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Combinatorial Strategies with Schwann Cell Transplantation to Improve Repair of the Injured Spinal Cord

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The immediate effect of spinal cord injury (SCI) is a mechanical trauma that results in direct damage at the lesion site followed by secondary responses leading to loss of adjacent neurons and glia. Consequently, SCI leads to paralysis and loss of sensation below the level of the injury, altered autonomic responses and, frequently, the development of abnormal sensation and pain. Substantial endogenous remodeling of the spinal cord occurs [6] as axons begin to sprout and cells, including inflammatory, endothelial and Schwann cells (SCs), invade the injury site [34], likely contributing to spontaneous improvement observed in humans. Despite this endogenous repair, it is modest and functional improvements are limited [47;55]. Because treatment options are inadequate, additional therapeutic interventions are needed. Some strategies to repair the spinal cord are focusing on neuroprotection, regeneration, and/or tissue replacement. First, strategies should be designed to limit the secondary spread of damage to adjacent axons, neurons and glia. Second, strategies should promote axonal remodeling to maximize the function of spared tissue locally. Lastly, strategies are needed to promote long distance regrowth of damaged axons by reducing inhibition and/or providing permissive substrates and trophic molecules.

The development of different injury models to mimic various aspects of human SCI, together with existing procedures to test behavioral recovery, have improved significantly our understanding of the pathophysiology of SCI and, importantly, have enabled investigation of a myriad of therapeutic interventions. Models are utilized to induce complete or incomplete SCI. Standardized devices are used to produce contusion or compression of the spinal cord, resulting in incomplete injuries with varying degrees of sparing depending upon the magnitude of the impact used [4;7;24;81]. Because standardization of the extent of injury is an essential component of these models, careful examination of the injury parameters and the behavioral recovery early after injury is

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essential to ensure the validity of the results observed in a specific therapeutic paradigm. Whereas contusion/compression models more accurately mimic the human injury, sparing of axons complicates the interpretation of axonal growth versus sparing. As a result, complete transection of the spinal cord is the favored model to study axonal regeneration.

A cystic cavity usually forms after SCI, and it is walled off from the surrounding spared rim of white matter by a glial scar [6]. At the margins of the lesion, injured axons terminate in dystrophic endings, indicating thwarted axonal growth [74]. The presence of axons within trabeculae crossing the lesion implies that, when provided with an appropriate substrate, some axons will grow into the injury site despite the scar [6]. The use of permissive substrates such as cells, extracellular matrix proteins or biomaterials appears necessary to span the lesion. Also, cellular transplants can replace lost neurons and/or glia, enhance tissue preservation via neuroprotection and support axonal regeneration.

In this chapter, we shall review experiments testing SC transplantation alone or in combination with known neuroprotective and neuroregenerative strategies. SCs from different sources will be described. Results of the use of SCs in three different injury models, complete transection of the spinal cord, lateral hemi-section and contusion, will be discussed. Whereas axonal regeneration is difficult to discern in the contusion model due to fiber sparing, it is relevant to human SCI in which there is usually some remaining tissue. Combinatorial approaches that have been successful to varying degrees will be highlighted; these generally involve combining neurotrophic factors (NTFs), scar modification, elevation of cAMP, reduction in myelin inhibition or olfactory ensheathing cell (OEC) transplantation with SCs. In addition, we shall discuss the rationale behind the success or failure of some combinatorial strategies and promising current treatments that may be amenable to combinatorial therapies.

Use of SCs for transplantation into the spinal cord

The ability of peripheral nerve (PN) bridges to support axonal regeneration in the CNS [74] laid the foundation for transplantation of SCs into the injured spinal cord. Unlike the CNS, which shows a limited ability to regenerate after injury, the PN milieu enables axonal growth and eventually varying degrees of target reinnervation [42]. In the CNS, PN grafts provide a permissive environment for axonal regeneration, mainly from adjacent sensory and propriospinal neurons [77] in the spinal cord as well as from a subset of brainstem spinal axons [22]. Thus, CNS neurons retain an intrinsic ability to regenerate axons when provided with the appropriate environment. SCs myelinate axons in PN. Following PN injury, SCs dedifferentiate and proliferate, remaining in bands of Bungner, into which axons regrow and are myelinated. Myelination of axons is necessary for appropriate axonal functioning, an advantage of transplanting SCs compared to other cell types.

