Original Article
Transplantation of bone marrow stromal cells enhances infiltration and survival of CNP and Schwann cells to promote axonal sprouting following complete transection of spinal cord in adult rats

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Abstract: This study aimed to investigate the roles of bone marrow stromal cells (BMSCs) in promoting axonal regeneration after complete transection of spinal cord in adult rats. Transplantation was done 9 days after injury. Only a few BMSCs were detected at the injury site 8 weeks after transplantation, yet there was robust growth of axons. The scarcity of surviving BMSCs may attribute to the adverse conditions in their ambient environment. In this connection, the immediate accumulation of a large number of macrophages/reactive microglia following BMSCs transplantation and subsequent cavitation of tissues may be detrimental to their survival. An unexpected finding following BMSCs transplantation was the marked increase in the nestin, GFAP, NF200, olig 3 and CNP positive cells at the injury site. Immunoelectron microscopy showed CNP cells were oval or fibroblast-like and had multiple perineurial-like compartments with long extending filopodia. The spatial relationship between regenerating axons and CNP-positive cells was also confirmed by double immunofluorescence staining. Our results suggest that transplantation of BMSCs elicits the influx and survival of local cells including CNP positive cells and Schwann cells into injury site, which provide structural support for the axon regeneration and remyelination after spinal cord injury.

Keywords: Bone marrow stromal cells, transplantation, CNP cells, Schwann cells, axonal sprouting

Introduction

The failure of damaged nerve fibers to regenerate is an underlying cause of permanent disability experienced by patients after spinal cord injury (SCI). A number of potential approaches have been employed to enhance the axonal growth and optimize functional recovery. These approaches include the manipulation of neuro-inhibitory environment of the spinal cord [1, 2], transplantation of cells or tissues that support the axonal elongation [3-6], delivery of neurotrophic factors to promote axonal growth [7], and maximization of the regenerative potential of endogenous progenitor cells [8-10].

To date, different types of cells including Schwann cells [11], olfactory ensheathing cells (OECs) [12-14], neural stem cells (NSCs) [15, 16], bone marrow stromal cells (BMSCs) [17, 18], and embryonic stem cells (ESCs) [19] have been used to support the axonal growth. This has resulted in progressive functional recovery in animals with SCI, suggesting the great potential of cell transplantation in the therapy of SCI.

BMSCs, referring to as mesenchymal stem cells, are multipotent adult progenitor cells derived from the bone marrow, and have the capability to differentiate into other cell types such as osteoblasts, adipocytes, and chondrocytes [20]. They also have the potential to differentiate into neural cell lineages, such as neurons and astrocytes, both *in vivo* [21, 22] and *in vitro* [23]. Furthermore, the secretion of growth factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), hepatocyte

growth factor (HGF), and glial cell line-derived neurotrophic factors (GDNF) by these cells has been demonstrated both *in vitro* and *in vivo* [24, 25].

Previous studies have indicated that transplanted BMSCs may "form guiding strands" for transected axons regeneration, reduce cavity formation, and promote functional recovery [21, 26, 27]. The fate of the transplanted BMSCs and their interaction with local cells have not been fully elucidated, although there is evidence showing that some of them may be integrated into the new environment and transdifferentiate into neurons [28] and glial cells [29]. Despite the voluminous studies, the sequential events especially at the cellular level at the lesioned site following the transplantation of BMSCs have remained elusive. By immunofluorescence staining and immunoelectron microscopy, this study aimed to investigate the fate of BMSCs and their role in promoting the axonal sprouting following transplantation into adult rats with complete transection of spinal cord.

Materials and methods

In the handling and care of all animals, the International Guiding Principles for Animals Research, as stipulated by the Council for International Organizations of Medical Sciences (CIOMS) and as adopted by the Laboratory Animal Centre, National University of Singapore, were followed. All efforts were made to minimize the number of rats used and their suffering.

Bone marrow stromal cells culture

BMSCs were separated from adult Wistar rats weighing 200-250 g. Rats were killed by an overdose anesthesia with 7% choral hydrate (500 mg/kg). Both femurs and tibias were collected and the bone marrow cavity was flushed with DMEM. The resultant medium was incubated in the presence of 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. After 24 h, non-adherent cells were removed by refreshing the medium. When the cell confluence reached about 100%, cells were passaged at a ratio of 1:3 following digestion with 0.25% trypsin/1 mM EDTA. After passaging for 4 times, the BMSCs were used for immunocytochemistry and transplantation.

