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Recent advances in regenerative medicine approaches for spinal cord injuries

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Abstract

Traumatic injury to the spinal cord leads to a loss of motor and sensory function below the level of injury. The lack of growth-associated proteins, local expression of inhibitory factors, and scar and cyst formation create an inhibitory environment in the spinal cord, which limits the regenerative capacity of endogenous or transplanted cells. Cell and drug delivery strategies, either alone or in combination, can induce changes in the local microenvironment at and around the lesion site to promote transplanted cell survival, integration, and/or endogenous repair. New biomaterial strategies also provide a platform for sustained delivery of otherwise unstable drugs.

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Introduction

Spinal cord injury (SCI) is a devastating condition affecting thousands of people each year. The global incidence of SCI is approximately 180,000 people annually as of 2015 [1]. Patients with SCI face high rehabilitation costs and limited treatment options [2], compounded by the insufficient ability of central nervous system (CNS) tissue to spontaneously regenerate following injury. This lack of tissue regeneration results in a lifelong loss of sensory and motor function. Consequently, SCI is a key focus area of tissue engineering strategies that aim to restore function and patient quality of life by promoting tissue regeneration (Figure 1). While many inhibitors to CNS regeneration have been uncovered, ongoing investigations into the pathophysiology of SCI continue to lead to novel therapeutic strategies. Here, we highlight recent scientific and clinical advances in cell transplantation and drug delivery strategies to promote tissue and functional recovery after SCI.

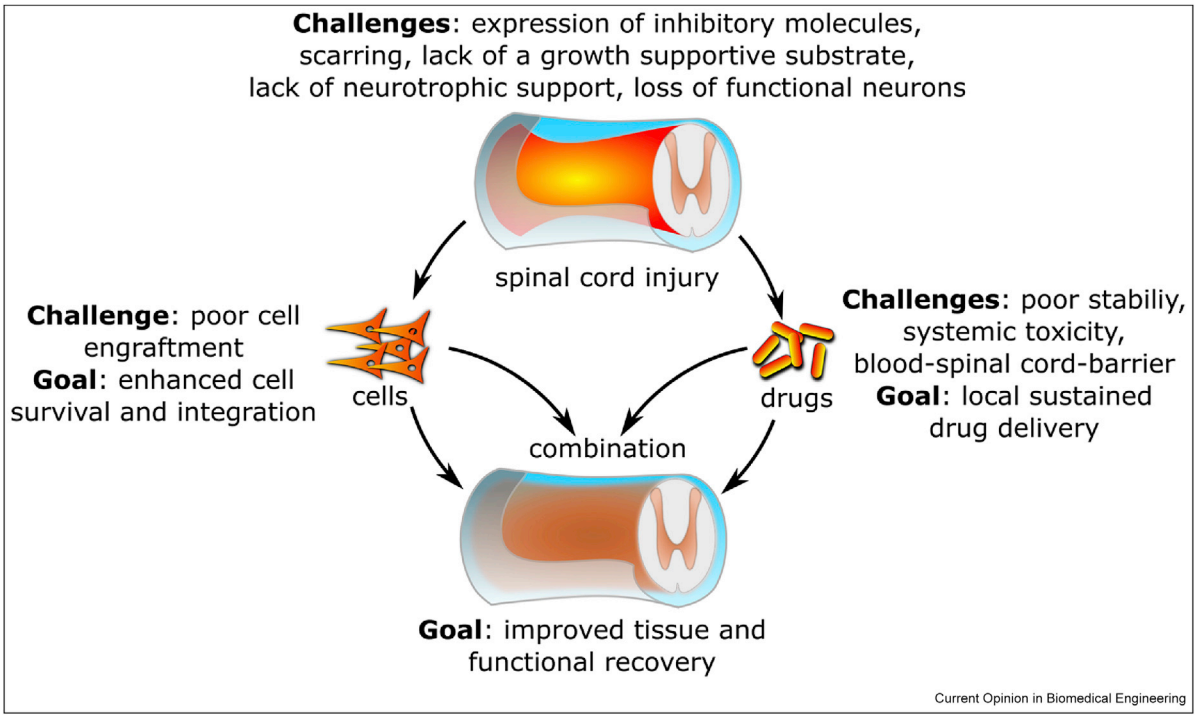
Overcoming the inhibitory injury environment