SCs possess additional properties that could enhance recovery after SCI [13]. They produce a variety of growth factors (including NGF, BDNF, and CNTF) which could stimulate the intrinsic ability of damaged neurons to survive and/or extend axons, a number of cell adhesion molecules (including integrins, N-cadherin, N-CAM, L1, contactin) and extracellular matrix proteins (such as laminin and collagens) which could support axonal

growth [10]. It is not surprising then that transplanted SCs fill cystic cavities, increase white matter sparing, myelinate axons of both PNS and CNS origin and promote axonal regeneration in a variety of SCI models [11;51;56;64;86;94].

Traditionally, PN is the most common source of SCs. SCs isolated from PNs can be induced to proliferate in tissue culture to obtain large numbers of cells [60]. The protocol has been optimized to obtain more than 95% purity, after which the cells can be expanded with mitogens for several passages to achieve the number needed for transplantation. Importantly, similar procedures can be used to prepare human SCs for transplantation [16;29;50] including from a spinal cord injured person's own sural nerve, incurring only a minimal sensory deficit while eliminating the need for immunosuppresion following SC transplantation.

In the following section, we will discuss the transplantation of SCs from different origins into complete or partial injuries. It is often difficult to compare these studies based on differences in injury models, surgical techniques, level of injury, restoration of fluid circulation by suturing the dura, among others, all of which could influence the results.

SC bridges in the completely transected spinal cord

Polymer channels filled with SCs in a matrigel suspension can bridge the two severed ends of the completely transected spinal cord and support axonal regeneration from both stumps [92], an important first step in repairing damaged circuits. Regenerated fibers, mainly of sensory and propriospinal origins, enter the SC implant where they associate with the SCs and become ensheathed or myelinated [92;94]. Axonal conduction across the transected cord has been shown [41;69]. Yet, at the level of the thoracic spinal cord, similar to PN grafts [76], SCs do not appear to provide the necessary (or sufficient) signals to promote regeneration of axons from the brainstem. Also, the utility of SC-matrigel grafts alone is limited by the inability of axons to exit the grafts, thus preventing the establishment of connections with the distal spinal cord. Given that brainstem axons can enter the distal spinal cord when PN grafts are placed adjacent to neuronal cell bodies [22], but not when placed remotely within the thoracic spinal cord [76], it seems unlikely that brainstem spinal axons are incapable of responding to SC cues. Instead, axonal regeneration is likely affected by the long distance between the neuronal somata and the grafts. Whereas SC grafts are permissive for axonal growth, additional strategies are needed to enhance regeneration of systems important for sensory, motor and autonomic function through and out of SC bridges.

SC implants in the partially injured spinal cord

Because most human injuries result in some spared tissue, it is essential to understand how SC transplants affect regeneration in partial injury models, and whether they can promote axonal growth in these conditions. Interpreting the results in incomplete models, particularly the ability of axons to regenerate, is complicated by the presence of spared fibers. Despite this confounding factor, important insights into the ability of SCs to repair the spinal cord can be obtained from these studies.

SC bridges in lateral hemi-section injury

Similar to SC bridges placed into the completely transected spinal cord, implantation of SC hemi-channels results in enhanced myelination and axonal regeneration, mainly from propriospinal and sensory neurons [95]. Axons entered the SC hemi-channels and extended up to 0.9 mm as early as 2 dpt, and up to 2.7 mm by 7 dpt [38]. A few tracer-labeled axons reached the caudal host/graft interface (3.1mm) by 14 dpt but were rarely observed beyond in the distal cord. Unlike the completely transected spinal cord, where axons do not reenter the host cord, some axons were found within the caudal cord at later time points [95]. In a longer term study, propriospinal axons extended into the caudal grey matter in 2/7 cases at 45–60 dpt and 2/3 of cases at 90 dpt [95]. Thus, time is a key factor in achieving axonal growth into the caudal cord. Reentry of axons into the distal cord also may be due, at least in part, to the partial injury. The presence of spared tissue is implicated in the enhancement of axonal regeneration because more axons grew into matrigel-only cables following hemi-section than after complete transection (∼550 vs. 126 propriospinal axons, respectively) [92;95]). In addition to the ingrowth of propriospinal and sensory fibers, some brainstem fibers responded following hemi-section, as detected by immunohistochemistry for raphespinal (5HT+) and coerulospinal (DβH+ or TH+) axons within the grafts, and retrograde labeling of neurons in the raphe, reticular, vestibular, locus coeruleus and red nuclei projecting to the grafts in 30% of the animals [95].

These studies raise important questions that warrant further study. Is the enhanced regeneration due to more spared tissue? Is the increased growth a result of regeneration of cut axons or sprouting of spared fibers? Does the exit of axons from the graft require survival times beyond the typically employed 4 – 6 weeks?

