

Commentary

## Are the long-term survival, proliferation, and differentiation of transplanted cells desirable in clinical application for spinal cord injury?

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### Abstract

Cell transplantation studies of spinal cord injury have a premise that the transplants should be integrated in the host spinal cord tissue, differentiate into neural cells, and re-establish neural circuits, leading to the improvement of locomotor functions. However, the long-term survival, extensive proliferation, and/or differentiation of transplanted cells are not necessarily desirable clinically, and may, on the contrary, cause serious problems regarding the safety of transplants. The excessive proliferation, migration, and/or differentiation of transplanted cells may deteriorate the histological as well as functional organization of the host spinal cord. The present communication will discuss the feasibility of using three kinds of cell as transplants, including bone marrow-derived cells (BMDCs), Schwann cells, and neural stem/progenitor cells (NSPCs).

BMDCs enhance tissue recovery and locomotor improvements; however, they disappear within 2-3 weeks after transplantation from the host spinal cord. This indicates that BMDCs do not serve as scaffolds for the growth of regenerating axons, but promote "endogenous" regenerating capacities of the host spinal cord, probably by secreting some trophic factors. This short-term survival of transplants, although appearing to be a disadvantage, guarantees the safety of cell transplantation. The transplantation of BMDCs is now at the Phase I/II stage of clinical application.

Schwann cells have been studied extensively as a transplant material for spinal cord injury. Schwann cells survive long-term, and moderately proliferate and/or migrate in the spinal cord. It can be said that Schwann cells become well integrated in the host spinal cord. Therefore, they are regarded as a safe transplant.

NSPCs proliferate, migrate, and differentiate extensively after transplantation in the host spinal cord. It is impossible at present to manipulate or control the proliferation/migration/differentiation of NPSCs to make them properly integrate in the host spinal cord. NSPCs are not considered safe for clinical application. BMDCs and Schwann cells are clinically relevant, while NS/PCs are clinically irrelevant.

**Key words:** spinal cord injury, bone marrow-derived cells, Schwann cell, neural stem/progenitor cell, clinically relevant, safety

Abbreviations :

BDNF : brain-derived neurotrophic factor, BMDC : bone marrow-derived cell, BMNC : bone marrow mononuclear cell, BMSC : bone marrow stromal cell, CNS : central nervous system, CPC : cell-processing center, CPEC : choroid plexus epithelial cell, CSF : cerebrospinal fluid, EGF : epidermal growth factor, ES : embryonic stem, FGF : fibroblast growth factor, GFAP : glio-filament-associated protein, GDNF : glial cell line-derived neurotrophic factor, GGF : glial growth factor, HGF : hepatocyte growth factor, IGF : insulin-like growth factor, iPSC : induced pluripotent stem cell, NSPC : neural stem/progenitor cell, SCI : spinal cord injury, TNF : tumor necrosis factor, NT-3 : neurotrophin -3, OEC : olfactory ensheathing cell, PDGF : platelet-derived growth factor

## Introduction

Many kinds of cell, such as bone marrow-derived cells (BMDCs) that include bone marrow stromal cells (BMSCs) and bone marrow mononuclear cells (BMNCs), Schwann cells, olfactory ensheathing cells (OECs) (Li et al., 1998), adipose-derived stem cells (Arboleda et al., 2011), skin-derived stem cells (Biernaskie et al., 2007), umbilical cord-derived stem cells (Yang et al., 2008), dental pulp-derived stem cells (Sakai et al., 2012), choroid plexus epithelial cells (CPECs) (Kanekiyo et al., 2015), and neural stem/projector cells (NSPCs), have been studied as transplants in experimental studies for the treatment of spinal cord injury.

NSPCs are derived from the embryonic/newborn CNS, induced pluripotent stem cells (iPSCs), or embryonic stem (ES) cells. All other cells are derived from adult somatic or umbilical tissues: BMDCs and adipose- and umbilical-derived stem cells can be categorized as mesenchymal stem cells, while Schwann cells, OECs, and CPECs belong to the peripheral or central nervous system.

BMDCs, Schwann cells, and OECs have been studied extensively with promising results, and are now at the Phase I/II stage of clinical application. On the other hand, NSPCs have serious problems regarding clinical application. NSPCs derived from ES cells have ethical problems, and those derived from iPSCs have tumorigenic problems. NSPCs survive long-term, proliferate, and differentiate into neural cells such as neurons and glial cells, after transplantation. These properties, although appearing favorable for transplant materials, pose critical problems in clinical application.

