Front. Med. 2019, 13(2): 131–137
https://doi.org/10.1007/s11684-018-0642-z

REVIEW

Combination of biomaterial transplantation and genetic enhancement of intrinsic growth capacities to promote CNS axon regeneration after spinal cord injury

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Abstract **The inhibitory environment that surrounds the lesion site and the lack of intrinsic regenerative capacity of the adult mammalian central nervous system (CNS) impede the regrowth of injured axons and thereby the reestablishment of neural circuits required for functional recovery after spinal cord injuries (SCI). To circumvent these barriers, biomaterial scaffolds are applied to bridge the lesion gaps for the regrowing axons to follow, and, often by combining stem cell transplantation, to enable the local environment in the growth-supportive direction. Manipulations, such as the modulation of PTEN/mTOR pathways, can also enhance intrinsic CNS axon regrowth after injury. Given the complex pathophysiology of SCI, combining biomaterial scaffolds and genetic manipulation may provide synergistic effects and promote maximal axonal regrowth. Future directions will primarily focus on the translatability of these approaches and promote therapeutic avenues toward the functional rehabilitation of patients with SCIs.**

Keywords spinal cord injury; biomaterial; extrinsic barrier; intrinsic regeneration capacity

Overview of spinal cord injury and current therapeutic strategies

A key pathological event in spinal cord injuries (SCI) is the loss of longitudinally projecting axonal tracts that interconnect the brain and the spinal cord. Two major hurdles prevent successful axon regeneration beyond the lesion site: (1) injury-induced gliosis and inflammation that result in a physical barrier and an extremely inhibitory microenvironment and (2) adult central nervous system (CNS) neurons that lack the intrinsic ability to regrow their axons after injury. To circumvent these barriers, substantial efforts have been considered toward the distinctive direction. In this review, we introduce recent proceedings on biomaterial substrate applications and genetic manipulations to enhance the adult neuronal regenerative ability. We hope that the combination of these procedures can provide synergistic effects and promote maximal axonal regrowth, thereby benefiting the design of therapeutic approaches toward functional rehabilitation after SCI.

Received November 20, 2017; accepted April 26, 2018
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Pathophysiology of spinal cord injury

Crush and contusion by forceful impact, which often result in dislocated or fractured vertebrae, are the two major causes of spinal cord damage among human patients [1]. Partial or complete functional loss, including motor and sensory impairment, bowel and bladder dysfunction, autonomic dysreflexia, and neuropathic pain, can occur depending on the severity of the damage caudal to the level of injury.

SCI pathophysiology is composed of two sets of mechanisms, namely, primary and secondary injury mechanisms. “Primary injuries” often refer to damages caused by a mechanical force that deforms or tears tissues. In most scenarios, the descending axons from multiple supraspinal nucleus are severed, which result in the degradation of the axon from its point of injury to its target, a process called Wallerian degeneration [2]. Wallerian degeneration gradually results in the swelling

of neurons, eccentric displacement of the nucleus, and the transformation of the Nissl substance (aggregations of basophilic molecules, such as rough endoplasmic reticulum and ribosomes) from aggregates to dispersed particles. These scenarios lead to neuronal death in some cases. Local motor and sensory neuron losses, along with damages to the propriospinal connections within the spinal cord, also account for the initial transformation after SCI. However, although traumatic injuries are potentially devastating by themselves, the changes are only the beginning of the loss of function.

Subsequent pathological changes, which are referred to as "secondary injuries," may progress to several months and appear to be equally or more devastating than the primary injury. Considering that damages ensue at the brain blood barrier (BBB), the infiltration of neutrophils, monocytes, and T-lymphocytes with the simultaneous activation of local microglia occurs rapidly after the crush or contusional injury [3]. For example, neutrophils enter the injury site within 4 hours after injury and produce oxidative and proteolytic enzymes that are beneficial to tissue repair but also cause damage to surrounding tissues [4]. Microglia penetrate the injury site immediately after injury, and some of these adopt a macrophage-like phenotype and release inflammation-promoting cytokines, nitric oxide, and reactive oxygen species along with blood-derived macrophages [5,6]. Microglia can also release TNFα and CD95 ligand, which cause the apoptosis of oligodendrocytes that express the CD95 receptor [7]. Incidentally, microglia have also been shown to promote the cleanup of damaged tissues and growth in T-cell-mediated manner [8]. However, the exact roles of T-cells remain unclear although studies have demonstrated their ability to promote [9] and inhibit recovery after SCI [10].