SC implants in contusion injury

A number of studies primarily performed by our group have demonstrated that SC transplants survive, are neuroprotective, provide a substrate for axonal growth, promote axonal sparing/sprouting/regeneration to regions caudal to the implant, and enhance locomotor function. Because the SCs were not labeled prior to implantation, it is difficult to know how many host SCs had migrated into the lesion, which is known to occur after SCI [6;14;31]. In the Takami et al. [86] study, a mean of 2125 SC-myelinated axons was found in lesion sites in control animals injected with culture medium only compared to a mean of 5212 myelinated axons attained in SC-implanted animals. More recently it has been demonstrated using genetically labeled cells that both transplanted and host SCs survive, fill the lesion [27;35;36;68] and myelinate axons with ratios (myelinated:unmyelinated axon ratio ∼1:7 [27]) similar to those observed following transection.

Transplanted SCs markedly reduce cystic cavitation [66;67;86]. SCs preserve neurons adjacent to the site of cervical SCI [80] and promote white matter sparing in some [80;86] but not all [67] studies. Sparing of lateral white matter or preservation of nearby neurons may account for the increases in reticulospinal axons and propriospinal neurons retrogradely labeled from below the injury site [80;86].

SC transplants also promote growth of axons into the grafts in contusion sites; about 10,000 – 45,000 total axons reach the graft midpoint [SC only controls in [27;67]. The origin of the majority of these axons has not been determined. Sparing in the contusion model prevents accurate assessment but, based on transection studies, it is expected that a large proportion arise from propriospinal and DRG neurons adjacent to the injury site. Some fibers arise from DRG as determined by CGRP immunohistochemistry and anterograde tracing from the sciatic nerve. Others are 5HT- and DβH-positive, suggesting some penetration of brainstem spinal axons, although the proportions of each phenotype and whether they enter the graft has varied between studies [27;67;68;86]. Overall, anterograde labeling of supraspinal axons has shown limited, if any, penetration of cortico-, reticulo-, and vestibulo-spinal axons into SC transplants [27;67;68;80;86].

SC transplants alone modestly improve behavioral function in some [3;80;86] but not all [27;67] studies. Whereas in all studies axons grew into SC grafts, increased white matter preservation was observed in studies with improved behavioral outcomes [80;86] as assessed by the Basso, Beattie, and Bresnahan (BBB) rating scale [4] following thoracic SCI or by forelimb and grip strength following cervical SCI [80], suggesting that the beneficial functional outcomes observed likely arise predominantly from white matter sparing rather than axonal growth into SC transplants.

Overcoming the limitations of SC transplants

Although SC grafts can promote tissue sparing, provide a permissive environment for sensory and propriospinal axons to grow into the transplanted area, and myelinate peripheral and central axons, by themselves SCs are not sufficient to promote either substantial supraspinal axon ingrowth or exiting of sensory and propriospinal axons from the graft into the host spinal cord. This may be due, in part, to the restriction of SCs to the site of injury, the formation of barriers and upregulation of proteoglycans when SCs encounter astrocytes [28]. Additionally, significant neurological deficits persist, indicating that SCs by themselves are not sufficient for spinal cord repair. A number of combination strategies, mainly targeting neuroprotection and/or regeneration, have been developed to improve repair. Recent reports suggest that some limitations of PN-derived SCs can be overcome by obtaining SCs from alternative sources.

Alternative sources of SCs

SCs used for transplantation are traditionally isolated from adult PNs. Recently, cells with a SC-like phenotype have been differentiated *in vitro* from bone marrow stromal cells (BMSCs) [45] or from skin-derived precursors (SKPs) [9] and examined after transplantation into the completely transected [45] or contused [9] spinal cord. In both cases, these non-PNS derived SCs were able to enhance the growth of descending brainstem axons, which correlated with modest behavioral improvements. Implantation of BMSC-SC bridges into the completely transected spinal cord enhances hindlimb movement without weight support. Transplantation of SKP-SCs into the contused spinal cord increased the number of animals (∼46%) achieving occasional forelimb-hindlimb co-ordination without increased sensitivity to noxious stimuli.

SKP-SCs resemble PN-derived SCs in terms of expression of SC markers, as well as the ability to fill the lesion and myelinate central and peripheral axons. Unlike PN-SCs they migrate and integrate better with host tissue (where they myelinate spared axons). The ability of SKP-SCs to integrate into host tissue better than PN-SCs might arise because cells are at different levels of maturation in the preparation and/or express a different subset of cell adhesion molecules that allow them to migrate over normally inhibitory substrates. Moreover, they may be able to modify the adjacent host tissue, specifically reducing reactive gliosis and neurocan expression or altering growth factor expression leading to enhanced tissue preservation [9]. Despite many unanswered questions and the need for further characterization and direct comparison with PN-SCs, other autologous sources may provide therapeutically beneficial SCs.