Immunocytochemistry

BMSCs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 30 min, and then washed three times with PBS (pH 7.4) containing 0.1% Triton X-100 (Sigma-Aldrich, MO, USA). After treatment in 1% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 h to block nonspecific antibody binding, BMSCs were incubated overnight at 4°C with corresponding antibodies. The secondary antibodies were Cy3-conjugated goat anti-rabbit, anti-mouse, and rabbit anti-goat IgG (1:200; Chemicon, USA). A mounting medium with DAPI (4',6 diamidino-2-phenylindole Vector Laboratories, Burlingame, CA, USA) was used to counterstain the cell nuclei. In negative control group, the BMSCs were incubated in a buffer or normal buffered serum instead of the primary antibody.

Surgical procedures

Adult female Wistar rats weighing about 250 g were used as recipients in this study. Rats were intraperitoneally anaesthetized with 7% choral hydrate (350 mg/kg), and then the spinal cord was completely transected at T10 level according to previously reported [30, 31]. The skin and muscles of the back were incised to expose the T10 vertebra. A T10 laminectomy was then done by using a microsurgery bone rongeur. With a spinal cord hook, the spinal cord was isolated and transected with a pair of microscissors, causing the two stumps completely separated and creating a 2-3 mm gap. A small piece of gelfoam was then inserted into the lesioned site for hemostasis. The gelfoam may also serve as a substratum for the infiltration of host-derived cells and as a new scaffold to support the survival of transplanted BMSCs at the lesioned site. After surgery, these rats were immediately kept under a heating lamp on the first day. Urine was evacuated thrice daily by direct compression of the lower abdominal wall until an autonomous bladder voidance reflex was present. To prevent the urinary tract infection, procaine penicillin G (20000 units, Sigma) was injected subcutaneously twice daily.

Preparation of cells and transplantation

In order to verify the survival and differentiation of transplanted BMSCs, BMSCs were labeled with 5(6)-carboxyfluorescein diacetate, succin-
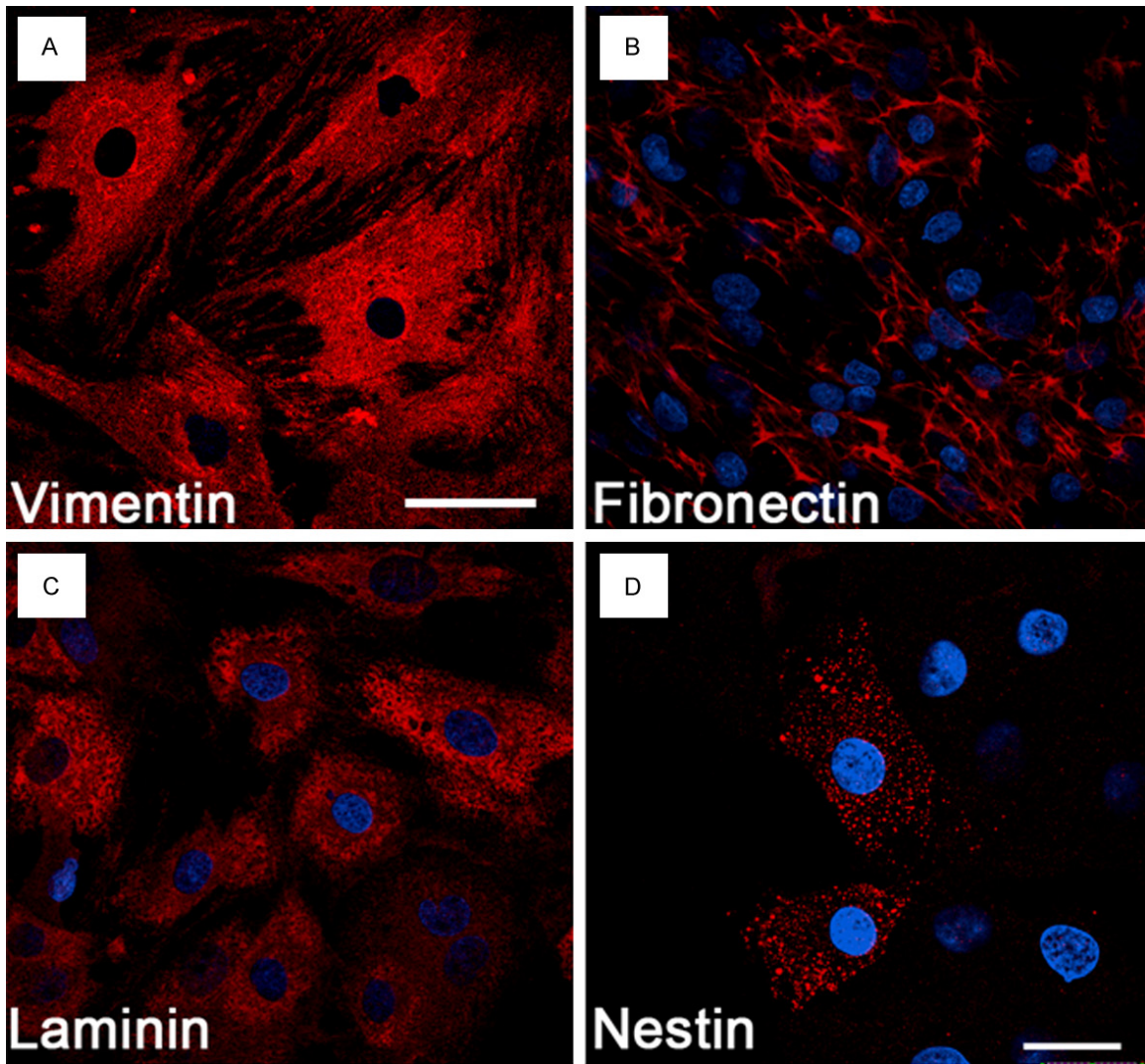
Figure 1. Characterization of BMSCs *in vitro*. Nearly all cultured BMSCs display vimentin- (A), fibronectin- (B), and laminin-immunoreactivity (C), the markers for mesenchymal cells after passaging 4 times. A subset of BMSCs also express nestin, a marker widely used for proliferating neural progenitor cells (D). Blue: DAPI positive nuclei. Scale bar, 50 μm (A-C) and 20 μm (D).