The inhibitory environment

Inhibitory molecules in the spinal cord after injury include those associated with myelin (i.e. myelin associated inhibitors, MAIs) and those associated with the extracellular matrix (ECM), such as chondroitin sulfate proteoglycans (CSPGs). Astrocytes and other reactive cell populations lining the injury site secrete CSPGs, which contribute to the formation of an ECM-rich glial scar. Although the glial scar limits the spread of secondary degenerative events [3,4], it also presents a physical and chemical barrier to regeneration at later stages. CSPGs prevent axonal regeneration and, in combination with the ECM molecule laminin, trap growth cones at the lesion site [4]. CSPGs are also found in perineuronal nets, which surround and stabilize mature neurons, restricting axonal sprouting and neuroplasticity [5]. Receptors for CSPGs include leukocyte common antigen-related receptor (LAR), protein tyrosine phosphatase (PTPσ) [6], and the Nogo receptors NgR1 and NgR3 [7].

Myelin-associated inhibitors, such as Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp), are released with oligodendrocyte necrosis and apoptosis in the form of myelin debris and primarily act through the Nogo receptor1 (Ngr1) to induce cytoskeletal rearrangement and subsequent axonal growth cone collapse [8]. Semaphorins and ephrins have also been identified as having inhibitory roles following SCI by negatively affecting axonal guidance [9]. Furthermore, activation of the Rho/ROCK pathway, a rise in intracellular calcium, phosphorylation of epidermal growth factor receptor (EGFR), and inhibition of Akt and Erk1/2 phosphorylation have all been implicated in MAI and CSPG mediated growth cone collapse [10]. It was also recently determined that spinal cord tissue caudal to the site of injury is in a

Figure 1



Challenges associated with spinal cord injury and its treatment. Repair after spinal cord injury presents several major challenges that can be overcome with cell therapy, drug delivery, or combination strategies. Several challenges persist with these treatment options, which are being addressed in current research.

chronic state of hypoxia, further contributing to the inhibitory environment [11].