Co-transplantation of SCs and OECs

Co-transplantation of different cell types can be used to overcome the limitations of one particular cell type and/or to promote an additive effect. To date, few studies have examined the benefits of transplanting SCs with other cell types that have been shown already to have a positive effect in models of SCI. One cell type that has been combined with SCs is the olfactory ensheathing cell (OEC). OECs have been of interest to students of CNS axonal regeneration because of their location in the body. They provide a channel for olfactory axons to grow throughout adulthood; because their parent sensory somata in the olfactory epithelium are continually replaced, olfactory fiber growth from these new neurons is needed to make contact with the olfactory bulb. OECs enable these axons to cross the pial-glial surface of the brain to enter the astrocyte environment of the bulb [72]. Thus, OECs appear to have unique properties that enable axonal growth from the peripheral environment into the central milieu throughout adulthood.

One of the unique properties of OECs compared to SCs is that they intermingle with astrocytes in vivo [49;79] as well as in vitro [62]. In contrast, SCs do not invade astrocyte territory and they form a barrier containing proteoglycans inhibitory to axonal growth when they confront astrocytes. These findings suggest that OECs combined with SCs may modify the SC-astrocyte interface to provide a permissive path for axons to cross the interface. In one study of OECs injected into the cord stumps bordering a SC bridge (in a complete transection model), the exit of axons from the SC bridge into host tissue was improved [75]. By introducing tracer above the bridge, labeled axons were observed on the bridge and leaving the bridge to enter the caudal cord.

OECs have been combined with SCs not only to modify inhibitory interfaces but also to foster axonal regeneration. Many investigators have transplanted OECs alone to examine this question. In an excellent review, Franssen et al. [26]; see also [72]] point out that 41 studies reported positive results while 13 showed limited or no effect of OEC transplantation. There are undoubtedly many factors that account for these divergent results as the studies are conducted differently in the respective laboratories. The lesion model, size and severity of the lesion, the source (olfactory bulb or lamina propria), preparation and purity of OECs, the age of the animal from which OECs are obtained (embryonic vs. adult), the post lesion time at which OECs are implanted, and especially, survival of the OECs

vary. OEC migration also may be a factor although how much they migrate is controversial [26].

It is as yet unclear to what degree OECs improve repair when they are co-transplanted with SCs beyond that observed following SC implantation alone. Transplantation of SC and OECs together has been compared to implanting each population alone. SCs (2×10^6), SCs and OECs (1×10^6 each) or OECs (2×10^6) were introduced in culture medium into a contusion site [86]. Eleven weeks post-transplantation (12 weeks post-injury), “peripheral type” myelinated axon counts in the SC/OEC implants were 3884 +/- 711 compared to 5212 +/- 1783 in SC implants and 2965 +/- 1110 in OEC implants. Most of the myelin in the combination graft could have been formed by SCs and, in the OEC implants, by endogenous SCs because there is substantial migration of host SCs into lesions; the myelinated axon count was 2125 +/-697 without any cell implantation in this experiment. Without reliable cell labeling before implantation, OEC survival without SCs could not be assessed adequately. OEC survival is improved when they are injected at either end of a contusion site into which SCs have been introduced [68].

When a retrograde tracer was injected caudal to the lesion/implant, and the neuronal somata of the axons reaching this level were labeled with the tracer, the numbers of labeled cell bodies in cervical and thoracic spinal cord and the reticular formation were significantly higher in SC and SC/OEC transplanted animals, than in OEC- grafted animals [86]. Because there is circumferential spared tissue after contusion, it is not known whether the axons below the lesion/implant were spared or regenerated. Occasional forelimb-hindlimb coordination attained in SC-grafted animals appeared to correlate with the higher numbers of spinal and supraspinal axons reaching spinal segments distal to the grafted area compared to OEC grafts. But, in another study [66] co-transplantation was advantageous in that there was sparing/regeneration of reticulospinal fibers caudal to the implant and these and serotonergic fibers were observed in the graft, in contrast to SC implantation alone.

