Scar Removal, Cell Transplantation, and Locomotor Training—Strategies to Improve Tissue Repair and Functional Recovery in Rat with Chronic Spinal Cord Injury

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Abstract

Great progress has been made over the past three decades with studies that have focused on the reversal of the symptoms of spinal cord injury (SCI). This progress has given hope that there is a possibility that tens of thousands of patients afflicted with this malady could gain both sensory and motor function again. According to estimates there are about 400,000 patients with chronic SCI who are confined to wheelchairs in the United States. However, significantly less attention has been lent to the study of chronic SCI. Although many approaches have been found to be beneficial leading to functional recovery from acute spinal cord injury, unfortunately, these interventions do not work on chronic SCI. Chronic SCI possesses a different pathophysiology that requires a different approach for effective treatment. One of the characteristic features of chronic SCI is the formation of a glial scar, which has been considered an obstacle for tissue repair and axonal regeneration. We believe that removal of the existing glial scar is necessary for tissue repair and axonal regeneration. In recent years our research work has been focused on glial scar removal, cell/tissue transplantation, and development of TANES (tail nerve electrical stimulation)-induced open field locomotor training to improve the locomotor outcome. These steps are synergistic and contribute to the final functional recovery. Results from our lab are encouraging and may be beneficial to further study of chronic SCI. In this article we will discuss the approaches we have administered and postulate their mechanisms.

Keywords: Chronic spinal cord injury; Scar ablation; Cell transplantation; Electrical stimulation; Physical therapy; Rat

Introduction

With the lapse of time post-spinal cord injury and the progression into a chronic injury, individuals show more or less endogenous tissue repair, spontaneous axonal regeneration, and slight functional recovery. For most cases, however, these restorations in anatomy and neurology are limited and slow [1-3], only active intervention can result in the effective tissue repair and functional improvement. Nevertheless, we have to face the difficult problems and resolve the obstacles to axonal regeneration and locomotor improvement, such as the removal of lesion scar, bridging the lesion cavity and stimulating axonal regeneration and functional recovery.

When traumatic injury occurs, spinal cord suffers from hemorrhage, ischemia, hypoxia and edema at the injury epicenter, resulting in death of neurons and glia, axonotmesis, destruction of synaptic connection, tissue necrosis, demyelination and finally the formation of lesion cavity and glial scar [4-8]. This is the anatomic and pathologic basis of the loss of sensation and locomotion below the level of injury. In addition, the initial, endogenous repaired tissue arises at the dorsal part of damaged cord segment at the early stage after contusion injury. The novel tissue contains invaded fibroblast, newly formed collagen fibers and blood vessels, invaded Schwann cells, regenerating axons, and myelin sheaths formed by Schwann cells [9,10].

The glial scar, which lines and walls off the lesion cavity from the spared tissue, consists of a few layers of reactive astrocytes, representing a combined physical and molecular barrier to axonal elongation [8,11-13]. The glial scar has become an important target for regeneration research in chronic spinal cord injury; however, to date almost no effective method has been described to destroy an existing glial scar in a chronic injury. Even though it appears the molecular barriers can be reduced by some antibodies or enzymes (such as Nogo, chondroitinase), or a derogatory event as the scar ages, the physical barrier still exists to block axonal elongation. In addition, the glial scar also blocks the tissue repair by cell or tissue transplantation [14,15]. Therefore, it is likely that only thorough removal of glial scar tissue can a permissive physical and biochemical environment for axonal elongation be established. Our recent findings illustrate that scar ablation does benefit tissue repair and axonal elongation.

However, tissue repair and axonal regeneration do not necessarily result in functional recovery in chronic spinal cord injury. This may be associated with the activity-dependent plasticity below the injury level. Plasticity is the ability of neurons to rearrange their anatomical and functional connectivity in response to environmental input, thereby achieving new or modified outputs, namely behaviors [16]. In the recent studies, plasticity and its shaping by physical activity are emerging as major contributors to functional recovery [17-19]. Studies have demonstrated that physical therapies including treadmill training and functional electrical stimulation may stimulate the formation of plasticity [20-26]. In this paper we will discuss the strategies to improve functional recovery from chronic SCI mainly based on our own studies and experience.
Our observations and others have demonstrated that the spinal cord has endogenous ability of repair following injury [1], although this ability is limited and slow. Following SCI (such as contusion induced by using the New York University impact device with 25 mm height setting), the whole gray matter and dorsal white matter were destroyed at the injury epicenter. Two weeks later the lesion cavity appears and the glial scar forms, which lines the spared tissue surrounding the dorsal root (arrowheads). D, regenerating axons (myelinated fibers) with H.E. staining are difficult to identify at the axonal zone (arrowheads), cellular zone, and fibrotic zones (arrows). DR: dorsal root; VR: ventral root. Magnifications: 4x (A), 10x (B), 20x (C), 40x (D). (Replicated from Zhang, 2011 with permission)