imidyl ester (10 μM CFDA SE; Molecular Probes) because CFDA SE can be inherited by daughter cells after cell division but not be transferred to adjacent cells [32]. Following harvesting, BM-SCs were incubated with 10 μM CFDA SE for 15 min, and re-suspended in a fresh warm medium for 30 min, resulting in CFDA SE acetate hydrolysis. After trypan blue dye exclusion test, the cell density was adjusted to 50,000 viable cells/μl with culture medium for transplantation.

It has been reported that, when compared with immediate transplantation, delayed transplantation enhances the survival of transplanted cells and exerts a beneficial effect on the functional improvement [33]. In the light of this, all experimental rats were maintained for 9 days before BMSCs transplantation or vehicle treatment. Then, a total of 36 rats were re-anesthetized with 7% choral hydrate 350 mg/kg). The injured site of the spinal cord was re-exposed. After irrigating with normal saline, 10 μl of BMSCs suspension was delivered into the injured site; in addition, two 5 μl cells deposits were injected, respectively, into the spinal cord 1 mm cranial and caudal to the lesioned site using a 50-μl Hamilton syringe (n=20). In control group, animals received an injection of
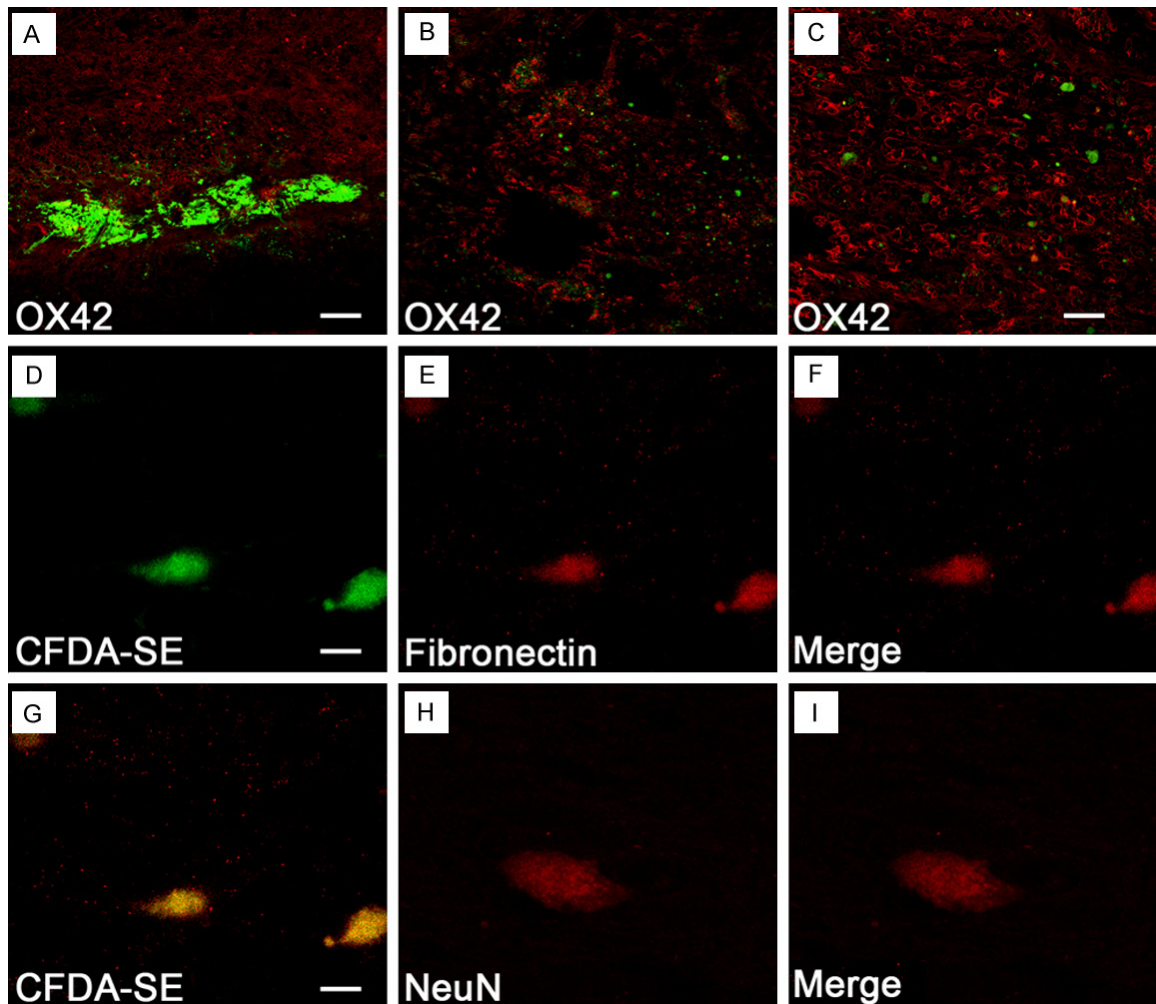
Figure 2. Survival and differentiation of BMSCs in the transected spinal cord at 8 w after transplantation. Although many CFDA-SE labeled BMSCs are surrounded by numerous OX42-positive macrophages at 2 w after transplantation (A), very few cells survive at 8 w at the lesioned epicenter (B). (C) Cells at a higher magnification (B). The BMSCs (D) surviving after transplantation retain fibronectin immunoreactivity (E, F), and a few (G) display faint NeuN-immunoreactivity (H, I). Scale bar, 200 μm (A, B), 100 μm (C), 20 μm (D-F) and 10 μm (G-I).