Removing inhibitory molecules

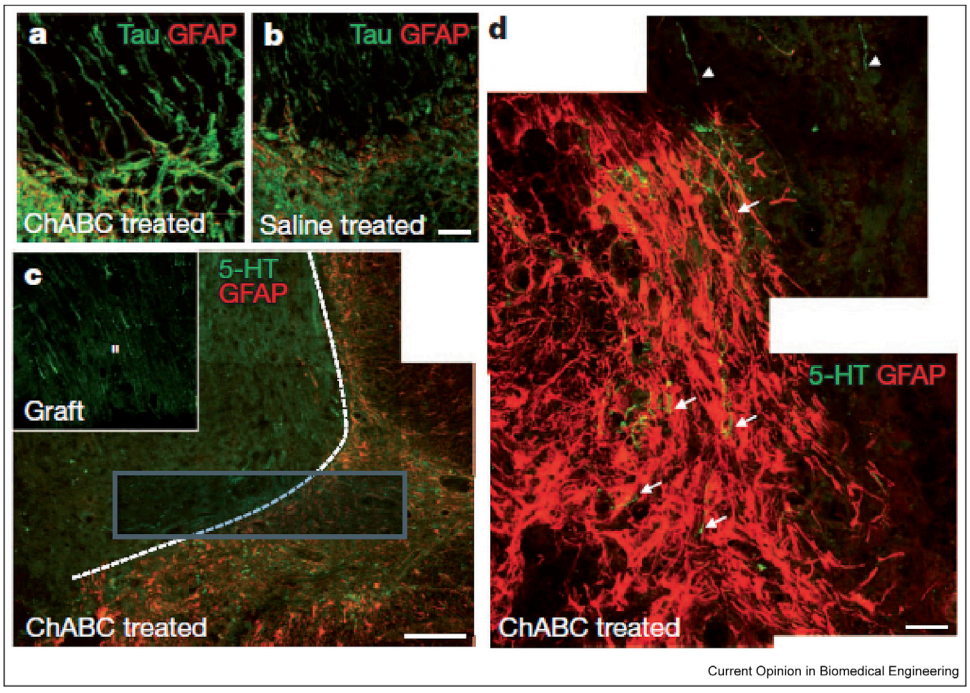
The bacterial enzyme chondroitinase ABC (ChABC) can be used to degrade the inhibitory glycosaminoglycan component of CSPGs within the glial scar and perineuronal nets, leading to axonal regeneration, some functional recovery, and increased synaptic plasticity (Figure 2) [5,12,13]. Due to the thermal instability of ChABC, initial studies required multiple invasive injections or continuous infusion of ChABC through osmotic mini-pumps, which are prone to clogging and infections. More recently, intrathecal injections of ChABC in hydrogel delivery vehicles have been used to prolong enzyme bioactivity and provide sustained delivery for days or weeks. For example, Sakiyama-Elbert and colleagues developed a fibrin hydrogel containing lipid microtubes for ChABC delivery [15] which have previously demonstrated efficacy *in vivo* [14], and PLGA microspheres for delivery of NEP1-40, a peptide that inhibits activation of the NgR1 receptor. These drug-loaded hydrogels demonstrated sustained drug release over 1−2 weeks *in vitro*, and decreased CSPG expression and increased axonal regeneration *in vivo*, 2 weeks after injury [15,16]. In another study, Pakulska, et al. developed an affinity-based delivery system, in which ChABC was expressed as a fusion protein with a Src homology 3 (SH3) domain and reversibly bound to a methylcellulose hydrogel decorated with SH3 binding peptides [17,18]. This delivery system provided sustained delivery of bioactive ChABC for 1 week *in vitro* and decreased CSPG levels for 2 weeks post-injury *in vivo*.

Blocking receptors of inhibitory molecules

Since some receptors and signaling pathways are activated by several inhibitory molecules, targeting the receptors or downstream pathways directly may be more effective than targeting the ligands. For example, targeting the NgR receptor led to greater behavioral recovery than targeting Nogo-A [19,20]. Similarly, Li, et al. inhibited ephrinB3 and sema4D using antibodies that were modified with collagen binding domains to enable sustained release from collagen hydrogels [21]. When combined with NEP1-40, this combinatorial strategy resulted in axon regeneration into the lesion site and improved motor skills. Although beneficial outcomes have been observed with NgR1 inhibition strategies, the results have been varied. Consequently, novel NgR1 antagonists [22] and NgR and Nogo gene silencing strategies [23,24] have also been recently developed as alternative approaches for inhibiting the NgR pathway.

Alternatively, inhibitory CSPGs can be counteracted by blocking the PTPσ receptor. In a recent report, Lang,

Figure 2



Treatment with chondroitinase ABC promotes axonal outgrowth. Rats with SCI were treated with peripheral nerve grafts and bolus injections of ChABC at a single timepoint. (A) ChABC treatment enhances axonal outgrowth both within the nerve graft and into the host tissue compared to (B) saline treatment. (C) The graft-spinal cord interface is denoted with a dashed white line. (D) ChABC treatment results in serotonergic fibers entering the CNS. Scale bars = 40 μm. Reproduced with permission from [13].

et al. demonstrated that PTPσ could be blocked using a peptide antagonist, leading to functional growth cone formation in an *in vitro* model of the inhibitory glial scar environment, and improved motor and urinary function following contusive SCI in a rat model [25].