**Endogenous Tissue Repair and Axonal Regeneration**

Our observations and others have demonstrated that the spinal cord has endogenous ability of repair following injury [1], although this ability is limited and slow. Following SCI (such as contusion induced by using the New York University impact device with 25 mm height setting), the whole gray matter and dorsal white matter were destroyed at the injury epicenter. Two weeks later the lesion cavity appears and the glial scar forms, which lines the spared tissue surrounding the cavity. At the same time a newly formed tissue, endogenous repaired tissue, can be seen at the dorsal part of the lesion cavity. It starts to form one week following SCI and is associated with the invasion of fibroblasts from the pia mater and Schwann cells from the peripheral nerves due to the destruction of glial limitans. The endogenous repaired tissue faces the lesion cavity, which is filled with macrophages, and is connected to the spared tissue with a few trabeculae that contain fibroblasts, blood vessels and even nerve fibers [9,10]. Both the glial scarring and the appearance of endogenous repaired tissue can be considered as response to the traumatic injury to the spinal cord and represents the beginning of spontaneous restoration of damaged tissue.

The endogenous repaired tissue, different structurally from normal cord tissue, contains fibroblasts, collagen fibers, blood vessels, small cysts, Schwann cells, and myelinated or unmyelinated axons. According to our own observation, the endogenous repaired tissue can be divided histologically into three irregular zones: fibrotic zone, cellular zone, and axonal zone (Figure 1) [9,10].

**Fibrotic zone**

The fibrotic zone is located near the dorsal border of the cord, and consists mainly of invading fibroblasts, collagen fibers, and newly formed blood vessels; it also contains some invading Schwann cells, some regenerating axons (myelinated or ensheathed by Schwann cells), macrophages and undefined bubble-like structures, which may be related to the degenerating axons. The axons, myelinated or unmyelinated, appear as a bundle, usually surround by a thin layer of fibroblasts. The blood vessels can be seen both inside and outside the nervous bundle. The whole zone looks like a kind of loose connective tissue, and it occupies the major area of endogenous repaired tissue at the early stage following spinal cord injury (Figure 1) [9,10].

**Cellular zone**

The cellular zone is composed of densely compacted, reactive young cells, and forms a clear U-shaped shell surrounding the fibrotic zone together with the pia mater at the dorsal border of the damaged cord. These young cells appear as immature Schwann cells and were often observed connecting to the cells from the dorsal roots, indicating that these young cells may migrate from the dorsal roots. The cellular zone also contains blood vessels and some myelinated axons detected by P0 antibody, but less than that in axonal zone in count. It seems that cellular zone may play a role in limiting tissue spreading of the fibrotic zone which contains numerous fibroblasts invaded from the surrounding connective tissue outside the spinal cord. At the earlier stage of tissue repair the cellular zone is thin and small, but it can become thick and expand in size with time or following intervention treatments (Figure 1) [9,10].

**Axonal zone**

Generally the axonal zone neighbors on the cellular zone ventrally and faces the lesion cavity. It has the smallest size in untreated spinal cord compared with the other two zones. It has a characteristic feature that consists mainly of myelinated axons positively stained by P0 antibody. The axons may form bundles with or without fibroblasts surrounding them. The bundles can be arranged loosely or densely, depending on the amount of the axons. At the later stage of the spinal cord injury, axons in this zone may increase in number. However, the increasing is limited and slow if the injured spinal cord receives no intervention treatment (Figure 1) [9,10].

**Blood vessels at the injury site**

Blood vessels are also distributed in all three zones, and some of the blood vessels including capillaries are located close to the regenerating axons. However, these blood vessels lack an integrated blood-brain barrier, demonstrated by the GFAP immunostaining, illustrating these blood vessels are not surrounded by the perivascular feet of astrocytes as seen in the uninjured spinal cord tissue or in the spared cord tissue of injury site [9,10]. However, the ultrastructure, permeability and function of these blood vessels at the injury site have not been yet well documented so far [27,28].