vehicle solution (n=16). The wound was closed, and animals were housed till sacrifice.

Tissue preparation and immunohistochemistry

Rats subjected to transplantation were allowed to survive for 2 (control: n=4; experiment: n=8), 4 (control: n=4; experiment: n=4) or 8 (control: n=8; experiment: n=12) weeks. At each time point, rats were deeply anaesthetized and perfused transcardially with Ringer's solution, followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The T8 to T12 spinal cord was collected, post-fixed in 4% paraformaldehyde overnight, and then cryoprotected in 20% sucrose for 1 day. The spinal cord was then cryosectioned horizontally at a thickness of 20 mm, mounted onto gelatin-coated glass slides and stored at -20°C until use. The spinal cord sections were processed as described above, and then incubated with a panel of primary antibodies overnight at room temperature. After incubation, these sections were treated with Cy3-conjugated goat anti-rabbit, anti-mouse, or rabbit anti-goat IgG (1:200; Chemicon, USA) for 1 h in dark at room temperature. After washing, the sections were mounted using the mounting medium containing DAPI (4',6 diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, USA). In the negative control group, sections were incubated in
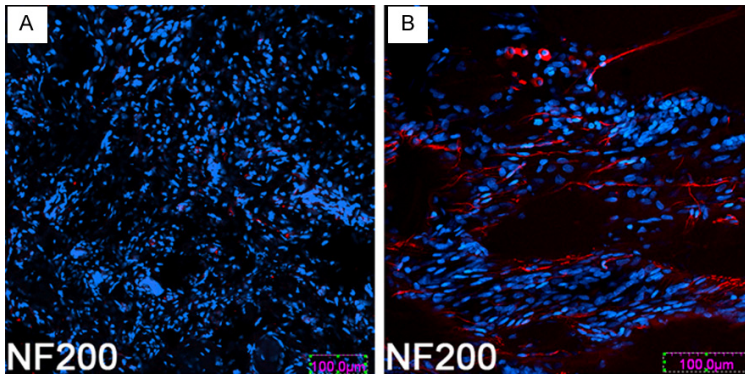
Figure 3. Transplantation of BMSCs enhances the sprouting of axons into the lesioned site. At 8 w after transplantation, few NF200 positive fibers sprouted into and through the lesioned site in control rats (A); however, a large number of NF200 positive fibers are observed in the experiment rats (B). Scale bar, 100 μm (A, B).
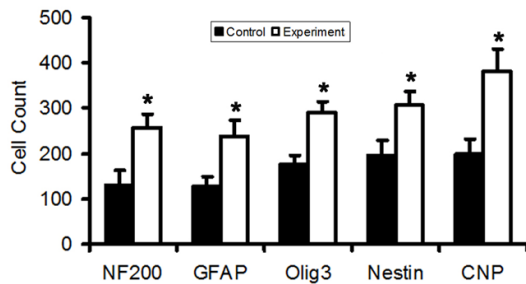

