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INVITED REVIEW

# The prospects of regenerative medicine combined with rehabilitative approaches for chronic spinal cord injury animal models

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## Abstract

Regenerative medicine has opened a window for functional recovery in acute-to-subacute phase spinal cord injury (SCI). By contrast, there are still only a few studies have focused on the treatment of the chronically injured spinal cord, in which cell-based regenerative medicine seems less effective. Since the majority of SCI patients are in the chronic phase, representing a major challenge for the clinical application of cell-based regenerative medicine. Although combined therapies for the treatment of chronic SCI have attracted attention of researchers and its potential importance is also widely recognized, there had been very few studies involving rehabilitative treatments to date. In a recent study, we have demonstrated for the first time that treadmill training combined with cell transplantation significantly promotes functional recovery even in chronic SCI, not only in additive but also in synergistic manner. Even though we have succeeded to outline the profiles of recovery secondary to the combination therapy, the mechanism underlying the effects remain unsolved. In this review article, we summarize the present progress and consider the prospect of the cell-based regenerative medicine particularly combined with rehabilitative approaches for chronic SCI animal models.

***Key Words:*** *transplantation; spinal cord injury; regenerative medicine; chronic phase; rehabilitation; treadmill training*

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## Introduction

Rapid progress in stem cell medicine is being realized in neural regeneration in various disease and injury animal models. With regard to spinal cord injury (SCI), researchers report remarkable recovery from sequelae such as motor paresis and spasticity, sensory disturbances, and urinary dysfunction with a variety of cell sources, like neural stem/progenitor cells (NS/PCs) and mesenchymal stem cells (MSCs), especially in acute-to-subacute-phase animals. By contrast, although there are more than 50-fold more SCI patients in the chronic phase than in the acute-to-subacute phase, only a few studies have focused on the treatment of the chronically injured spinal cord, in which cell regenerative medicine seems less effective (Shinozaki et al., 2016). In this review, we summarize our recent study on combination therapy for chronic SCI using transplantation of NS/PCs as well as treadmill training (TMT) (Tashiro et al., 2016) and consider the prospect of combining regenerative medicine with other treatment approaches.

## Early, Subacute, and Chronic Phases of SCI

The drastic microenvironmental transition loosely defines the time-phases of injured spinal cords. In the "hyper-acute phase", which is before 1 day post-injury (DPI) in the rodent model, there are still little inflammatory changes induced. Although it seems not practically treated in the clinics, most of experimental studies demonstrate good therapeutic effect of cell replacement therapy. The consecutive "acute phase" is characterized as the inflammatory reactions by the up-regulation of various cytokines, harmful neurotransmitters, free radical and nitric oxide, those are mediated by the haemorrhage, the destruction of blood-brain barrier (BBB) and the infiltration of inflammatory cells. Although neuronal plasticity may be potentially resided, those harmful factors

interfere the effect of regenerative therapy around 2–4 DPI. It is followed by the "sub-acute phase", in which the glial scarring is not yet progressed and the neuronal plasticity is still retained, besides the inflammation is reduced, shaping a time window with good therapeutic response around 7–14 DPI. However, as the glial scar and the cystic cavity formed around the lesion, it gradually transits to a "chronic phase" by 28–42 DPI (Nakamura and Okano, 2013). Although the spontaneous recovery is dried up during this transitional phase called "late-subacute/early-chronic phase", the plastic response to treatments is still preserved to some degree. After this phase, the injured spinal cord becomes literally refractory to various therapeutic approaches including cell transplantation in the definite "chronic phase" later than 42 DPI (Tashiro, 2016). Although few researchers have reported different characteristics of "delayed-chronic phase" later than 90 DPI, this is still not clarified sufficiently (Kumamaru et al., 2013).

## Therapeutic Time Window for Cell Transplantation

The refractory state of the chronically injured spinal cord is characterized as a microenvironmental change including glial scar formation around the epicentre of the lesion, which interrupts the migration of grafted cells and results in lower regeneration of neural circuits and remyelination, the secretion of axonal growth inhibitors like semaphoring 3A that restricts the regenerative potency, and a decrease in the anti-inflammatory M2 macrophages, but there is no remarkable change in the expression of cytokines and growth factors that influence the survival rate and the differentiation of grafted cells. The phase-dependent transition of these factors affects the therapeutic time window for transplantation therapy in SCI (Nishimura et al., 2013).