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**Figure 1:** Endogenous repaired tissue in cross section from injury epicenter (6 weeks after 25 mm contusion; H.E. staining). A, the repaired tissue located at the dorsal part of damaged cord, connects bilaterally to the spared tissue, and neighbors the lesion cavity, which is surrounded by the glial scar (arrows) and spared tissue. B, under higher magnification, the repaired tissue can be divided into three different zones: fibrotic, cellular, and axonal. The fibrotic zone is surrounded by a U-shape cellular zone. The axonal zone does not appear clearly due to its small amount of axons at this moment. C, the cellular zone, connecting to both spared tissue (*) and fibrotic zone, consists mainly of Schwann cells migrating from dorsal root (arrowheads). D, regenerating axons (myelinated fibers) with H.E. staining are difficult to identify at the axonal zone (arrowheads), cellular zone, and fibrotic zones (arrows). DR: dorsal root; VR: ventral root. Magnifications: 4x (A), 10x (B), 20x (C), 40x (D). (Replicated from Zhang, 2011 with permission)
Central canal and ependyma-derived cells

At the rostral and caudal ends of injury site, the central canal was intact morphologically, but it was surrounded by numerous reactive, young cells and was rich in blood vessels. These ependyma-derived cells usually form cellular clusters or rosettes with accessory lumina. Among these cells few of P0-positive myelin sheaths were observed. Interestingly, in sagittal section, the ependyma-derived cells were found filling up the remote lesion cavity or forming cord-like structures to move toward the lesion epicenter. In addition, some ependymal cells are shown to contain immunoreactive product of glial fibrillary acidic protein (GFAP), illustrating that they may be a type of young astrocytes or have potential to differentiate into mature astrocytes [9]. These astrocytes move to the injury epicenter and participate in the formation of glial scar, but do not express chondroitin sulfate proteoglycans (CSPGs), a family of inhibitory molecules [29].

Removal of Existing Glial Scar by Photochemistry

Following spinal cord injury, reactive astrocytes hypertrophy and proliferate and migrate to and accumulate at the margin of the spared tissue, forming the glial scar [12,30,31]. According to our observation, glial scar and gliosis are two different concepts, which are often obscure in some published papers. Gliosis is an increase in size and number of astrocytes, which may connect each other to form a network by their processes in the vicinity of injury site, but they do not necessarily express CSPGs, although they show significant enhancement of GFAP due to the hypertrophy induced by SCI [32]. Glial scar is a membrane-like structure composed of two or three layers of reactive astrocytes, which are densely compacted at the margin of the spared tissue (Figure 2) [12]. The reactive astrocytes of glial scar strongly express both GFAP and CSPGs.

Removal of glial scar is necessary for tissue repair and axonal elongation. A recent study has shown that regenerating axons from a microtransplanted dorsa root ganglion are able to grow almost everywhere with the zone of Wallerian degeneration and reactive quiescent and that axon repulsion might be caused by the mechanical site prior to transplantation [36-38], indicating that both scar ablation and cell transplantation are necessary to the treatment of chronic SCI.

Surgical removal is not feasible for glial scar. While some approaches have been demonstrated to effectively reduce or prevent the scar formation in the acute injury model of central nervous system (CNS), so far no approach has been used to effectively remove an existing glial scar in a chronic injury model, especially in a chronically contused spinal cord. Because the glial scar in contused spinal cord is irregular in shape, it is not feasible to do so by surgical removal or laser surgery. We have successfully removed, at least partially, an existing glial scar by using photochemical method with rose Bengal [8,39].