Despite advances in the sustained delivery of sensitive proteins and the development of new therapeutics, it remains unclear at what time point and for what duration molecules that counteract the inhibitory environment should be delivered, such that overall healing is not negatively impacted. Further investigations are necessary to establish the optimal timeframe for delivery of protein therapeutics, especially in clinical settings.

Enhancing endogenous repair mechanisms

Promoting axonal regeneration with growth factors

Glial scar formation, MAIs, and CSPGs are not the only factors that deter axonal regeneration. The overall balance is further shifted towards a growth inhibitory environment due to the lack of neurotrophic support and an ongoing inflammatory response. Consequently, strategies that stimulate axonal regeneration following SCI continue to be investigated. Numerous neurotrophic factors have demonstrated efficacy in promoting regeneration in preclinical models of SCI, including neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) [26]. Both NT-3 and BDNF activate tropomyosin related kinase (Trk) receptor signaling pathways, leading to increased axonal sprouting and neuroprotection. While endogenous levels of NT-3 and BDNF are typically low in healthy spinal cord tissue, Trk receptor expression increases in both neuronal and non-neuronal cells following injury [27].

Therapeutic use of neurotrophins is limited by their short half-life *in vivo* and difficulty crossing the blood-spinal cord barrier (BSCB). Damage to the microvasculature is linked with changes in the permeability of the BSCB, which can be affected by both physical disruption and inflammatory signals. Increased BSCB permeability peaks within hours post-injury, and can persist at a lesser degree for up to 1–2 weeks, coinciding with the timeframe of revascularization [28]. Since the BSCB presents a significant barrier to systemic drug delivery, a better understanding of the BSCB will aid in establishing an accurate therapeutic window for improved treatment efficacy.

Biomaterials that can be injected in a minimally invasive manner into the intrathecal space of the spinal cord can overcome the limitations of the BSCB and provide sustained local delivery of bioactive molecules to the

spinal cord. For example, intrathecal injection of a composite delivery system, in which NT-3 was encapsulated within poly(lactic-co-glycolic acid) (PLGA) nanoparticles dispersed throughout a hyaluronic acid/methylcellulose hydrogel, enabled NT-3 delivery over 28 days and induced axonal regeneration and functional motor recovery [29]. Interestingly, both NT-3 and BDNF have recently been shown to electrostatically bind to PLGA nanoparticles within hydrogels, enabling effective growth factor delivery without harsh encapsulation processes [30].

Other affinity-based delivery systems have also been developed to prolong growth factor delivery. For example, Han, et al. employed a collagen hydrogel to deliver BDNF tagged with a collagen binding domain, which promoted axonal regeneration and motor and sensory recovery in a canine model of SCI [31]. Alternatively, lentiviral vectors have been delivered *in vivo* to support continuous BDNF and NT-3 secretion by endogenous cells within and surrounding the SCI lesion site [32,33]. These lentiviral-based strategies also exhibited improved axonal regeneration and functional recovery in various animal SCI models. However, lentiviral delivery carries the risk of vector-related side effects, variable immunogenicity, and potential for oncogenesis, which are compounded by lack of specificity to the intended tissue. To mitigate these risks, lentiviral delivery could be combined with biomaterial scaffolds to increase lentivirus retention and efficacy within the injury site. Emerging gene editing strategies such as CRISPR/Cas9 may also present the possibility of efficiently driving overexpression of neurotrophic factors in spinal cord tissue [34,35].