Neuroprotective strategies

Secondary damage leads to the death of neurons and glia adjacent to the primary injury site. Neuroprotective strategies target pathways involved in secondary injury to prevent or mitigate this loss and enhance tissue sparing, thus preserving function. Recently, using genetically labeled cells demonstrated that the majority of transplanted SCs die early after implantation when transplanted into the acute, sub-acute or chronic injured spinal cord [3;35;36;68], primarily via necrosis [35]. Thus, the use of neuroprotective agents at the time of transplantation should be considered for both limiting the initiation of secondary tissue damage and enhancing SC survival and function.

Several pharmacological agents reduce post-traumatic spinal cord degeneration, including methylprednisolone sodium succinate (MP), dexamethasone, naloxone, and monosialoganglioside (GM1), among others [32]. Some of these agents, for example MP and GM1, appear to have a dual role in neuroprotection and regeneration, which may complicate interpretation of results. An emerging example of this duality is cyclic AMP (cAMP), a second messenger involved in CREB-dependent gene transcription that has been implicated

in axonal regeneration [33]. In addition, cAMP-dependent genes are involved in protection against cellular stress [20;61].

After contusive SCI, significant neuroprotection is observed after prolonged administration of Rolipram, a phosphodiesterase 4 inhibitor that prevents the breakdown of cAMP, but only if it is delivered immediately after injury and not after a 7 day delay [67]. Indeed, acute delivery of Rolipram alone significantly increases both the number of SC-myelinated axons within the lesion and white matter sparing, as assessed by counts of spared centrally myelinated axons. These histological results correlate with reduced hindlimb exorotation and improved base of support as well as fewer footfall errors on a gridwalk. Additionally, acute Rolipram treatment alone accelerated the rate of spontaneous recovery, as indicated by the significant improvement in the BBB score observed at 4 and 5 weeks but not at early and late time points [67].

The addition of SCs did not result in enhanced histological or behavioral outcomes over acute Rolipram treatment alone. Yet, a significant improvement in the BBB was observed 3-8 weeks post-transplantation when db-cAMP also was injected into the spinal cord (triple combination) compared to injury only. Delaying Rolipram administration until the time of SC transplantation at 1 week post-injury did not appear to be neuroprotective, as it neither enhanced white matter sparing nor increased the number of retrogradely labeled reticulospinal axons over injury alone. This suggests that Rolipram, like most neuroprotective agents, has a narrow time window in which it can modulate the injury environment. Whereas the effects of Rolipram as a neuroprotective agent for SCs was not the subject of this study, given the strong neuroprotective effects observed following acute delivery, it will be important to test whether cAMP improves the survival of the transplanted cells, the ability of transplanted cells to myelinate or whether the effects of the increase in SC-myelinated axons following cAMP treatments arises from increased ingrowth of host SCs.

As mentioned, many of the neuroprotective agents used also may affect regeneration. cAMP was primarily studied for its regenerative abilities after SCI. It should be pointed out that both acute and delayed Rolipram treatment in combination with SCs and db-cAMP increased the number of total axons in the graft and 5HT+ raphespinal axons projecting into both the SC transplant and the caudal spinal cord beyond. The increase in 5HT+ fibers or retrogradely labeled raphe neurons in all groups receiving Rolipram acutely suggest that Rolipram was able to enhance axonal growth of at least one fiber system. The number of labeled raphe neurons was significantly larger when the cAMP levels were highest (in the triple combination) than in the Rolipram/SC combination [67]. Further examination of this and other axonal tracts is needed to further understand the role that Rolipram plays in regeneration and to what extent the histological and behavioral effects are due to regeneration in addition to neuroprotection.

The most extensively studied and controversial neuroprotective agent for SCI is MP, a corticosteroid that modestly improves neurological function in humans when administered at high doses within the first 8 hrs after SCI [23;32]. Although a steroid, the high effective doses of MP required for efficacy indicate non-steroid actions. The beneficial effects of MP

are mainly attributed to its anti-inflammatory properties through inhibiting lipid peroxidation and reducing edema [32]. When administered at the time of transection and implantation of SC bridges, MP promotes host tissue sparing, as determined by increased tissue preservation within the rostral spinal cord stump, and enhances the size of the SC bridge [19]. The latter suggests that MP promotes transplant survival, which correlates with an increase in myelinated axons within the tissue bridge. In addition to the neuroprotection, MP promotes axonal regeneration into both rat and human SC bridges [19;30]. In addition to the propriospinal and sensory axons observed normally in SC cables, MP promotes brainstem spinal axon regeneration, as determined by both tracing and the presence of 5HT+ and DBH+ axons within the grafts [19]. The presence of more axons within SC grafts after MP treatment may arise from increased preservation of neurons within the spinal cord stumps, reduced glial scarring or, alternatively, increased growth factor levels resulting from improved transplant survival. Similar to the increased regeneration observed with SC hemi-channels, the MP data suggest that preserving tissue adjacent to the site of SCI may be one of the most effective ways to promote regeneration. Thus, additional neuroprotective agents should be considered in conjunction with SC transplants.