Figure 4. Host-derived cells at the lesioned site. The number of cells positive for nestin, GFAP, NF200, Olig3 and CNP is significantly different between experiment group and control group (P<0.05).

a buffer or normal buffered serum instead of the primary antibody.

Cell quantification

Sections stained with NF200, GFAP, Olig3, Nestin and CNP were used for cell counting. Three sections from the injured spinal cord were used for cell counting in each rat at 8 weeks after transplantation. A total of three fields were randomly selected from each section. Cell counting was carried out under a microscope at the magnification of ×200. Student's t-test was used to compare the cell counts between experiment group and control group. A value of *P* less than 0.05 was considered statistically significant.

Electron microscopy

Four rats receiving BMSCs transplantation were killed at 2 and 8 weeks, respectively; at each time point, two rats were used for routine electron microscopy and the others for immunoelectron microscopy. For routine electron microscopy, rats were killed by perfusion with a mixed aldehyde solution containing 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M PB. For immunoelectron microscopy, rats were killed by perfusion with Ringer's solution and then with 4% paraformaldehyde containing 0.1% glutaraldehyde in 0.1 M PB. The T8-T12 spinal cord was removed and 100-μm vibratome sections were obtained (transversely and horizontally). For immunoelectron microscopy, sections were then incubated overnight at room temperature with mouse anti-CNP (dilution 1:200; Chemicon, USA). The treatment with secondary antibodies was done with anti-mouse ABC kit (Vector Laboratories, Burlingame, CA). The peroxidase reaction was visualized using the 3, 3'- diaminobenzidine (DAB) containing 0.1% nickel as a peroxidase substrate. Post-fixation was performed in 2% osmium tetroxide. Tissue blocks were embedded in araldite mixture. Ultrathin sections were double stained in uranyl acetate and lead citrate or lead citrate alone and observed under a CM120-biotwin or EM208S electron microscope.

Double immunofluorescence staining

In order to clarify the relationship between cells and regenerating fibres, double immunofluorescence staining was carried out with primary antibodies. Secondary antibodies used were FITC-conjugated goat anti-rabbit and Cy3-conjugated goat anti-mouse antibodies. The procedures for immunofluorescence staining were those described above.

Result

Characterization of MSCs in vitro

BMSCs were subjected to immunocytochemistry after passaging 4 times. Virtually, all of these cells expressed vimentin, fibronectin and laminin, markers of mesenchymal cells (**Figure 1A-C**). A subpopulation of BMSCs expressed
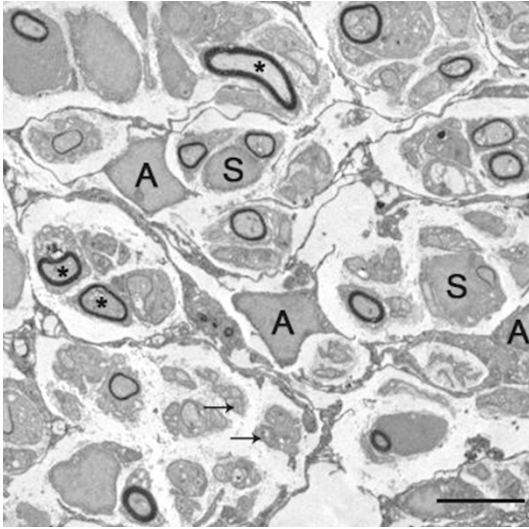
Figure 5. Astrocyte-like CNP positive cells (A) with emanating filopodial processes form multiple peri-neurial-like compartments containing a varying number of myelinated (asterisks) and unmyelinated (arrows) axons. The axons are associated with Schwann cells (S). Two weeks after BMSCs transplantatioin. Scale bar, 5 µm.
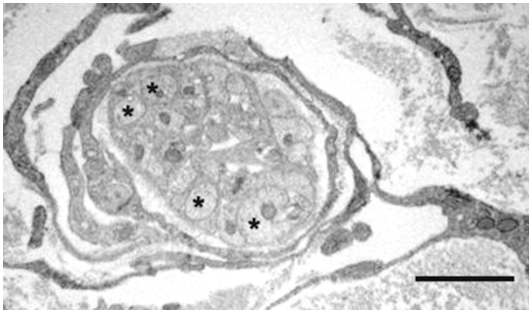

