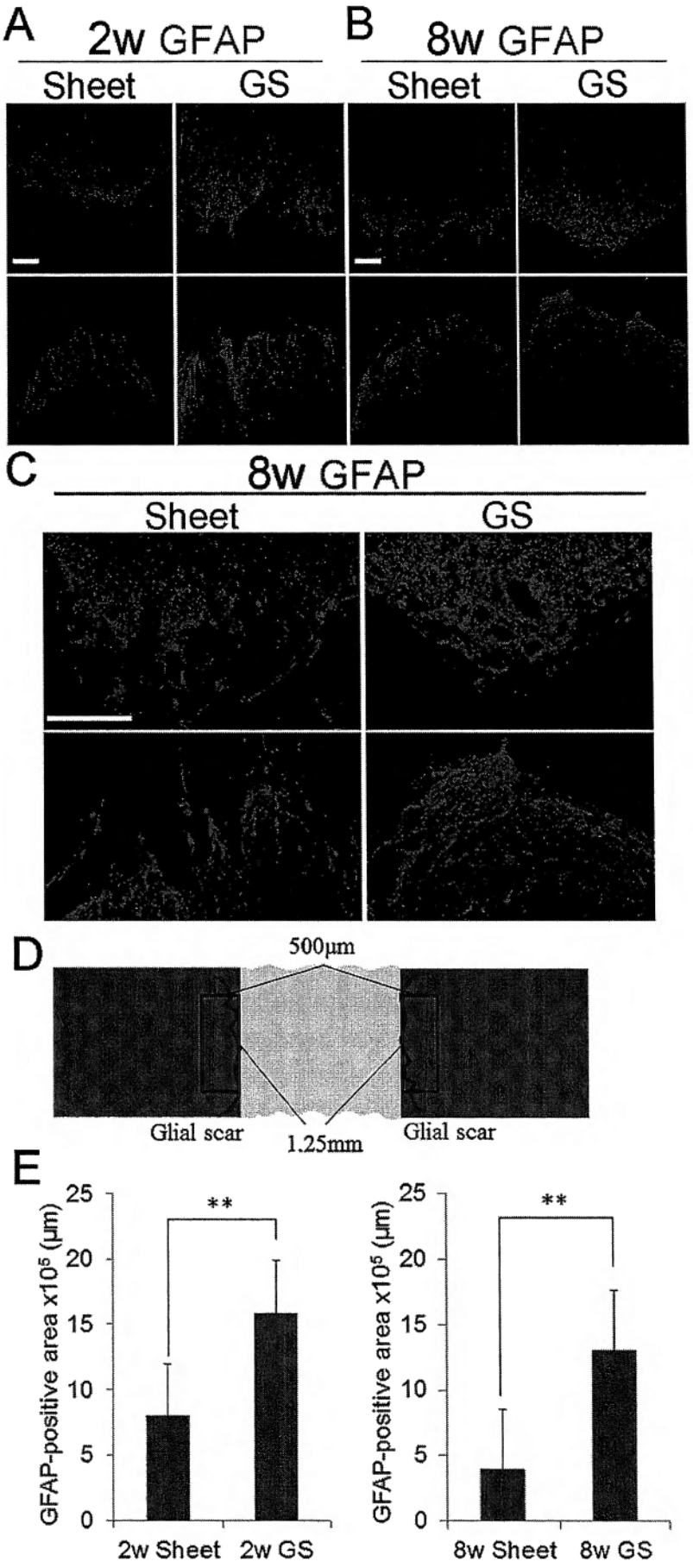
A
2w GFAP
Sheet
GS
B
8w GFAP
Sheet
GS
C
8w GFAP
Sheet
GS
D
500μm
Glial scar
1.25mm
Glial scar
E
GFAP-positive area x10⁵ (μm)
25
20
15
10
5
0
**
2w Sheet
2w GS
GFAP-positive area x10⁵ (μm)
25
20
15
10
5
0
**
8w Sheet
8w GS

Figure 5