The present communication deals with three kinds of cell: BMDCs and Schwann cells as somatic cells, and NSPCs as embryonic/artificially manipulated stem cells. We discuss their positive and/or negative properties of them from the point of view of transplant safety.

We believe that the cell transplantation studies aiming at the regeneration of tissues or organs have significance only when they are clinically applicable under the condition that the transplants are safe and they promote functional improvements of recipients.

## 1. BMDCs

BMDCs include BMSCs and BMNCs. BMSCs are cells that adhere to the dish in cell culture of bone marrow perfusate. BMNCs are nucleated cells obtained without culture after erythrocytes are removed from the bone marrow perfusate.

The effects of BMSCs on the repair of an injured spinal cord and locomotor improvement were studied by Ohta et al. (2004). This pioneering work adopted a new method for cell transplantation: BMSCs were infused through the 4<sup>th</sup> ventricle into the CSF. The cell infusion was done immediately after contusion injury of the spinal cord. Infused cells, after flowing through the CSF, attached to the surface of the spinal cord, some of which invaded the spinal cord lesion. These cells disappeared before 3 weeks after infusion from the spinal cord. Locomotor functions were clearly improved, and cavity formation in the spinal cord lesion was suppressed. It was suggested that host spinal cord axons near the lesion did not undergo secondary degeneration, but were spared in BMSC-transplanted rats. These findings indicate that BMSCs did not serve as a scaffold of regenerating axons, but probably released some trophic factors, which were effective for tissue repair, and axonal outgrowth and preservation, in the spinal cord injury.

This study opens up a new field for the treatment of spinal cord injury: BMSCs are safe and effective transplants, because they do not survive long-term in the spinal cord, while enhancing locomotor recovery. In addition, the transplantation of BMSCs through the CSF means that the lumbar puncture technique can be used for cell

transplantation in clinical usages.

In the next study, we injected BMSCs directly into the spinal cord lesion 2 weeks after injury (subacute spinal cord injury, Ide et al., 2010). Transplanted BMSCs survived as cell assemblies for 1–2 weeks in the lesion, which remained as an astrocyte-devoid area, appearing as empty cavities on immunostaining for GFAP. However, it was found that such astrocyte-devoid spaces are filled with extracellular matrices including collagen fibers. Numerous axons associated with Schwann cells grew out through such extracellular matrices in astrocyte-devoid areas (Fig. 1). These axons were myelinated by Schwann cells,

displaying peripheral nerve-like structures. This indicated that these axons might not be spared ones, but regenerated ones in the spinal cord lesion. There was no finding indicating the blocking of axonal extension at the border of the lesion. No astrocytic scar was formed at the border. Cavity formation was reduced in cell-transplanted rats. Locomotor behavior improved to 9–10 points as assessed by the BBB scale, while it remained at 5–6 points in the control. This study demonstrated that, BMSCs, even though they disappeared 1–2 weeks after transplantation, promoted axonal regeneration and locomotor improvement, probably by secreting some trophic