The secondary injury response is therefore characterized by severe inflammatory responses, which often leads to the formation of a fluid-filled cyst that spreads both rostrally and caudally from the injury site [11]. Moreover, activated astrocytes, along with oligodendrocytes, microglia, meningeal cells, and oligodendrocyte precursors that surround the cavity, form a scar [12]. This glia scar is thought to not only provide a physical barrier but also generate inhibitory factors, such as chondroitin sulfate proteoglycans (CSPGs) that prevent regenerating axons to penetrate the injury site [12–14]. CSPGs have a protein core conjugated with sulfonated glycosaminoglycans, which are composed of alternating residues of N-acetylgalactosamine and glucuronic acid. CSPGs are normally found in the extracellular matrix (ECM), and their suggested roles in the nervous system include effects on cell adhesion and migration [15,16]. Several CSPGs are found in the scar, and their expression is up-regulated in glial cells and along the periphery of the injury site. They include NG2, versican, aggrecan, phosphacan, tenascin-C, and brevican [17–19]. Their ability to halt the growth of axons has been attributed to both the glycosaminoglycan side chains and the protein core with the possibility of differential effect depending on whether the molecule is membrane-bound or secreted [20]. Interestingly, recent findings challenged this view by showing that astrocytic scars are not responsible for the generation of CSPGs, and they can even facilitate the regeneration of damaged axons under certain conditions [21].

Extracellular matrix structures in spinal cord at pre- and post-injury

Unlike those in systematic tissues (i.e., cartilage), the ECM of CNS is enriched with glycoproteins and proteoglycans rather than fibrillar collagens and fibronectin. The major components of the CNS ECM are laminin, fibronectin, and collagen, which form the basal laminae along with hyaluronan (HA), tenascin link proteins, and CSPGs, which are mainly found in the perineuronal net matrices.

The ECM composition undergoes dramatic changes at post-SCI [22]. For injuries where the dura remains intact (mainly, contusive-type SCI), the activated glia cells are considered the major composition of the scar matrix. Furthermore, the penetrating injuries incur significant fibroblast invasion. In either case, CSPGs are significantly upregulated in an extremely inhibitory environment. In addition, depending on the severity of the immunoreactive responses, the ECM near the epicenter often undergoes lytic necrosis, which often causes fluid-filled cysts and further creates a physical gap in the spinal cord.

Providing growth-supportive environment through biomaterial approaches

The secondary injury that causes gliosis and inflammation creates an extremely inhibitory microenvironment for the regenerating axons, if any, which penetrate the lesion site and enable synaptic connections to the downstream targets. To circumvent this problem, a scaffold composed of appropriate biomaterials will not only bridge the gap of the lesion for the regrowing axons but also engineer the local environment toward the growth-supportive direction, usually by combining with stem cell transplantation.

In accordance with biodegradability, mechanical strength, and elasticity, hydrogels are mostly used as permissive scaffolds. Hydrogels can provide a matrix for the penetration of the regrowing axon by forming a unique three-dimensional porous structure. Such structure is also suitable for embedding growth factors or stem cells, and the microenvironment around the lesion site can be converted from a growth-inhibitory to a permissive setup. The common materials used to develop hydrogels include ECM molecules (fibrin, collagen, and fibronectin)

and other natural polymers (alginate, agarose, and chitosan) [23].

Collagen is probably the mostly widely used biomaterial in CNS bioengineering repair due to its excellent biocompatibility and biodegradability [24–28]. Combined with different growth factors, the outcome of using collagen as a scaffold material is promising. For example, the insertion of a linear collagen matrix that contains the brain-derived neurotrophic factor (BDNF) fused with the collagen binding domain not only alleviates neuroinflammation but also enhances functional recovery in the partial or complete SCI model in rodents [24,25] and higher-order mammals such as dogs [27]. In addition, collagen matrix can also inhibit glial scar formation [23,26,28].

Fibrin, a fibrous protein required for blood clotting, is also extensively chosen as a scaffold in SCI bioengineering. For example, PTEN silencer in the motor cortex is combined with salmon fibrin and then injected into the lesion site of dorsal hemisectioned rats, and findings showed significant recovery of functional motor skills [29]. One of the most successful cases involved the utilization of fibrin as a matrix for growth factor cocktails to support the embedding of neural stem cells (NSCs). The implantation of such fibrin matrices can sufficiently induce long-distance supraspinal axonal growth and achieve a certain level of functional recovery for severe cases of post-SCI models (C3 full transection), probably through the relays mediated by axons that emanate from differentiated neurons [30]. Interestingly, a source of NSCs was not only limited to rats but also included the iPS-induced cells from an 84-year-old healthy human being [31]. These results shed light into the clinical implications of fibrin matrix-guided stem cell transplantation in SCI patients. However, from a mechanistic perspective, follow-up studies have shown that long-distance supraspinal axon regeneration only occurs when the spinal cord with lesion is filled with caudalized (spinal cord) but not rostralized (hindbrain or telencephalon) NSCs, which also supports the concept of "homologous reconstitution" [32].

Chitosan, a polysaccharide, is rich in the exoskeleton of crustaceans and insects. Several examples have demonstrated that chitosan is useful as a biomaterial and promotes functional recovery after SCI. Li *et al*. was the first to show that a chitosan tube filled with collagen and implanted in a transected spinal cord not only prevented glial scar tissue formation but also facilitated axonal regeneration in rats, which ultimately led to functional recovery [33]. Li *et al*. also discovered that neurotrophin-3 (NT-3)-coupled chitosan implantation after SCI could attract endogenous NSCs, and subsequently enrich and differentiate them at the lesion site in rats. These newly formed neurons served as relay units for the descending and ascending axons, and they realized motor and sensory behavioral recoveries [34]. Transcriptome analysis also confirmed that such treatments promote neurogenesis and angiogenesis, along with the reduction of inflammatory responses at the lesion center, thus providing novel insights into the underlying mechanisms of biomaterial transplantation-mediated functional recovery [35].