Promoting axonal regeneration with small molecules

Several small molecules have been shown to enhance tissue repair in the spinal cord. Perhaps the most interesting new therapeutic avenue in this area is the novel use of anti-cancer drugs. Epothilone B and Taxol are two clinically approved drugs that stabilize cellular microtubule networks, effectively inhibiting cancer cell division. However, when delivered systemically or intrathecally following SCI, epothilone B and Taxol inhibit scar formation by reducing fibroblast migration and TGF-β signaling, resulting in axonal regeneration and functional recovery [36,37]. Although administration of Taxol requires continuous intrathecal delivery, which may hinder clinical translation until sustained delivery strategies are developed, epothilone B can freely cross the BSCB and thus can be optimized for systemic delivery. Small molecules can also be used to overcome hypoxia that occurs following SCI. Inhibition of either monoamine receptors or L-amino acid decarboxylase (AADC) has been shown to counteract the effects of hypoxia and improve locomotor function [11]. Ultimately, sustained local delivery strategies will be necessary to improve drug efficacy and reduce potential systemic side effects associated with anti-cancer drugs.

Stem cell recruitment and reprogramming

Neural stem/progenitor cells (NSCs) play an important role in the healing response following SCI. A study by Frisen and colleagues used lineage tracing to demonstrate that FoxJ1^{+} cells within the central canal migrated to the lesion in response to SCI, differentiating primarily into astrocytes within the injury site and oligodendrocytes in adjacent regions [3,38]. Since then, several studies have aimed to enhance the natural homing abilities of endogenous NSCs to injury sites and divert their differentiation from astrocytes to neurons. One attractive target for enhancing NSC homing is stromal cell-derived factor-1α (SDF-1α), which can modulate cell migration through the CXCR-4 pathway. To this end, Liu, et al. recently demonstrated that the SDF-1/CXCR-4 axis is essential to NSC migration and proliferation [39]. Moreover, continuous SDF-1α infusion has been shown to increase endogenous cell proliferation and vessel formation following SCI [40]. However, local SDF-1α delivery strategies are still under development and require further refinement. For example, local sustained delivery of SDF-1α using PLGA nanoparticles from an injectable hyaluronic acid/methylcellulose hydrogel did not influence NSC behavior or recovery [17].

In vivo cell reprogramming offers a different avenue for modulating endogenous healing by diverting a subpopulation of NSCs towards the neuronal lineage to replace lost neurons. Su et al. demonstrated that *in vivo* reprogramming of astrocytes to neurons in the spinal cord could be achieved through the delivery of only two components — the transcription factor Sox2 and the histone deacetylase inhibitor, valproic acid [41]. This strategy generated astrocyte-derived neurons 4–8 weeks post-treatment. Given the importance of the astrocytic scar in potentiating the CNS healing cascade [42], it may be beneficial to stimulate gradual or incomplete turnover of astrocytes into neurons, as the need for the protective scar lessens and the injury environment becomes more amenable to regenerating axons.

Cell transplantation

Cell fate following SCI

Significant cell loss occurs during SCI, resulting in an expansive region of tissue necrosis that extends beyond the original injury site. The lack of growth-permissive substrates and neurotrophic support in the lesion site hinders axonal regrowth, and repair is further abrogated by mature neurons, which downregulate Trk receptors and upregulate receptors for MAIs and CSPGs [43]. Consequently, one of the key challenges in treating SCI

is restoring cellularity and re-establishing the complex neuronal network.

Promoting cell survival and integration

Cell transplantation aims to replace lost neurons and other neural cell types following SCI. Since survival of transplanted cells is typically low, recent work has focused on improving cell engraftment and survival. Many biomaterials used for drug delivery are also suitable for cell transplantation, providing a simple strategy for co-delivery of cells and growth factors that can facilitate cell survival and integration [8].

Tuszynski's group established basic trophic requirements for successful NSC delivery and engraftment, demonstrating excellent integration of rat and human NSCs into fully transected spinal cords in rodents using a fibrin delivery vehicle containing a combination of 9 growth factors and a neural cell death inhibitor [44,45]. A large portion of transplanted NSCs differentiated into neurons, which formed synapses with all major known spinal projections and integrated into the neuronal network, promoting functional recovery [45,46]. Recently, the original cocktail was further reduced to 3 crucial growth factors (BDNF, fibroblast growth factor 2, and vascular endothelial growth factor) and the neural cell death inhibitor MDL 28170, while maintaining the ability to promote NSC survival, integration, and axonal outgrowth (Figure 3) [47]. Interestingly, the anatomical origin of NSCs influences their ability to promote axonal outgrowth, with cells harvested from the spinal cord promoting greater axonal regeneration than cells harvested from the brain [45].