Regeneration strategies

The re-establishment of functional connections after transplantation is a multistep process in which the axons should 1) circumvent the inhibitory milieu and enter the transplant, 2) exit the transplant into the host cord, 3) extend through host tissue to find the appropriate targets and 4) make functional synapses with the targets. Overall, significant axonal growth can be achieved by enhancing the inherent ability of axons to regenerate and/or by decreasing the inhibitory environmental signals that prevent the growth of axons through a transplant and the host tissue. Interestingly, axons closely associate with substrates expressing both inhibitory and stimulatory molecules [43], suggesting that axonal regeneration can take place in situations where both signals are present. Hence, for a therapeutic intervention to work, it needs to shift the balance between these two opposing signals, decreasing the response of the axons to the inhibition and/or increasing the response to growth.

Growth factors

NTFs are strong candidates for combination therapies, given their known ability to promote neuronal survival [37], axonal regeneration/sprouting and neuroprotection [85], and SC differentiation [17], and to enable growth of neurites on inhibitory substrates [15]. Administration of NTFs after SCI has provided information about the response of certain neuronal populations to specific factors. For these studies, it appears that the efficacy of a particular NTF is influenced by the method of delivery, the site of administration, the specific axonal system assessed, the expression of NTF receptors after injury and whether or not a substrate is provided for growth. Here we will limit our discussion to the role of NTFs on regeneration in the context of SC transplantation.

The use of individual NTFs after SCI can promote regeneration although their benefits are limited. This might be due partially to the tracts examined, as the response of particular axon tracts depends on the repertoire of receptors expressed as well as the signals elicited by the NTF [44]. Among the most studied NTFs in the field of SCI repair are neurotrophin 3

(NT3), nerve growth factor (NGF), glia derived neurotrophic factor (GDNF) and brain derived neurotrophic factor (BDNF), all of which exert specific (and sometimes overlapping) effects on different populations of cells. NT3 treatment promotes sprouting of corticospinal tract (CST) fibers [82] and growth of sensory axons but this response is limited and disorganized [12;73]. GDNF enhances neuroprotection and regeneration after SCI but not recovery of electrophysiological parameters [40], for which remyelination of the regenerated axons is essential. BDNF, on the other hand, does not appear sufficient to promote sprouting of CST fibers [82] or growth of ascending sensory axons across a lesion site [12;73]. Taken together, the results obtained with NTFs alone are promising but suggest the need for additional interventions.

Delivery of NTFs to the injured spinal cord can promote regeneration of axons onto SC implants beyond that observed following transplantation of SCs alone [93]. In general, NTFs enhance propriospinal, sensory and supraspinal axonal growth, although different populations of axons respond differently to NTFs either alone or in combination. Propriospinal neurons readily extend axons into SC grafts after GDNF, NT3 and/or BDNF treatment [2;27;39;59;93]. Sensory neurons appear more responsive to certain combinations, specifically BDNF or NGF [59;87;91]. The enhanced response of sensory and propriospinal neurons may arise from the site of NTF delivery being close to the neuronal cell body, whereas differences in response to specific NTFs is probably due to differing levels of their receptors [1]. The response of supraspinal fibers seems more restricted, influenced at least in part by the long distance between the site of delivery and the neuronal soma. Brainstem neurons rarely extend axons into SC grafts alone, whereas when combined with NTF treatment some but not all brainstem nuclei respond [59;93]. The failure of some brainstem neurons as well as cortical neurons to respond to NTFs is unlikely due to unresponsiveness of the tracts because NT3, BDNF or NGF supports the growth of CST axons towards spinal cord tissue in an organotypic co-culture system [46] and NT3 promotes sprouting of CST fibers after injury *in vivo* [82]. Although local upregulation of NTF receptors takes place after injury [52;88], differential expression of the repertoire of receptors will influence the response to a particular NTF and could account for the failure of specific brainstem and cortical neurons to respond. Assessing the overall effects of NTFs is complicated because it is unclear from these studies how far the labeled axons grow and whether the observed regeneration translated into functional connections with host neuronal circuits. Yet, under a number of paradigms tested, NTFs clearly show the capability to promote long-distance regeneration into SC grafts.