![Figure 2: Glial scar and scar ablation. Glial scars with sharp edge are pointed out by arrows in A (H.E. staining) and B (GFAP immunostaining). Five minutes after being injected into the lesion cavity, rose Bengal (in pink) was absorbed by the superficial cells with the morphology of astrocytes (larger arrows in C) of the glial scar. Macrophages (MΦ) within the lesion cavity and some other astrocyte-like cells (small arrows) underneath the superficial cells were also stained in different degree, but no cell in deeper spared tissue was stained by the rose Bengal. Following illumination rose Bengal-stained cells of the glial scar were obviously damaged (area pointed out by arrows in A (H.E. staining) and B (GFAP staining)).](image)

Photochemical method with rose Bengal is feasible for contusion model. Rose Bengal is a xanthene derivative that is only slightly toxic, but it can be photoactivated by light irradiation, resulting in a lethal toxicity to involved cells [40,41]. We injected the rose Bengal solution into the lesion cavity 6 weeks after contusion injury, and then illuminated the injury site of the cord. Histological and immunohistochemical evaluation suggested that at least partial glial scar tissue was ablated by the illuminated rose Bengal (Figure 2). Such scar ablation did not show prominent harm to the spared tissue, electrophysiological conduction, and neurological outcome, but induced Schwann cells migration to form new myelin sheaths in the spared tissue. These findings indicate that the photochemical method is feasible for ablation of the glial scar which surrounds an irregular lesion cavity in shape. The scar ablation might provide a permissive environment for the regenerating axons when enriched by cellular or drug therapy [42,43]. More importantly, this method to remove the glial scar in rat contusion model may be translated into human clinical
application. Most SCI model in humans is contusion, and our purpose of study on glial scar ablation has been geared to clinical application.

Tissue Repair And Axonal Regeneration By Scar Ablation And Cell Transplantation

So far few treatment strategies applying cellular transplantation to the chronically injured, especially contused, spinal cord have yielded significant functional improvement in animal experiments [44,45]. However, in the cellular transplantation therapies, lamina propria (LP)-derived olfactory ensheathing cells (OECs) and LP have been considered as leading cell or tissue candidates for transplantation to bridge the gap in injured spinal cord due to their potential reparative properties and function [46–49].

Significant changes of tissue structure. In the recent studies, we transplanted olfactory mucosa-derived LP alone or in combination with cultured OECs immediately after scar ablation into the lesion cavity 6 weeks after contusion injury at spinal cord segment T10 of female Long-Evans rats. Sixteen weeks after LP/OEC transplantation, the repaired tissue was found to expand and fill the lesion cavity, resulting in marked reduction even disappearance of the lesion cavity. In fact, transplanted LP cannot be distinguished morphologically from the repaired tissue at the injury site, where numerous Schwann cell-like, young cells were observed, indicating that the transplanted tissue or cells have successfully integrated into and become a part of the repaired tissue. More importantly, no apparent border can be found between the repaired tissue and spared tissue, suggesting that the repaired tissue has substantially merged with the spared tissue. In addition, the typical glial scar detected by GFAP immunostaining was not found as seen in the injury control group or in the group of LP transplantation without scar ablation; this further confirms the merging of two different tissues (Figure 3) [35].

Axonal regeneration promoted by combination treatments. In our previous experiments we used monoclonal antibody P0 to label the myelin sheaths formed by Schwann cells at the injury site. P0 (myelin glycoprotein P-zero) is a major component found in PNS myelin sheaths and is characteristically absent from CNS myelin sheaths normally. A monoclonal antibody against P0 has been developed that labels the myelin sheaths with the characteristics of peripheral nerves [50–52]. The number of P0-positive myelin sheaths was counted under the light microscope to determine the increase in number of regenerating axons. The counting area includes the repaired tissue and the spared cord tissue on the transverse sections at the injury epicenter. In rats receiving scar ablation and transplantation, almost no border can be distinguished between the spared tissue and repaired tissue, therefore all P0-positive myelin sheaths within the counting area were included as a total number.

According to our previous observation, Schwann cells and Schwann cell-formed myelin sheaths start to appear at the endogenous repaired tissue two weeks after contusion injury. Some of Schwann cells may migrate from the endogenous repaired tissue to the spared tissue probably through the trabeculae between the repaired tissue and the spared tissue. Some of Schwann cells might migrate from the ventral root and myelinate the axons in the spared tissue of ventral portion of the cord. P0-positive myelin sheaths possibly formed by the endogenous Schwann cells were found in the endogenous repaired tissue, transplant, and spared tissue. These myelinated fibers may include regenerating axons or demyelinated axons [8,9].