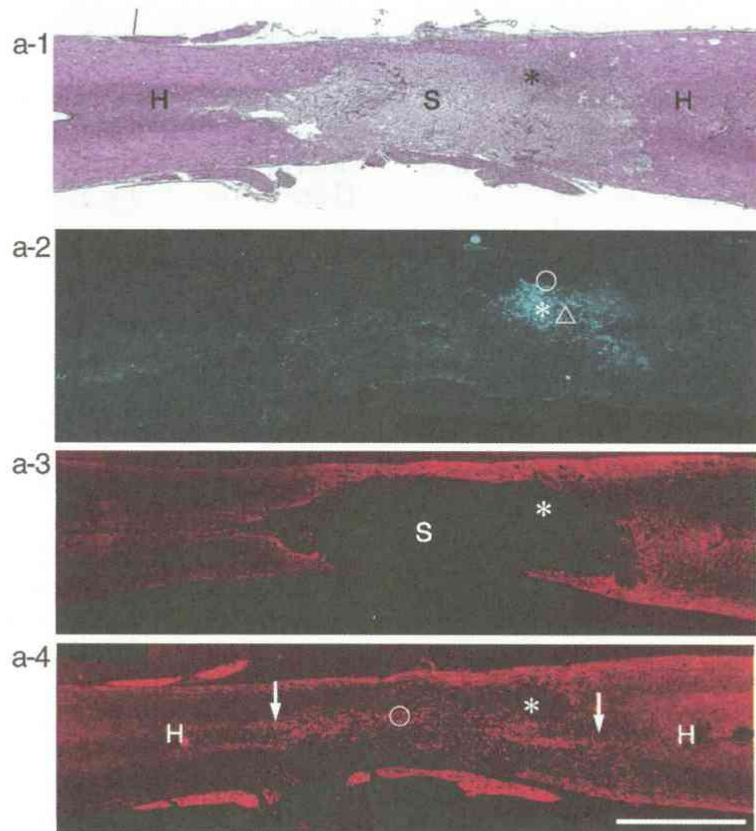


Fig. 1 The spinal cord was contusion-injured at T8–9 in adult rats, and BMSCs were transplanted into the spinal cord lesion at 2 weeks post-injury. Rats were fixed at 1 week post-transplantation, and horizontal sections of the spinal cord lesion were observed. Left to right: rostro-caudal direction. a-1: HE staining. The lesion is filled with tissue (S) different from the spinal cord parenchyma (H). An asterisk indicates the site of engrafted BMSCs shown in a-2. a-2: The section adjacent to a-1. Engrafted BMSCs are found as cell assemblies (\*). a-3: GFAP immunohistochemistry (red) in the section adjacent to a-2. A large astrocyte-devoid area (S) extends rostro-caudally. Engrafted BMSCs (site indicated by asterisk) are located at the border of the astrocyte-devoid area. a-4: Immunohistochemistry for neurofilaments in the section adjacent to a-3. Numerous axons (red) extend in a bundle along the total length of the astrocyte-devoid area shown in a-3. Numerous regenerating axons extend through the lesion of the spinal cord injury. There is no finding suggesting the blocking of extension of growing axons at the transition zones (arrows) on the rostral or caudal side. The asterisk indicates the site of engrafted BMSCs. H shows spinal cord parenchyma. Scale: 2 mm  
From Ide et al. (2010).

factors.

We further advanced the experiment: BMSCs were infused through the CSF three times (once weekly) in subacute (1 and 2 weeks after injury) and chronic (4 weeks after injury) groups (Nakano et al., 2013). Similar to a preceding study (Ide et al., 2010), numerous axons associated with Schwann cells extended through the extracellular matrices (connective tissues) in the astrocyte-devoid areas of the spinal cord lesion. In the 1-week post-injury group, a small number of BMSCs were found to have survived at 2 days after injection within the spinal cord lesion, but none were found at 7 days. No BMSCs were found in the spinal cord lesion 2 or 7 days after injection in the 2- or 4-week post-injury groups. In all 3 groups, BBB scores increased from 1-4 points before injection to 9-11 points at 4 weeks after the initial injection, while they increased only slightly from 1-4 to 3-5 points in the control. These findings indicate that, like in the preceding study, BMSCs exerted their effects not by serving as scaffolds for regenerating axons, but by secreting trophic factors effective for the regeneration and extension of axons in the spinal cord lesion.

It should be noted that, even in the control group, there were a few regenerating axons extending through the astrocyte-devoid area. This indicates that the spinal cord has "endogenous" abilities to repair the lesion by forming extracellular matrices, through which regenerating axons extend.

In addition to the study of BMSC transplantation, we studied the transplantation of BMNCs in rats with SCI (Yoshihara et al., 2007). Mononuclear cells were separated from bone marrow infusate by density-gradient centrifugation. The separated cells include those that are positive for CD90 (Thy-1), CD45 (for leukocytes), CD29 ( $\beta$ 1-integrin), CD 117 (c-kit), CD34 (for hematopoietic stem cells), CD31 (for endothelial cells) and CD11/b (for macrophages). One hour after contusion injury of the spinal cord, a cell suspension was injected into the 4<sup>th</sup> ventricle. Injected cells were found in the choroid plexus and on the spinal cord surface at 3 days, but were no longer observed at 7 days after transplantation. The BBB scores increased to 15 points at 5 weeks post-transplantation, while they were 10 points in the control.

Cavity formation was suppressed and blood vessel formation was promoted.