Axons do not exit SC implants without additional intervention. We currently do not know how much growth is required to promote recovery of function, but the axons will need to leave the graft to release neurotransmitter, excite the central pattern generator and/or form connections with host neuronal circuits. In addition to their ability to promote regeneration into SC grafts, NTFs enable axons to reenter the host spinal cord in some cases. Infusion of BDNF, NT3 or a combination of BDNF/NT3 caudal to SC hemi-cables lures propriospinal axons up to 6.5mm into the host cord towards and beyond the source of NTFs by 1 month after injury [2]. Axons leaving the permissive milieu of the SC bridge face the inhibitory environment of the interface and the white matter beyond. Of interest, pre-exposure of

cerebellar neurons to BDNF counteracts the inhibition by myelin-associated glycoprotein, one of the major blockers of regeneration found in myelin, and supports axonal growth over an inhibitory environment via a cAMP-dependent mechanism *in vitro* [15]. Hence, it is possible that levels of cAMP (and other signaling intermediates) in the regenerated axons were elevated before reaching the caudal spinal cord, thus counteracting the effects of the inhibitory milieu. Indeed, strategies aimed at maintaining high levels of cAMP enhance fiber ingrowth into SC grafts [67]. Moreover, a direct comparison of BDNF and NT3 in the study by Bamber and colleagues [2] reveals that axons orient differently in the caudal spinal cord, with more terminal branching after NT3 treatment and more parallel growth after BDNF delivery. Because each NTF likely has unique effects on axons, their combination might be synergistic.

SC grafts secreting NGF attract coerulospinal axons, but mainly axons of sensory origin [87;91]. Although regeneration of CGRP-positive fibers can result in sensory recovery of thermal and pressure nociception [73;78], it is possible that aberrant sprouting could lead to functional abnormalities such as chronic pain [21] or autonomic dysreflexia [48]. Thus, the effects of NGF and SC transplantation have been viewed with caution although, to our knowledge, a direct correlation between these two parameters in the context of SC transplantation has not been established. Interestingly, analysis of postmortem human spinal cord has revealed that the regenerated axons found at different time points after SCI associate with SCs expressing higher levels of NGF receptor [90], suggesting a role of NGF in the regeneration attempt.

It should be noted, however, that not all combinations have an additive or synergistic effect. For example, the combination of SC grafts with either bFGF [58] or a mixture of insulin-like (IGF-1) and platelet-derived (PDGF) growth factors [63] did not result in enhanced regeneration of fibers, compared to SCs alone. A commonality between these two approaches is that the growth factors were mixed with the matrix at the time of preparation, and the levels or activity of the factors after implantation were not evaluated *in vivo*. In addition, more and larger cavities were present in both studies, as well as a stronger endogenous glial response, which could influence axon growth negatively. This reinforces the idea that signals rendering the axons insensitive to, or neutralization of, the inhibitory milieu are essential for regeneration.

An important aspect that has not been examined extensively is the role of NTFs in the survival and differentiation of the grafted SCs. It appears that the size of the SC transplant is larger when cells genetically modified to produce high levels of NTFs are used. Transplants of SCs expressing either D15A, a bi-functional NTF that mimics the actions of NT3 and BDNF [27], or NGF [91] are enlarged compared to control grafts, which correlates with greater axon ingrowth. Whether the reason for the accompanying increase in cell number is due to increased cell survival, proliferation or migration into the graft is not yet known.

Overall, the studies discussed in this section demonstrate that the combination of SC transplants and NTFs can enhance propriospinal and brainstem spinal axonal growth through a SC graft spanning the injury site. And, yet, the short-length regeneration together with the modest response of some supraspinal tracts highlight the necessity to continue the search for

optimal combination therapies. Perhaps altering the glial scar and/or reducing myelin inhibitors or raising cAMP levels could be additive to NTF and SC graft combinations.

Modification of the glial scar

Despite robust growth into SC grafts, few axons exit the bridges, most likely due to astrocytic scarring and accumulation of proteoglycans at the host-graft interface [38;70]. Thus, additional approaches are needed to target the inhibitory signals from the glial scar in order to promote reentry of axons into the caudal spinal cord. Reduction in proteoglycans by chondroitinase ABC (Ch'ase) treatment favored the exit of axons caudal to SC implants in complete [25] and incomplete [18] transection models. The majority of axons exiting SC/OEC/Ch'ase grafts arose from propriospinal neurons in the thoracic and, to a lesser extent, the cervical spinal cord as well as from raphe, reticular and vestibular brainstem nuclei [25;89]. Modification of adhesion interactions has been explored in an attempt to modulate the interaction of SCs with the glial scar. When SCs expressing PSA-NCAM (a negative regulator of cell-cell interaction) were transplanted into the spinal cord, ∼7% of 5HT+ fibers entering the grafts were seen exiting into the host cord, which correlated with a modest improvement in locomotion [65]. The significance of PSA-NCAM in these results is not completely clear because few cells express it at 7 days and beyond. Despite this, altering cell-cell adhesion to modify SC interactions with the host tissue is an interesting approach that warrants further study.