Quantitative analysis of P0-positive myelination demonstrated the effect of combination treatments on axonal regeneration. In injury alone control rats the number of P0-positive myelin sheaths did not have a significant change at the last 16 weeks (1,750±254, n=6) compared with that found 6 weeks after injury (1,966±433, n=5). The number of P0-positive myelin sheaths increased slightly in rats with LP (2,268±385, n=4) or LP plus OEC (2,584±198, n=5) transplantation without scar ablation, although the difference was not statistically significant when compared with the SCI control animals. Rats subjected to scar ablation alone did not have an increase in number of P0-positive myelin sheaths (1,759±215, n=6) when compared with injury alone control rats. The group that received scar ablation and LP transplantation showed more P0-positive myelin sheaths (3,043±404, n=8) with a significant difference when compared with the control animals. A further increase in the number of P0-positive myelin sheaths was observed in the group receiving scar ablation and transplantation of both LP and OECs (4,001±569, n=7) [35]. Besides the myelin sheaths, there may be additional unmyelinated fibers, which can be observed under the electron microscope, ensheathed by endogenous Schwann cells and transplanted OECs [46,53,54]. All these data demonstrate that combination of scar ablation and transplantation of LP/OEC significantly promotes the axonal regeneration and myelination.

Possible mechanisms

Recent studies showed that Schwann cells but not OECs myelinate the regenerating axons (OECs may ensheath axons, which become unmyelinated fibers), and the OECs stimulate the endogenous Schwann cells to migrate into the injury site and myelinate the demyelinated or regenerating axons, promoting the axonal regeneration.
regeneration and repair of damaged cord tissue [46,54-57]. Our previous study also showed that GFP-labeled OECs from transplanted LP into the lesion cavity did not completely integrate into the spared tissue, in spite of that the tissue repair is clearly seen at the injury site. It may be true that these transplanted OECs do not directly involve in the tissue repair, but stimulate through neurotrophins the endogenous Schwann cells or young cells from the proliferation of ependymal cells to make the cellular zone spreading and stimulate these young cells to become mature cells and myelinate the regenerating axons. This can be verified by the fact that 8 weeks after LP transplantation, the lesion cavity significantly decreased in size or completely disappeared by being filled with solid tissue or cells (not macrophages) in most cases, but the original size of transplanted LP may be smaller than that of the lesion cavity. Moreover, the transplanted LP usually does not have high density of OECs, indicating that densely compacted cells in cellular zone may not be originated directly from transplanted OECs, but may be originated from proliferation of endogenous Schwann cells stimulated by transplanted OECs [9,10,35].

However, remarkable tissue repair and axonal regeneration did not necessarily result in significant locomotor recovery in rats with chronic SCI. The reasons remain unclear, but may be associated with poor neural plasticity below the injury [35].

**Functional Recovery by TANES-Induced Open Field Locomotor**

**Training**

**Tail nerve electrical stimulation (TANES):** Physical therapy has become a commonly used strategy for functional recovery from spinal cord injury, and electrical stimulation is one of the most widely applied methods [18,58,59]. Recently we developed a technique, TANES-dependent physical therapy, which can directly trigger the activation of the central pattern generator (CPG) below the lesion level through the tail nerves, resulting in temporary body weight-supported plantar stepping with characteristic features of active, alternative movement of left-right hind limbs and coordination of front-hind limbs in rats with chronically contused spinal cord [60]. This is similar to an early study in which the spinally transected rats were induced to exhibit a strong tendency to step with alternating limbs during tail pinch [61]. The TANES may also have an effect analogous with that of epidural stimulation which enabled the SCI patient to achieve full weight-bearing standing and stepping with assistance during stimulation [59]. The noninvasive TANES seems to show more beneficial effects on locomotor training over other approaches such as treadmill training and functional electrical stimulation [62-68] (Figure 4).

**Effect of TANES on functional recovery:** In the recent studies, a number of promising outcomes have been reported, which include restoration of connections, remyelination, revascularization and sparing of tissue from secondary damage. However, the desired final outcome is recovery of function, especially for the chronic spinal cord injury; so far neither approach has led to a completely effective repair strategy [69-74]. We used TANES as an open field locomotor training method to promote the functional recovery in rats with chronic contusion injury. Six weeks after contusion injury, rats received glial scar ablation and LP/OEC transplantation followed by TANES-induced open field motor training. The electrical stimulation was produced by using a physical therapy instrument (Type J18A1, China; it has been demonstrated no risk and no side effect in the application in clinic and home). The open field motor training lasted 20 min per session, 5 sessions a week, for a total of 16 weeks.