Among several studies focusing specifically on chronic SCI in rodents, only a few, in which transplantation was carried out at the relatively early stage of approximately 21 to 30 DPI, report significant recovery. By contrast, the rest of the studies, in which the intervention was administered after 42 DPI, all conclude ineffectiveness of the intervention, as far as we know. Based on these previous studies, the time window seems to close by the end of the subacute phase to the early-chronic phase, and then, during the definite chronic phase in the rodent model, the SCI becomes refractory to transplantation as a single therapy. This is therefore a major challenge for the clinical application of cell transplantation for most SCI patients in the chronic phase.

## Combination Therapies to Treat Chronically Injured Spinal Cord

On these grounds, combination therapies that include cell transplantation and administration of neurotrophic factors and medication have been investigated for chronic SCI. In particular, medicine-based treatments have attracted attention, as represented by the use of chondroitinase ABC (C-ABC) infusion to degrade chondroitin sulfate proteoglycans, which are major constituents of the glial scar, and a semaphorin3A inhibitor to block a major axonal growth inhibitor, for which a silicon sheet preparation was recently developed for use as artificial dura mater (Zhang et al., 2014). Because the factors that interfere with recovery are quite complex, various preparations of medications can have remarkable superiority. So far, studies have revealed that a combination of C-ABC and neurotrophic factors with NS/PCs and C-ABC with TMT can both induce significant locomotor recovery in rats with chronic SCI (Karimi-Abdolrezaee et al., 2010; Shinozaki et al., 2016). Although it has not yet been investigated for chronic SCI, semaphorin3A inhibitors may also be promising. By contrast, there are few studies on combination therapies that involve rehabilitative intervention, and there were none on combination therapies involving cell transplantation in the chronic phase until our recent study.

## Combination Therapy of Transplantation and Rehabilitation

Although some rehabilitative interventions are inappropriate due to the difficulty in employing them for motor-impaired SCI animals, bipedal treadmill training (TMT) is a method that can be easily employed with bodyweight support, particularly in rats with SCI, in order to effectively rehabilitate their paretic limbs (Tashiro et al., 2014). TMT has been used for rats with SCI to facilitate their functional recovery after transplantation of a variety of cell sources, including NS/PCs, MSCs and a combination of Schwann cells with olfactory ensheathing cells. However, surprisingly, only a few articles treating the acute-to-subacute SCI were available, nevertheless, their potential importance as combination therapy candidates is widely recognised.

Therefore, the molecular mechanism underlying this beneficial effect has not yet been fully elucidated, but the following effects are reported: (1) TMT enhances neuronal plasticity below the lesion; (2) TMT improves NS/PC survival in the host's spinal cord; (3) TMT facilitates NSC differentiation into neurons and oligodendrocytes, with unchanged astrocyte differentiation and fewer un-differentiated cells; (4) TMT attenuates cellular stress from reactive nitrogen species and reactive oxygen species; and (5) IGF-1 signalling contributes to points (2) to (4) (Hwang et al., 2014). By contrast, it remains to be determined how the combination influences the expression of neurotrophic factors, the formation of regenerative fibres, synaptogenesis, the inhibitory capacity of spinal network and motor control in these early phases. Moreover, it is also important to clarify whether or not adverse effects are induced by the combination with rehabilitation with regard to spasticity, hyperalgesia and/or allodynia. Moreover, none of these mechanisms had been clarified until our recent study.

The reason why there are only a few numbers of studies is partially explained by the fact that the experimental animal species suitable for cell transplantation and rehabilitation are different. Generally, research on cell transplantation is performed using mice because of their smaller size and amenability to genetic manipulation, as well as the availability of bioluminescence *in vivo* imaging (BLI) for transplanted cells. On the other hand, rehabilitative intervention, like TMT, is far more established for rat models because of their calmer temperament, greater endurance and larger body size, which allows for