Regenerating axons were observed within the lesion. The CSF harvested 3 days after cell infusion showed increased HGF and decreased

TNF- $\alpha$ . BMNCs disappeared before 7 days post-transplantation. These findings indicate that BMNCs might release some trophic factors into the CSF, leading to axonal regeneration, blood vessel formation, and the suppression of cavity formation.

Based on these studies, we advanced to the clinical application of BMSCs for patients with SCI. The fact that BMSCs do not survive long-term to be integrated into the host tissue, but they still promote the locomotor function and axonal regeneration, indicate that they are safe and effective as transplants for the treatment of SCI. The first transplantation to a patient was performed in 2006 (Saito et al., 2008), and a total of 5 patients received BMSC transplantation up until 2009 (Saito et al., 2012). To our knowledge, the patient who received BMSCs in 2006 was the first case of BMSC transplantation for spinal cord injury in Japan. Cell transplantation was performed by lumbar puncture in all 5 patients. There was no adverse effect, and patients showed varying levels of recovery in locomotor and sensory functions, revealing that the transplantation of BMSCs by lumbar puncture is promising for the treatment of patients with SCI.

We advanced to the use of BMNCs for clinical application. BMNCs do not need to be cultured before transplantation. This is an advantage of BMNCs over BMSCs. BMNCs can be separated immediately after obtaining them from the iliac bone of the patient, and transplanted by lumbar puncture without culturing in an expensive Cell Processing Center (CPC). So far, 10 patients have received BMNC transplantation by lumbar puncture. Six months after transplantation, patients were assessed regarding sensory and motor functions. There was no adverse effect in any of the 10 patients. Patients showed varying degrees of improvement in motor, light touch, and pin prick scores (Suzuki et al., 2014). This was a pioneering study showing that the BMNC transplantation by lumbar puncture is effective and feasible for patients in a hospital with no CPC facilities. BMNCs are promising for the treatment of patients with SCI.

## 2. Schwann cells

Since the epoch-making, elegant study by David and Aguayo (1981) demonstrated that transplanted peripheral nerve segments provide a favorable environment for the growth of regenerating axons in the CNS, Schwann cells have been regarded as a key cell to provide an

appropriate environment for the growth of regenerating axons in the CNS. They showed that Schwann cells also worked as scaffolds for the growth of regenerating axons also in the central nervous system, indicating that Schwann cells may be effective transplants for spinal cord injury. Since then, many studies have been performed using Schwann cells as transplants to promote nerve regeneration in the spinal cord injury (William and Bunge, 2012).

Schwann cells were obtained from adult peripheral nerves. Small nerve explants were allowed to undergo axonal and myelin breakdown, and enzymatically dissociated. Dissociated Schwann cells were treated with glial growth factor (GGF) and forskolin, and cultured for 10 weeks. Thus, Schwann cells were derived from the adult nerves with no genetic manipulation.

The problem with Schwann cells is that regenerating axons growing through the assemblies of transplanted Schwann cells do not exit the transplants in the spinal cord lesion (Kanno et al., 2015). It is assumed that axons are blocked from extending through the glial scar formed at the border of the lesion. It is considered that chondroitin sulfate in the scar tissue inhibits the growth of regenerating axons. This disadvantage was overcome through the digestion of chondroitin sulfate by chondroitinase ABC (Chau et al., 2003), by the local injection of growth factors (aFGF, GDNF), and/or co-transplantation of Schwann cells with other types of cells such as BMDCs, OECs, and NSPCs (Deng et al., 2015).

It was shown that “modified” or “activated” Schwann cells that were generated at the proximal as well as distal stumps of transected peripheral nerves have beneficial effects on axonal regeneration, when transplanted in the spinal cord lesion (Senoo et al., 1998).

It was reported that 60–90% of the transplanted Schwann cells survived over 3 weeks after transplantation (Pearse and Bunge 2007). Schwann cell proliferation peaked at 2 weeks, decreased thereafter, and ceased at 12 weeks post-transplantation. Some Schwann cells had migrated along the central canal for up to 5 mm at 4 weeks post-grafting (Wang and Xu, 2014).

Schwann cells show moderate proliferation and migration, but no differentiation after transplantation. Schwann cells are well integrated in the host spinal cord tissue. Therefore, they are considered safe as transplants, and available for clinical applications.