Biomaterial delivery vehicles can also be utilized to protect cells from the inflammatory response and inhibitory SCI environment. Hydrogels containing crosslinked hyaluronic acid and gelatin can protect human NSCs transplanted into immunocompetent rat spinal cords for up to 2 weeks post-transplantation [48]. To reduce cell death due to a lack of an adhesive substrate (anoikis), a hyaluronan/methylcellulose hydrogel was modified with the fibronectin-derived peptide sequence GRGDS and platelet derived growth factor A (PDGF-A). This strategy improved early cell survival and long term differentiation of grafted oligodendrocyte progenitor cells compared to transplantation in media [49].

Recently, there have been several reports of ectopic cell deposits in healthy regions of the rat spinal cord following NSC transplantation into spinal cord lesions [50,51]. The cause of this phenomenon and whether it will present a safety concern in clinical trials has been widely debated [50] and will likely require further systematic investigation. To mitigate this concern, biomaterial strategies could be employed to attenuate the proliferative capacity of transplanted cells. For example, hyaluronan/methylcellulose hydrogels used for progenitor cell delivery to the injured spinal cord attenuated cell proliferation and teratoma formation [49].

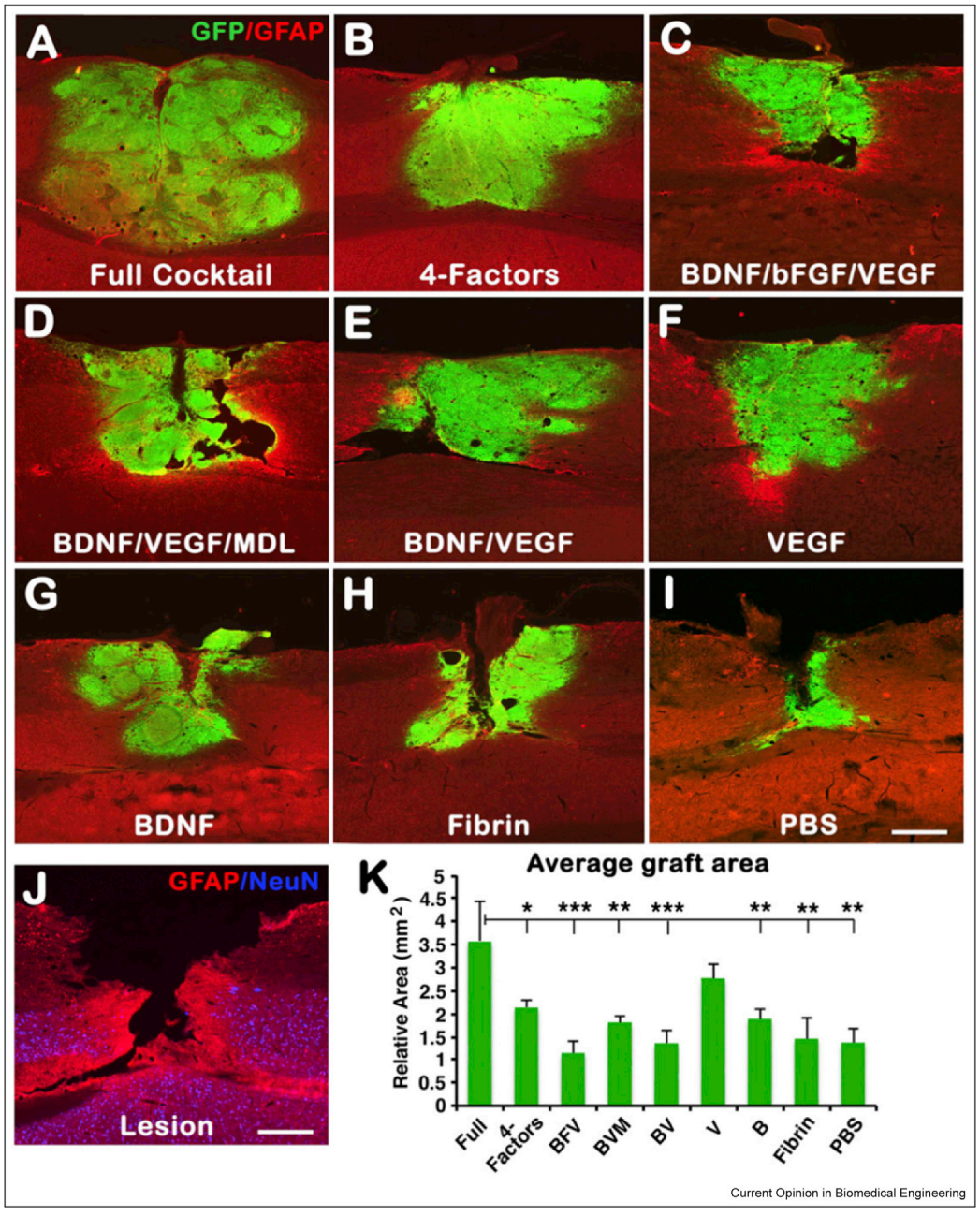
Ultimately, the following must be considered for effective cell transplantation: cell maturity and lineage commitment, timing of delivery, delivery vehicle, and supply of pro-survival factors.