Figure 6. Multilayered perineurial-like sheath encloses a bundle of unmyelinated axons (asterisks). Scale bar, 2 µm.

nestin, a marker widely used for proliferating neural progenitor cells (**Figure 1D**).

Survival and differentiation of MSCs in vivo

Although a large number of transplanted BM-SCs labeled by CFDA-SE were observed at 2 w after transplantation (**Figure 2A**), they markedly reduced in rats surviving for a long time, and only a few were detected at the lesioned site at 8 w after transplantation (**Figure 2B**, **2C**). The surviving cells were localized mainly in areas either rostral or caudal to the epicenter and appeared to have integrated into the host spinal cord. At different time points, the surviving BMSCs were often distributed near or sur-
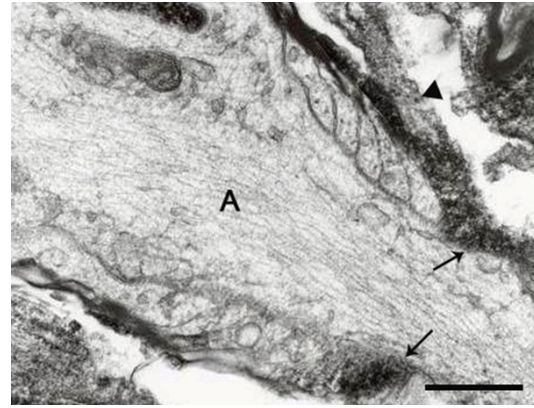

Figure 7. CNP is localized in the paranodal loops as well as in a filopodia (arrowhead) contacting an axon (A). Scale bar, 0.5 µm.

rounded by reactive macrophages as confirmed by OX42 immunofluorescence staining (**Figure 2A-C**). Indeed, many reactive macrophages were found to infiltrate the lesioned epicenter at 2 w after transplantation in both control and experiment rats. Besides the infiltration of reactive macrophages, another distinct feature at the lesioned epicenter was the formation of cavities beginning at 4 w after transplantation in both control and experiment rats. In experiment rats, however, the cavities noticeably reduced in size as they were traversed by strands of nerve fibers (**Figure 2B**, **2C**). The surviving CFDA-SE labeled BMSCs were oval or spindle-shaped, had long processes and expressed fibronectin (**Figure 2D-F**) and NeuN (**Figure 2G-I**). However, none of them showed nestin, vimentin, laminin, GFAP, CNP, and Olig3 immunoreactivities after transplantation.

MSCs transplantation promotes axonal sprouting into the lesioned site

Although very few BMSCs were found at 8 w after transplantation, all rats receiving BMSCs transplantation showed robust growth of NF-200 immnopositive axons into the lesioned site (**Figure 3B**); similar fibers were barely observed in control rats (**Figure 3A**). However, a variable number of regenerating axons were found to sprout into the lesioned site where BMSCs were transplanted.

MSCs transplantation enhances the infiltration and survival of host-derived cells at the lesioned site

In agreement with Hokari [34], a large number of nestin- and GFAP-positive cells appeared at
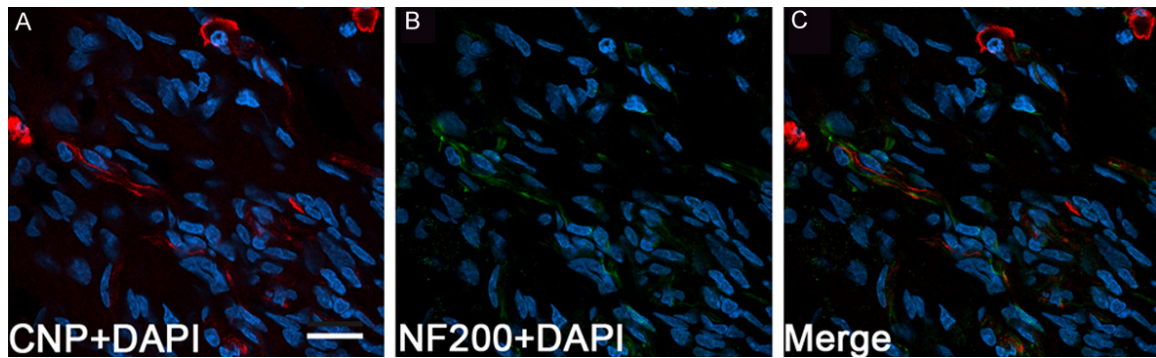
Figure 8. Spatial relationship between regenerating axons and CNP-positive cells. At 8 w after transplantation of BMSCs, many CNP positive cells and their long extending fibers (red, A) show close spatial relationship (C) with NF200 positive fibers (green, B). Blue: DAPI positive nuclei. Scale bar, 20 μm (A-C).