Reduction in myelin inhibition

Fibers regenerating through a SC graft generally do not grow long distances into the host cord and locate preferentially in the grey matter [18]. A number of inhibitory molecules are present in CNS myelin, including Nogo, myelin associated glycoprotein, oligodendrocyte-myelin glycoprotein and a subset of ephrins. Neutralization of these molecules directly or modification of their downstream signaling events were able to enhance axonal growth in different models [8;71;84]. Combination strategies to promote exiting of axons from SC grafts should be tested with agents that counteract myelin inhibitory signals. This goal has not been explored extensively, except for a report by Guest and colleagues [29] where only CST axons were examined; the effect on propriospinal or brainstem spinal axonal regeneration is not known. Most of the myelin inhibitors identified so far elicit common downstream signaling events that center on the activation of the Rho pathway through the Nogo receptor complex. Indeed enhanced axonal regeneration in the optic nerve and in various models of SCI has been achieved by targeting the Rho pathway through Rho kinase inhibitors [57]. The advantage of this approach is that with a single molecule the inhibition could be neutralized, reducing the need to use a variety of agents to counteract each myelin protein or its receptors. This is an exciting area that needs to be further explored.

Relationship between enhanced neuroprotection/regeneration and functional recovery

Despite partial success in neuroprotection and regeneration strategies described above, restoration of locomotion needs further improvement. Although frequently assessed, the percent of spared white matter is not the best predictor of functional outcome, but rather

identification of the tracts that remain intact within the spared white matter would be more valuable [83]. In rodents, sparing of neurons in the ventral medulla [5] and the ventrolateral funiculus (VLF) [53;83] seems to be essential for the initiation of stepping movements, as assessed by the BBB scale. Contained within the VLF are the coeruleo- and reticulo-spinal tracts and the long-descending propriospinal systems. Growth of these tracts into SC grafts has been observed in response to various combinations. Whereas serotonergic fibers are among the tracts that respond most readily to SC combination therapies, and their growth is a good marker for long-distance regeneration, in what manner their growth correlates with functional improvement after SCI remains to be elucidated [25;67]. Descending systems in the dorsal spinal cord, including the CST and rubrospinal tracts, seem to be more involved in the control of fine movements, as assessed by the gridwalk test [54;83]. Usually CST fibers do not respond to SC combinatorial strategies in contrast to the rubrospinal tract.

Concluding remarks

The SC is a promising candidate for cellular transplantation to repair the injured spinal cord. Studies have shown consistently that SCs promote axonal growth, particularly from sensory and propriospinal origins adjacent to the lesion. Moreover, SCs are able to myelinate the ingrowing axons and re-establish axonal conduction. SC transplants are limited in that few long-tract axons enter and few axons exit the grafts. Using SCs in combination with neuroprotective agents, molecules that modify the glial scar or NTFs can enhance the ingrowth of long descending axons as well as the exit of fibers. An example of a molecule that may affect both neuroprotection and regeneration is cAMP which in combination with SC implants is able to improve functional recovery. Nonetheless, appropriate restoration of function has not yet been achieved. Thus, development of additional combination strategies is needed. An ideal combination would enhance transplant survival, host tissue sparing, axonal growth across the lesion and neutralization of the inhibitory signals that prevent axonal regeneration at the implant-host cord interface and distal to the lesion. Despite the complexity of repairing the injured spinal cord and the work that remains to be done, important milestones have been achieved and provide a sound foundation for future discoveries.

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Abbreviations

BMSCs	bone marrow stromal cells
BDNF	brain derived neurotrophic factor
Ch'ase	chondroitinase ABC
CNS	central nervous system
CST	corticospinal tract
cAMP	cyclic AMP
DRG	dorsal root ganglia
GM-1	monosialogangloside
GDNF	glial derived neurotrophic factor
MP	methylprednisone
NGF	nerve growth factor
NTF	neurotrophic factor
NT3	neurotrophin 3
OEC	olfactory ensheathing cell
PN	peripheral nerve
SC	Schwann cell
SCI	spinal cord injury
SKPs	skin-derived precursors