With TANES-induced open field locomotor training, the locomotor function and spinal cord conduction of rats was significantly improved compared with the control animals, assessed by using Basso, Beattie, and Bresnahan (BBB) open field locomotor rating scale (BBB score: 11.46 ± 0.47 vs 9.38 ± 0.32, p<0.01, n=12), horizontal ladder rung walking test (p<0.01, n=12), qualitative kinematic analysis, and electrophysiological tests (somatosensory evoked potentials, p<0.05, n=12; Hoffman reflex, p<0.05, n=12) in our lab. In addition, we also found that the TANES-induced open field locomotor training promoted axonal regeneration including those located in brain stem associated with locomotor control, such as red nuclei, lateral vestibular nuclei, reticular formation, raphe nuclei, and locus coerulei, verified by retrograde tracing. We have noted that the supraspinal axonal regeneration may have a positive relationship with BBB scores (r=0.89, p<0.001, n=23) [43,75] (Figure 5).

**Figure 4:** Immediate effect of TANES on locomotion. Representative images, saved from the video record for the same rat with chronic SCI, show typical body-weight supported stepping induced by TANES. Before TANES the rat’s left hind limb was able to partially support the body weight in stance, but the right hind limb was unable to do so (A, arrowhead indicating the plantar face up). When TANES started the same right hind limb became strong and powerful, enabling the rat to fully stand up and step with body weight-support (B, arrow indicating the plantar face down) and alternate movement of two hind limbs. Note that the cords (pink in color, B) connect the instrument of physical therapy (not shown) and the tail through two electrodes by clamping the tail. (Replicated from Zhang, 2010 with permission).

In addition, transection at T8 was performed 22 weeks after injury. There was a marked decrease in BBB score after transection, but 2 weeks later, the BBB score in the trained rats (6.83 ± 0.17) is still higher than that of control groups (2.83 ± 1.59, p<0.05) [43].

Plasticity may be the key for functional recovery. Plasticity is the interface between physical and neural activity. Spontaneous injury-induced plasticity anatomically includes regenerative sprouting from damaged and intact neurons, synaptogenesis and synaptic remodeling, and functionally includes changes in neuronal excitability and inhibition, conduction velocity and synaptic efficacy [16,76]. Distributed plasticity underlies recovery of foot kinematics by generating new patterns of muscle activity that are motor equivalents of the normal ones [77]. Physical exercise has profound effects on cellular and molecular function [78-80]. A recent study has shown that the quality of hind-limb stepping in rat with spinal cord transection is dependent on the amount of treadmill training that is imposed after transection surgery [81], this may be related to the formation of...
plasticity, and this intrinsic plasticity can allow functional recovery in the absence of significant regeneration [23]. In our previous study [42], we have found transplantation of LP/OEC to the injury site following scar ablation did not directly result in markedly functional recovery although robust axonal regeneration were found at the injury site, implying that it may need plasticity triggered by physical therapy.

Activity-dependent plasticity occurs in the spinal cord throughout life. Driven by input from the periphery and the brain, this plasticity plays an important role in the acquisition and maintenance of motor skills and in the effects of SCI and other CNS disorders [82]. After a complete loss of supraspinal control by cord transection, a higher degree of BBB score still remained in trained rats when compared to untrained rats. This may be related to the activity-dependent plasticity promoted by TANES, which results in the maintenance of motor skills, although there is no more supraspinal input or control. We consider this maintenance of motor skills as evidence of transformation of temporary ability to walk into permanent ability. The activity-dependent plasticity may be the morphological basis of this transformation [43,75].

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Possible Mechanisms of Locomotor Recovery from Chronic SCI

**Role of CPG in functional recovery:** CPG is a local neural system (network) in animal and human spinal cord, mediating coordinated activity between groups of agonist and antagonist limb muscles on opposite sides [63,64,83]. The CPG can generate patterns of rhythmic activity for locomotion even in the absence of external feedback or supraspinal control, but normally it is modulated by supraspinal and peripheral inputs [84,85]. Evidence from animal experiments showed that CPG may be located in rat lumbar (L) segments L1-2, and controlled by the descending locomotor command from cortex and brainstem. In turn, the CPG produce rhythmic output, through interneurons, onto rhythmic elements distributed throughout the lumbar enlargement [86,87]. The CPG also receivesafferent feedback originating from the muscles via proprioceptive afferents (groups Ia, Ib and II) as well as cutaneous afferents (Aα, Aβ) [88]. In our studies, since contusion injury fell on thoracic (T) segment T10, the CPG located in L1-2 and the connection between CPG and motor neurons in lumbar segments should remain largely intact, in despite of that the descending locomotor command pathways, at least mostly, from the cortex and brainstem have been cut off. In addition to spinal cord reflexes, which by definition are single-phased motor response to sensory input, the spinal cord is able to generate complex, rhythmic behaviors in the absence of both supraspinal and movement-related (proprioceptive) information [64].