the lesioned site following BMSCs transplantation when compared with corresponding controls. Our results showed a concomitant increase in host-derived CNP, NF-200 and Olig3 positive cells in the same area of rats killed at 8 w. Similar cells were almost undetectable in the control group. The number of cells positive for nestin, GFAP, NF200, Olig3 and CNP was significantly different (P<0.05) between experiment group and control group (**Figure 4**).

Relationship of CNP positive cells with sprouting axons and other host-derived cells

Many studies have reported the roles of Schwann cells in the remyelination and axonal regeneration after SCI [35-38]. In order to clarify the relationship between host-derived myelinating cells (Schwann cells and oligodendrocytes) and regenerating axons after BMSCs transplantation, immunoelectron microscopy was performed using their common marker CNP. Immunoelectron microscopy confirmed that there were many CNP positive cells at lesioned site at 2 and 8 w after BMSCs transplantation. These cells had long extending filopodia forming a single- (**Figure 5**) or multi-layered (**Figure 6**) perineurial-like lamellae delineating bundles of axons and associated Schwann cells into numerous compartments. In a fortuitous longitudinal section of an axon, a CNP positive filopodial process was in direct contact with the axolemma at the Ranvier node as well as in close approximation with the paranodal loop (**Figure 7**). The spatial relationship between regenerating axons and CNP-positive cells was also confirmed by double immunofluorescence staining (**Figure 8**).

Discussion

Although axonal regeneration is extremely limited in the central nervous system of adult mammalians, spontaneous axonal sprouting and functional improvements may follow a partial lesion of the spinal cord [39, 40]. Recent studies using different animal models and stem cells transplantation have shown an enhanced axon sprouting after SCI. There is strong evidence suggesting that stem cell transplantation facilitates the axonal sprouting, yet the relationship between the transplanted cells and regenerating axons, especially at cellular level, remains elusive. For example, it remains to be clarified if cells (such as olfactory ensheathing cells, [OECs]) used for transplantation could transdifferentiate into so-called perineurial-like cells and Schwann cells at the lesioned site [41]. It has been reported that invading Schwann cells rather than implanted OECs are solely responsible for the myelination of axons at the lesioned site [41, 42]. Indeed, according to the study of Ramer [42] et al the transplanted OECs were undetectable at the lesioned site.

In the present study, a complete transection of the spinal cord was prepared in which a 2-3 mm gap was created between two stumps of the spinal cord, and the possible axonal regeneration was investigated after BMSCs transplantation. At 8 w after transplantation, very few BMSCs labeled with CFDA SE and expressing fibronectin were detected and appeared to integrate into the host spinal cord at the lesioned site. Meanwhile, a large number of OX42-positive macrophages accumulated at

the lesioned site. Reactive microglia/macrophages are known to secrete a plethora of inflammatory cytokines such as TNF-α and IL-β [43] that may be detrimental to the survival of BMSCs at the lesioned epicenter. This would explain the scarcity of BMSCs at later time points. Another possible explanation for the marked reduction of BMSCs is the formation of large cavities that are probably not helpful for the survival and growth of transplanted cells. It has been reported by Li et al [44] that the transplanted OECs decreased in number over time, and that they were undetectable at the lesioned site at 13 w after transplantation, suggesting that the lesioned epicenter was not helpful for the survival of transplanted cells. The occurrence of some surviving BMSCs expressing NeuN suggests their potential of transdifferentiating into neurons. On the other hand, none were labeled with antibodies against GFAP, laminin, vimentin, nestin, Olig3, and CNP [34]. In addition, there was no strong evidence that the BMSCs were spatially related to the regenerating axons. This, along with their rarity in number, argues against the possibility that the cells are directly involved in the myelination and maintenance of sprouting axons.