Restricting cell fate

Numerous cell types at different stages of differentiation have been investigated for spinal cord repair, ranging from pluripotent stem cell-derived cells and NSCs [52,53] to committed progenitors of neurons, astrocytes, and oligodendrocytes [49]. The capacity of each cell type to self-renew and differentiate leads to different advantages and challenges with their use. Immature cells typically exhibit higher proliferation and greater plasticity, allowing them to differentiate into multiple neural cell types. Thus, immature cells can facilitate robust reconstitution of spinal cord lesions, but can also cause tumor formation if left unchecked. Transplanted NSCs also differentiate preferentially into glia, but not functional neurons [54]. To encourage NSC differentiation into neurons, several strategies have been employed. For example, the ratio of oligodendrocytes and neurons derived from transplanted NSCs has been recently hypothesized to be cell dose-dependent, with higher cell numbers resulting in more neuronal differentiation [55]. Furthermore, both MAI and CSPGs have been implicated in the regulation of stem cell fate. To this end, it has been shown that the EGFR is involved in MAI and CSPG-mediated glial differentiation of NSCs [56]. Binding of MAIs and CSPGs to their receptors elevates intracellular calcium levels and triggers the EGFR signaling pathway. Interestingly, blocking EGFR through the delivery of EGFR antibodies leads to an increase in neuronal differentiation of endogenous and transplanted NSCs [56–58], demonstrating a potential new pathway through which NSC fate can be modulated.

Lineage-committed cell types have also exhibited promise in cell transplantation strategies for SCI, providing the opportunity to specifically choose a desired neural cell type. Schwann cells are promising candidate cells for transplantation into the spinal cord due to their role in myelination and neuronal protection in the peripheral nervous system [59]. Several studies have demonstrated effective Schwann cell transplantation in animal models of SCI, leading to axonal regeneration and functional recovery [60,61]. Consequently, clinical trials to evaluate Schwann cell transplantation in humans are underway. Oligodendrocyte progenitor cells (OPCs) have been investigated for SCI treatment for similar reasons, and several groups have observed effective remyelination of axons *in vivo*

Figure 3



Effect of growth factors on survival and integration of NSCs. (A–J) NSCs were delivered to injured spinal cords in rats in PBS, fibrin, or fibrin containing different combinations of growth factors. GFP signal demonstrates survival of NSCs within the lesion. (K) The full 9-factor cocktail as well as the reduced 4-factor cocktail resulted in the highest filling of the lesion site. Scale bar = 1000 μm. Reproduced with permission from [47].