It has been reported that simulating the rat tail, which induces tonic afferent input, can increase the strength and rate of locomotion produced by CPG, and make the pattern more consistent [89]. Recently, electrically epidural stimulation of L2 segment was shown to induce bilateral stepping patterns with body weight support on a treadmill in adult rats with spinal cord transection on T7-9 [90], suggesting that the CPG without supraspinal information but activated by electrical stimulation, is able to produce and send signals to the leg muscles. We believe that rats in our studies followed the same mechanisms. When CPG was activated by TANES via both cutaneous and proprioceptive afferents through the tail nerves, in turn, the CPG send signals to the motor neurons at the anterior horn, leading to the alternative contract of hind-limb muscles and the coordination of front- and hind-limbs. However, after neurons at L1-2 were destroyed by transection of spinal cord, there was no more movement of hind limbs during the electrical stimulation through the tail nerve, negatively indicating the important role of CPG in locomotor function [60].

**Effect of neural re-growth on functional recovery:** Our studies showed that 2 or 3 weeks after applying the TANES-induced open field locomotor training, rats either with or without cell transplantation started to show their functional improvement, indicating that this fast improvement of neurological outcome may be directly associated with the activity-dependent plasticity promoted by physical therapy, but not with the axonal regeneration. However, 7 or 8 weeks later, the BBB scores of rats without cell transplantation, i.e. without significant regeneration of axons, stay on a plateau without further raising till the end of experiment (22 weeks after initial contusion injury) , while rats with scar ablation and cell transplantation continue to gain higher BBB scores. This fact implies that without obvious tissue repair and axonal regeneration as an anatomic foundation, the role of physical therapy is limited [43,75]. This point of view has been supported by a study of cat spinal cord injury [91], and some of cases in the clinic trial recently [92]. It is likely that only being based on the tissue repair and axonal regeneration, the effect of physical therapy on the functional recovery be reliable and permanent, therefore a strategy which not only promotes tissue repair and axonal regeneration, but also induces the spinal plasticity will be considered as an effective, perfect strategy for the final functional recovery from spinal cord injury, especially chronic spinal cord injury.

**Discussion**

It is widely accepted that no single approach will prove sufficient for successful regeneration—a methodology combing the most effective individual therapies is required [93]. Our combination treatments including scar ablation, cell transplantation, and physical therapy have shown to significantly improve the functional recovery in chronically contused rat spinal cord, indicating that this novel method of TANES-induced open field locomotor training, may have tremendous potential for achieving improvement in functional outcomes in patients with chronic SCI. In conclusion, our studies and other recent
studies demonstrate that: 1) spontaneous tissue repair, axonal regeneration and functional recovery are limited and very slow in rats with chronically injured spinal cord; 2) glial scar ablation benefits the tissue repair and axonal regeneration promoted by cell/tissue transplantation; 3) TANES-induced open field locomotor training may significantly improve the locomotor functional recovery with or without markedly axonal regeneration; 4) TANES-induced open field locomotor training based on the tissue repair and axonal regeneration results in better recovery of locomotor function in chronic model.

Gial scar represents a physical and molecular barrier to axonal regeneration and has become an important target for regeneration research in chronic spinal cord injury. Our method to ablate the glial scar with photochemistry has been demonstrated effective and safe without additional impairment to the locomotion, spared tissue, and electrophysiological conduction. When glial scar tissue becomes quiescent with time in chronic spinal cord injury, the inhibitory CSPG levels may prominently reduce even disappear, however, the mechanical constraints on axonal elongation still exist in the glial scar tissue composed of dense astrocytes [33,34]. Many other methods, such as the use of enzyme chondroitinase ABC, can destroy the molecular barrier [94], but still leave the physical barrier intact in structure. These kinds of methods are analogous to cutting the grass, but retaining the roots which can re-grow under an appropriate condition. Our method is aiming at not only cutting the grass, but also removing the root. In addition, our results have shown that after scar ablation and cell transplantation, when repaired tissue is integrated into the spared tissue, no room is left for the re-grown glial scar. We believe that scar ablation with photochemistry is reasonable and feasible, and we are working on moving it forward the clinical trial.