It was reported that there was a marked infiltration of endogenous Schwann cells into the

host spinal cord tissue (Hill et al., 2006). This finding is in line with that of our study that regenerated axons are surrounded by Schwann cells in the BMSC transplantation. This finding poses the question of how many transplanted Schwann cells contribute to myelin sheath formation in the host spinal cord lesion. Schwann cells surrounding regenerating axons in the Schwann cell-transplanted spinal cord are presumably a mixture of transplanted and endogenous ones.

Functional recovery after Schwann cell transplantation was not so prominent in rats with SCI (Wang and Xu, 2014).

### 3. Neural stem cells

In our previous study, NSPCs were obtained from the fetal central nervous system, and injected into the spinal cord lesion (Wu et al., 2001). NSPCs survived, effectively integrated, and migrated extensively. They were differentiated into neurons and astrocytes by 4 weeks post-transplantation. NSPCs were suggested to be a potent, promising candidate in cell transplantation for SCI. When they were transplanted through the CSF (Wu et al., 2002), NSPCs attached to the surface of the intact as well as injured spinal cord, and migrated into the spinal cord tissue. These findings were interesting from the point of view of basic science; however, we wondered at that time whether NSPCs with the potent properties of migration and proliferation were safe as transplants from a clinical point of view. Are NSPCs applicable for clinical cases? For further examination, NSPCs obtained from the fetal hippocampus were infused into the CSF. NSPCs attached to the surface of the spinal cord, proliferated, and occupied a large area of the spinal cord 3 weeks after transplantation (Bai et al., 2003) (Fig. 2). They survived long-term, and proliferated and migrated extensively. From these findings, we thought that NSPCs were not safe as transplants from a clinical point of view. As stated above, we think that regeneration studies are of significance only when they can be applied clinically. Therefore, we discontinued the study of NSPCs as transplants for spinal cord injury.

Lu et al. (2012) reported a study using NSPCs derived from ES cells for spinal cord injury in rats. NSPCs were suspended in a fibrin matrix containing growth factors such as BDNF, NT-3, PDGF, IGF, EGF, bFGF, aFGF, GDNF, and HGF. This mixture of NSPCs and growth factors was



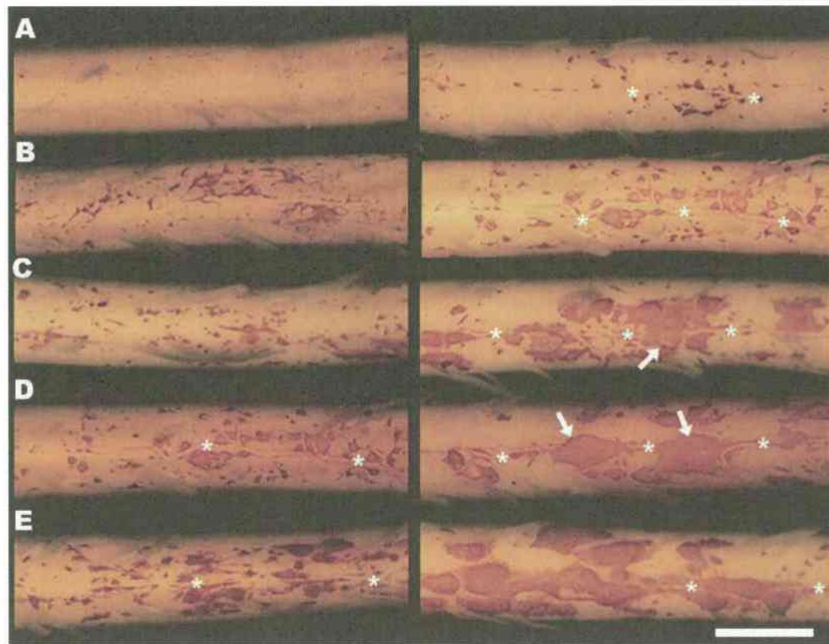


Fig. 2 Neurospheres were formed by culturing dissociated hippocampal cells of E16 fetuses of GFP-transgenic rats. Neurospheres containing NSPCs were injected into the CSF via the 4<sup>th</sup> ventricle of normal, intact rats. This micrograph shows the whole view of thoracic segments of spinal cords at 3 (A), 5 (B), 7 (C), 14 (D), and 21 days (E) after cell injection. Pictures in the left column show the dorsal surface, and those in the right column show the ventral surface of the spinal cord. GFP-labeled NSPC clusters attached to the spinal cord surface, and increased gradually to cover a large area of the spinal cord surface at 3 weeks after cell injection. NSPCs will continue to proliferate to cover the entire surface of the spinal cord. NSPC clusters were formed around blood vessels (\*), and further expanded over them (arrows). Scale : 2.0 mm  
From Bai et al. (2003).