[62,63]. However, endogenous remyelination, at least in rodents, is efficient, and the necessity of cell transplantation strategies to further improve myelination has been questioned [64].

Since sophisticated cellular reprogramming and differentiation techniques are now available, rapid generation of functional somatic cells can be more easily achieved. Kim et al. recently demonstrated that OPCs directly reprogrammed from fibroblasts could contribute to axon remyelination and recovery of motor skills after contusion SCI [65]. In another study, Butts, et al. reported a novel method to derive excitatory interneurons from pluripotent stem cells that were shown to integrate into a non-injured spinal cord within 2 weeks post-injection [66]. Similarly, extensive work by Weinrug and colleagues on generating oligodendrocytes and neurons through direct reprogramming and differentiation

techniques has led to robust methods of generating new cell populations that can be investigated for SCI treatment in the future [67–69].

Additional cell transplantation strategies for SCI may become available as the range of cell types from which to choose diversifies. In the future, cell transplantation strategies could be coupled with methods to restrict cell fate, such as instructive biomaterials or EGFR modulation, to ensure that the desired cell types are replenished in the injury site.

Current state of clinical trials for spinal cord injury treatment

Several promising therapeutic strategies for SCI are currently in various stages of clinical trials. Most of these clinical trials, which are focused on functional recovery mediated by tissue regeneration, are investigating the safety and efficacy of cell transplantation for SCI [70–72], although clinical trials to develop effective drug delivery strategies are also underway [73].

Positive results observed in rodent and non-human primate SCI models using NSCs have led to several companies initiating clinical trials with allogenic human stem cell lines; these include a Phase I study using spinal cord-derived NSCs for chronic SCI initiated by Neuralstem and a Phase I/II study by Asterias Biotherapeutics involving dose escalation of embryonic stem cell-derived OPCs. Recently, Asterias Biotherapeutics reported positive preliminary results on both the safety and efficacy of their cell product, which has been successfully delivered at doses of up to 10 million cells per patient, and is currently being tested at higher doses (20 million cells per patient).

While the Neuralstem and Asterias Biotherapeutics trials are still underway, the Stem Cells Inc. Phase II efficacy trial for NSC delivery was terminated in May 2016 after minimal biological effects were observed. Interestingly, there appeared to be an inconsistency between cells processed according to good manufacturing practices (GMP) and those processed for research studies [74,75]. In a head-to-head comparison, clinical grade cells exhibited reduced engraftment compared to research grade cells, and only mice transplanted with research grade cell lines exhibited functional recovery [75]. While the reasons behind the differences in cell product efficacy are still unknown, this study highlights the need for more comprehensive quality control testing of cells.

Schwann cell transplantation is also being investigated for acute and chronic SCI. Clinical trials using Schwann cells are primarily being conducted by the Miami Project to Cure Paralysis. Phase I clinical trials have been completed in acute SCI and are underway for chronic SCI, revealing no adverse events associated with autologous Schwann cell transplantation [71,72].

Conclusions

The last few years have seen an increase in the number of potential therapeutic targets available for treating SCI, as well as an improvement in the biomaterial strategies available for effective cell and drug delivery. These advances can be partly attributed to an increased understanding of the pathophysiology of SCI and use of combinatorial strategies that target multiple aspects of the injury, including overcoming the inhibitory environment, replenishing neurotrophic cues, and replacing lost functional cells. Although significant work is required to move these novel therapeutic strategies into the clinic, recent focus on the origin and fate of transplanted cells, improving sustained delivery of sensitive proteins, and the efficacy of clinically used cell lots will advance SCI treatment options.

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Conflicts of interest

None declared.

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** of outstanding interest

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