The potential of stem cells has fascinated SCI researchers for decades. Around the world, The enthusiasm of clinicians and desperation of SCI patients thrust cell transplantation approaches into the translational spotlight, as SCI individuals traveled around the globe to receive these unproven treatments. However, many questions need to be addressed, such as cell survival, integration, and migration and so on [45]. After spinal cord injury, invasion of Schwann cells from the outside of spinal cord is necessary and a natural response to the damage of spinal cord; the endogenous Schwann cells are responsible for the tissue repair and the formation of new myelin sheaths. However these intrinsic abilities are limited and slow. Probably, transplanted OECs are not directly involved in the tissue repair and myelin sheath formation, but they might drive endogenous Schwann cells to do so. Recently, more and more studies demonstrate that the OECs, by secreting nerve growth factors, stimulate the host Schwann cells to migrate to the injury site, indirectly promoting the tissue repairing and remyelination [46,55,57,95]. Taken together, for the possibility of autologous transplantation and further clinical application (including easy to obtain the cells) OECs would be one of the best candidates for cell therapy, thus we propose here that it may not be necessary to transplant Schwann cells into the spinal cord, because transplanted OECs can induced numerous host Schwann cells migrating to the injury site without a survival problem as transplanted Schwann cells have.

During the endogenous restoration after spinal cord injury, another cell type, ependymal cells also involve in the tissue repair. It has been reported that ependymal cell population largely contains adult spinal cord stem cells. After spinal cord injury they may proliferate and differentiate into astrocytes, oligodendrocytes or other type of cells, contributing to the tissue repair [29,96-99]. The astrocytes differentiated from the ependymal cells may contribute to the formation of glial scar, but do not express inhibitory CSPGs [29]. We also found numerous ependyma-derived cells forming rosette or cord-like structures to fill up the remote lesion cavity or move toward the lesion epicenter. This is evident that ependyma-derived cells are directly involved in the tissue repair, and may be associated with certain factors or signals produced by injury. Should we find out a similar way to enforce these in vivo activities of ependymal-derived cells, it would be very helpful to speed the tissue repair. It is important for us to believe that philosophically external causes become operative through internal causes; various treatments (approaches) we utilized for the spinal cord injury are considered as external causes which can promote an essential change, functional improvement, through the internal causes, i.e., intrinsic environment for tissue repair, axonal regeneration, and functional recovery.

Compared with the epidural stimulation and similar treatments [59], the TANES is a noninvasive stimulating method and may be easier to operate if it could be applied in clinic in future. For the treadmill training with body weight support, the robotic device was commonly used to train rats to step bi-pedally [67,68,81]. Basically, this kind of device causes the movement of rat hind-limbs related to the passive contract of leg muscles. In contrast to this, our TANES-induced open field locomotor training results in positive contract of leg muscles triggered by the activation of motor neurons at the spinal cord. The stimulating cycle may primarily involve stimulated tail muscles and skin sending signals to the CPG through the cutaneous and proprioceptive afferents, then activated CPG sends signals to the motor neurons at the gray matter of spinal cord, in turn, the motor neurons command the leg muscles to contract via motor fibers. In this cycle, cutaneous receptors, proprioceptors (spindles), CPG, interneurons, motor neurons, efferent elements, and neuromusysynaptic connection are all activated. This can explain what we observed during the electrical stimulation that once the stimulation started, the rat is able to immediately stand up and walk around with plantar stepping alternatively. We think the positive movement of hind limbs induced by TANES should be more effective theoretically to promote the activity-dependent plasticity than done by passive movement of hind limbs induced by other approaches. A question needing to be addressed is how to transform this temporary ability, weight supporting stepping, into a permanent ability, i.e., normal locomotor movement. This is a topic to be further studied [60,75].

Taken together, the combination strategies of scar removal, LP/OEC transplantation, and TANES-induced open field locomotor training to improve functional recovery from chronic SCI have been demonstrated to be effective and feasible in rat model. These may represent the hope of patients with chronic spinal cord injury and especially the glial ablation and TANES-analogous physical therapies or locomotor training methods are worthy of moving to the clinical trial.

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