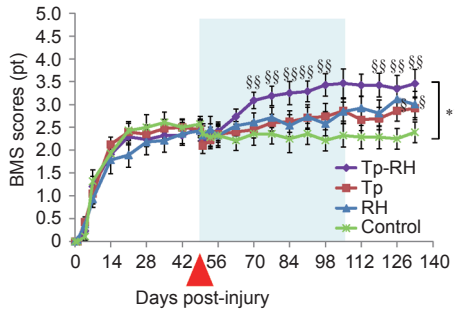


**Figure 1 Behavioural manifestations in each group.**
Summary of previous study showing the time course of locomotor functional recovery rated in the Basso Mouse Scale (BMS) in adult female C57BL/6J mice with severe thoracic cord contusive injury treated by Tp-RH (combination therapy of NS/PCs transplantation and treadmill training), Tp (transplantation single therapy), RH (treadmill training single therapy), and control (no treatment followed by 1 week of conditioning treadmill training) (Tashiro et al., 2016). NS/PCs: Neural stem/progenitor cells.

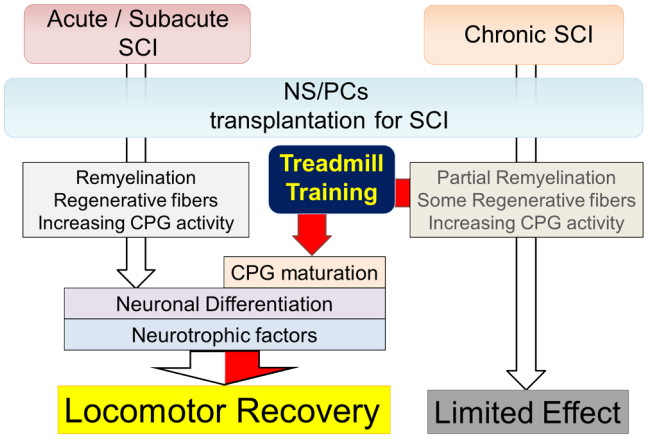


**Figure 2 A scheme summarizing the mechanisms of locomotor recovery brought about by each intervention.**
The effects of NS/PCs transplantation and treadmill training therapies on functional recovery in chronic and acute-to-subacute SCI model animals in the previous study (Tashiro et al., 2016). Although transplantation single therapy has only the limited effect of partial remyelination and increased CPG activity without appropriate inhibition, the addition of treadmill training further facilitates neuronal differentiation and CPG maturation, leading to significant locomotor recovery. CPG: Central pattern generator; NS/PCs: neural stem/progenitor cells; SCI: spinal cord injury.

easier handling. Thus, the lack of an optimized animal model well-suited for both training and cell transplantation may be a remarkable issue to be resolved in this area. We addressed this problem by employing a novel method introduced by Fong et al., namely partial body weight-supported bipedal treadmill-gait training for mice with SCI (Fong et al., 2005).

## Challenges to a Combination of Rehabilitation and Cell Therapy for Chronic SCI

Aiming for the clinical application of regenerative medicine for chronic SCI patients, we investigated the effect of combination therapy with the transplantation of mouse NS/PCs and rehabilitation using 80 severely thoracic cord-contused C57BL/6J mice in the chronic phase. A four-armed design with groups composed of combination therapy (Tp-RH), transplantation single therapy (Tp), TMT-rehabilitation single therapy (RH) and a control group (Control) was applied. TMT was used for two purposes during two different periods: 1 week of conditioning training was conducted for all animals from 42 DPI, and 8 weeks of intervention training was conducted for the RH and Tp-RH groups after the NS/PC vehicle injection. NS/PCs were transplanted at 49 DPI as neurospheres three passages after they were harvested. The cell source was the striata of E14 transgenic mice established from C57BL/6J that ubiquitously express ffLuc-cp156, which is a fusion protein of a yellow variant of Aequorea GFP and firefly luciferase, which enables the assessment of transplanted cell viability using BLI. In particular, no intervention was performed during the acute to "early-chronic" phase in this study.