injected into the transected lesion at T3, and observed for 7 weeks after transplantation. Transplanted NSPCs survived, and filled the spinal cord lesion. They differentiated into astrocytes and neurons. NSPC-derived neurons extended axons over a long distance up to C8 rostral and L1 caudal to the T3 site. Axons were myelinated, and formed synapses with host neurons. In a re-examination of this study, Sharp et al. (2014) stated that the transected lesion of the spinal cord remained as a partition, and there was no ingrowth of host axons into the transplants of NSPCs. There was no functional recovery.

In another paper, Lu et al. (2014) used human iPSC-derived NSPCs as transplants. The spinal cord was transected at C5, and NSPCs in fibrin matrices containing a growth factor cocktail, as described above, were transplanted 2 weeks after transection. Observation was performed 3 months after transplantation. NSPCs filled the lesion cavities, differentiated into neurons, and extended numerous axons rostrally up to the midbrain and even to the cortex. Many axons also extended caudally up to T6, or even to the T12 level. No myelination was noted on regenerated

axons extending through the white matter of the host spinal cord. No functional recovery was observed.

Concerning “safe” and “unsafe” iPSCs, NSPCs derived from murine iPSCs were transplanted into the spinal cord lesion of mice. NSPCs differentiated into neurons, astrocytes, and oligodendrocytes. Oligodendrocytes formed a myelin sheath on axons. No tumor formation was found on the transplantation of NSPCs derived from “safe” iPSCs for 5 weeks post-transplantation, whereas those derived from “unsafe” iPSCs showed robust teratoma formation (Tsuji et al., 2010). It is impossible to evaluate the safety of NSPCs in such short a time as 5 weeks post-transplantation. The functional recovery was not so marked as to offset the concern about the tumorigenicity of iPSC-derived NSPCs.

Concerning the risk of the tumorigenicity of iPSC-derived NSPCs, the ablation of tumors was studied through immunoregulation. NSPCs derived from tumorigenic iPSCs were transplanted into the intact spinal cords of immunocompetent mice with or without immunosuppressant treatment. All transplants survived in the group receiving

immunosuppressant treatment. Most of them developed tumors at 3 months after transplantation. Tumors were rejected within 42 days after the discontinuation of immunosuppressants. The authors concluded that the immune rejection could be used as a “fail-safe” system against potential tumorigenicity after the transplantation of iPSC-derived NSPCs to treat SCI (Itakura et al., 2015). This study clearly shows the risk of iPSC-derived NSPCs for spinal cord injury. For the use of iPSC-derived NSPCs a high hurdle must be overcome before they can be applied as a transplant for spinal cord injury clinically. Similarly, tumor formation was reported in 103 days after the transplantation of human iPSC-derived NSPCs (Nori et al., 2015).

Lee-Kubli and Lu (2015) observed that iPSC-derived NSPCs grew out axons extensively, resulting in the innervation of ectopic targets. They thought that ectopic innervations could potentially be manipulated and shaped with axon-guidance strategies. In addition, cell proliferation, migration, and differentiation should be appropriately manipulated in clinical application. At present, however, there is no effective method to manipulate/control the ectopic innervation, proliferation, migration, and differentiation of NSPCs in spinal cord lesions. Lee-Kubli and Lu (2015) stated as follows: “Ideal methods of iPSC reprogramming and differentiation remain to be developed for clinical translation, and further experimentation is necessary at the pre-clinical level.” Mothe and Tator (2013) stated that “NSPCs are multipotent cells that self-renew and are committed to the neural lineage, and thus, they are especially suited to SCI repair. NSPCs may differentiate into neural cells after transplantation into injured spinal cord, replace lost or damaged cells, providing trophic support, restoring connectivity and facilitating regeneration.” This view of NSPCs is too optimistic. This statement means that NSPCs cannot be used for clinical purposes until the methods are developed, by which the behaviors of NSPCs can be properly manipulated and controlled.

Locomotor recovery is the essential parameter for the clinical application of cell transplantation. The critical problem is that NSPC transplantation is not necessarily effective for locomotor improvement. NSPCs will remain irrelevant clinically until their proliferation, migration, differentiation, and axonal extension can be effectively controlled for the safety of recipients.

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