The present study showed the robust axonal sprouting across the lesioned site despite the fact that it contained only sporadic BMSCs. As similar features were not evident in control rats, it is concluded that transplantation of BMSCs did facilitate the regeneration of fibers but it remains to elucidate the mechanisms. The combined use of BMSC and OEC may provide an improved approach for the treatment of SCI [45]. Hence, the involvement of BMSCs in promoting or facilitating the axonal sprouting, if any, might be indirect. To this end, it is desirable to examine other cellular changes during the axonal sprouting. It is significant to note the appearance and rise of numerous nestin and GFAP positive cells at the lesioned site following the MSCs transplantation, a feature that has also been reported by Hokari et al [34]. This study confirmed this and added fact that there was a concomitant increase in host-derived nestin-, GFAP-, NF200-, Olig3 and CNP positive cells. The coincident occurrence of above-mentioned cells, in particular, the close spatial relation between CNP cells and regenerating fibers, strongly indicates that they functionally link to the regeneration process.

As drastic increase in CNP positive cells at the lesioned site following BMSCs transplantation is a novel and major finding of this study, we further performed immunoelectron microscopy to investigate the role of these cells in nerve regeneration. 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) is expressed as two isoforms with an apparent molecular weight of 46 kDa (CNP1) and 48 kDa (CNP2), which are produced by alternative ribosomal initiation at two different AUG codons, thus differing each other only by the 20-amino acid extension at the N terminus [46]. In addition to the myelinating cells such as oligodendrocytes and Schwann cells [47, 48], CNP is also expressed in various cell types such as retinal cells [49], liver cells [46], Purkinje cells [50], hippocampal neurons (soma and dendrites) [51], neural stem cells [52], astrocytes [53] and glioblasts [54] indicating more general cellular functions. Despite its lengthy history, relatively little is known about the function of CNP. Recent biochemical evidence indicates that CNP interacts with mitochondria and cytoskeletal proteins in certain non-glial cells, and serves as a membrane anchor for tubulin [55]. In addition, study also revealed that CNP over-expression in cultured cells increased the outgrowth of filopodia [56]. Recently, Cnp1, a gene encoding both CNP1 and CNP2 in the oligodendrocytes, was found to be essential for the axonal survival but not for myelin assembly [57], because CNP1 knockout mice developed axonal swelling and neurodegeneration throughout the brain, but the ultrastructure, periodicity and physical stability of myelins were remained unchanged in these mice. Our results were consistent with those reported by Lappe-Siefke [57].

Immunoelectron microscopy showed the extensive prolongation of filopodia from the CNP positive cells which may be linked to the contents of enzyme known to interact with microtubules [58] as well as housekeeping function in organelle transport [57].

The present results revealed that host-derived CNP positive cells formed perineurial-like compartments containing regenerating axons and associated Schwann cells, a feature also reported after OECs transplantation. Indeed, the CNP and Schwann cells displayed features resembling the so-called A and S cells, respectively, as reported by Li et al [59, 60]. Although

there is still controversy on whether the OECs can transdifferentiate into so-called perineurial-like cells and Schwann cells at the lesioned site [41], on the basis of the configuration of filopodial processes of CNP cells and their spatial relation with axons presently described, it is likely that they provide structural support in the form of compartments or tunnels for fiber growth. Some CNP positive processes were in close apposition to the axolemma suggesting their direct interaction with some factors for nerve growth. However, this remains to be further elucidated. Despite the above mentioned, our results showed the CNP may function to form perineurial-like compartments and remyelination of axons by Schwann cells.

Although BMSCs may not be directly associated with axonal sprouting, their possible involvement in promoting infiltration and survival of CNP and Schwann cells should be considered. We speculate that some trophic factors [61] secreted by the transplanted BMSCs may provide an optimal environment for the infiltration and survival of CNP and other host-derived cells.

This study shows that very few transplanted BMSCs actually survive and integrate into the local spinal cord following transplantation. The scarcity of surviving BMSCs may be attributed to the adverse environment into which the cells were transplanted. The transplantation site was filled with a large number of macrophages/reactive microglia and the presence of cavities in the spinal cord may not be helpful for their survival. The most dramatic feature following BMSCs transplantation in this study is the presence of a large number of host-derived CNP positive cells. The CNP positive cells form many perineurial compartments delineating a varying number of axons, strongly suggesting their support, perhaps both structurally and developmentally, for the fiber regeneration. It is also evident that Schwann cells are key cells involved in axon remyelination. The mechanistic link between BMSCs and host-derived cells is vital for a better understanding of sequential events promoting axonal growth. Likewise, the source of CNP and Schwann cells as well as their roles and mechanisms in guiding the growth of regenerating axons should be further studied.

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