To outline the profiles of recovery secondary to the combination therapy, we carried out cross-sectional analyses on various aspects of the changes induced in each group. Although no significant changes were observed in transplanted cell viability, residual spinal volume or spared fibres within the lesion epicentre in our investigations, the following new insights were gained. NS/PC transplantation both promotes the recovery in spinal conductivity and upregulates excitatory central pattern generator (CPG) activity, and TMT facilitates the suppressive regulation related to coordinated rhythmic motor control and the amelioration of spasticity, respectively. Although no significant locomotor recovery was detected in either of the Tp and RH single therapy groups, in comparison with the control group, the above-mentioned results indicate that even each single therapy induced potentially beneficial changes in chronically injured spinal cord (**Figure 1**). Furthermore, neuronal differentiation and the phenomenon of appropriate inhibition recovery and/or synaptic regeneration, which we have termed "CPG maturation", were specifically promoted by the combination therapy. Overall, significant locomotor recovery was observed in the mice of the Tp-RH group, compared with that in the control mice, due to the not only additive but also synergistic effect of transplantation and rehabilitation. We have summarized the mechanisms in a scheme in the previous study (**Figure 2**). On the other hand, no significant difference was functionally detected between the Tp-TMT and TMT groups in our study; thus, some other treatment is still required for the clinical application of cell transplantation in chronic SCI (Tashiro et al., 2016).

## What is the Key to the Beneficial Changes of Rehabilitation-Transplantation Combination Therapy?

Although we have outlined the changes brought about by the interventions, the mechanisms behind them remain to be determined. Our current hypothesis is that neurotrophic factors and rehabilitation-specific effects are contributing factors. Summarizing the previous reports, while combined infusion or genetic expression of neurotrophic factors facilitate the recovery induced by transplantation or TMT single therapy, both NS/PC transplantation and TMT also have the

potential to independently supply neurotrophic factors to the injured spinal cord (Houle and Cote, 2013; Kumamaru et al., 2013). Therefore, such trophic support can enhance the intrinsic ability of these interventions to interact with one another and thereby induce locomotor recovery.

Next, the functional recovery secondary to TMT can be explained by following rehabilitation-specific effects: 1) activity-dependent neuronal plasticity and modification and 2) the treatment of disuse and learned non-use. Voluntary and/or instrumental training can promote both task-specific and use-dependent neuronal plasticity and modification, involving the formation of new neuronal circuits, the reinforcement of locomotor networks in a more selective and stable manner, the strengthening of synaptic connectivity with long-term structural change, the limiting of maladaptive plasticity, spinal fixation through the peripheral sensory input and the reorganisation of the cortical network. Furthermore, we hypothesize that chronic SCI animals are in a state of disuse and learned non-use, triggering dysfunction and less recovery, which could further suppress the impaired hindlimb activity, thereby latently masking the beneficial effects of transplantation. Interestingly, in our study, locomotor function gradually improved in the transplantation single therapy group, in which animals were exposed to 1 week of conditioning training, which disagrees with previous reports on transplantation therapy for chronic SCI. Because even 1 week of TMT intervention stimulates the endogenous expression of BDNF, which further induces spinal learning (Gomez-Pinilla et al., 2007), conditioning training as we incorporated it may be effective in improving the behavioural states of SCI animals to some degree (Tashiro et al., 2016).

## Perspectives on Regenerative Medicine in Combination with Various Therapeutic Modalities

There are still elements needed to augment recovery in chronic SCI other than transplantation and rehabilitation. Notably, there was almost no evidence of recovery observed around the lesion epicentre in our intervention, suggesting that unfavourable factors, such as glial scarring and/or axonal growth inhibitors developed specifically in the chronically injured spinal cord, strongly interfere with recovery at the exact site of transplantation. Therefore, the addition of further treatments that target these factors using C-ABC and/or semaphorin3A inhibitors in combination with NS/PC transplantation and rehabilitation represent a promising future strategy.

Although there are almost no reports on the mechanisms underlying the changes induced by the combination therapy of TMT and cell transplantation in detail, such studies would be a major step in exploring more effective approaches and preventing adverse effects. Therefore, further investigation into the neurotrophic factors, electrophysiological changes and neural plasticity is needed, as well as an examination of adverse effects related to pain. In particular, profiling the changes in the expression of neurotrophic factors will provide us with the most desirable composition of such factors to be used in a combination therapy.

In conclusion, we have demonstrated that rehabilitative treatments can be a third therapeutic option to facilitate locomotor recovery after NS/PC transplantation, even in chronic SCI. TMT promotes functional recovery not only additively but also synergistically with cell transplantation through neuronal differentiation and CPG maturation. Therefore, a comprehensive regenerative approach, involving medication and rehabilitation as well as the cell replacement therapy, will have a remarkable effect on the treatment of the chronically injured spinal cord, which is refractory to each of these interventions alone.

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