Unlike fibrin and collagen, agarose can be fabricated into stable template scaffolds according to the organization of the descending and ascending axon tracts. Through this approach, individual channels can segregate axons within bundles of functionally related fascicles and ultimately facilitate axon regeneration [36]. Subsequent studies showed that when these template agarose scaffolds are seeded with bone marrow BDNF-secreting stromal cells, they can significantly promote long-distance growth of PNS- and CNS-injured axons in rats [37,38].

Natural or synthetic polymers (degradable or non-degradable) have their own advantages and disadvantages. Natural polymers are accessible and biodegradable. In addition, they are appropriate for integrating implanted cells. However, natural materials are difficult to sterilize and often easily degrade. Meanwhile, the adoption of sterilization and functional parameters, such as biodegradation rate, can be controlled for synthetic biomaterials. The disadvantages of using synthetic biomaterials include low biocompatibility and insufficient recognition signals relative to the use of natural materials.

Promoting intrinsic regenerative capacity of adult CNS

Engineered biomaterials provide promising avenues that attenuate the inhibitory environment surrounding the lesion site at post-SCI. However, accumulating evidence indicates that manipulating the inhibitory environment in many cases is insufficient in inducing supraspinal axon regeneration. For example, neither the single deletion of Nogo nor the triple deletion of Nogo, MAG, and OMgp (i.e., the three myelin-associated inhibitory glycoproteins) [12] can consistently lead to robust CNS axon regeneration and functional recovery [39,40]. The studies showed that the low intrinsic growth capacity of adult CNS neurons is a significant limiting factor of axon regeneration and cannot be easily overcome through the regulation of the inhibitory cascade alone.

During development, the axon elongates rapidly when the newly formed growth cone navigates the changing extracellular environment. However, once the axon reaches its target, the axon outgrowth mode transforms into a synaptic formation in maturation mode to establish the functional synaptic connections. Consistent with this developmental process, CNS axons lose their growth ability. For example, retinal ganglion cells (RGCs) show a dramatic reduction in axon elongation ability during the birth period [41].

Therefore, an effective strategy for restoring adult neurons to a robust growth state is to initially identify the key molecules whose developmental changes in the expression are responsible for the intrinsic loss of axonal growth capacity. One such molecule is the cyclic AMP (cAMP). Neurons that express high levels of cAMP effectively grow axons on myelin [42]. The age-related sharp declines in the levels of cAMP correlate well with the onset of the myelin's inhibitory effect on axon outgrowth. However, the mechanisms by which cAMP can affect axonal growth, probably through the direct activation of the dual leucine zipper kinase DLK, are not fully understood [43].

A high-throughput gene profiling study revealed that the distinct expression patterns of Kruppel-like factors (KLFs), a set of zinc-finger transcription factors, correlate with the developmental decline of axonal growth capacity in RGCs [44]. For example, KLF4 and KLF9 are upregulated in adult RGCs relative to embryos, whereas KLF6 and KLF7 are downregulated. Moreover, the deletion of the KLF4 gene substantially promotes optic nerve regeneration in adult RGCs [44], whereas the overexpression of KLF7 promotes the regenerative growth of adult CST axons [45]. In addition, the overexpression of the transcriptional factor Sox11 can reprogram adult non-α-RGCs to a growth-competent state [46].

Apart from KLFs, mammalian target of rapamycin (mTOR) levels are also significantly decreased in mature RGCs, and much more in injured adult RGCs and damaged corticospinal neurons [47–49]. The activation of mTOR in adult RGCs and corticospinal neurons by the genetic or shRNA-mediated deletion of PTEN, a tumor suppressor gene [50], leads to increased global translation activities, which are required for optic nerve and CST regeneration [47,49,51]. Moreover, apart from PTEN removal, subsequent studies showed the overexpression of extracellular protein osteopontin (OPN) with a growth factor, such as the insulin-like growth factor (IGF) or BDNF, can activate mTOR in injured RGC and promote axon regeneration [52]. Furthermore, robust CST regrowth with relevant behavioral recovery was observed among mice with post-lesion AAV-OPN/IGF1 treatment after T10 lateral hemi-section [53]. When voltage-gated potassium channel blockers, such as 4-aminopyridine and its derivative, are applied to enhance axon conductance, the regrown axons achieved a certain level of functional recovery [53,54]. These findings contribute to the translatability of possible mTOR activation at post-injury. Therefore, the switch from robust growth to inactive growth states during development can lead to changes in multiple pathways and involve the expression of many different molecules.

Perspectives

Functional recovery is extremely limited after severe SCI in mammals due to extrinsic and intrinsic barriers. The implantation of biomaterial scaffolds and the enhancement of CNS growth capacity play complementary roles. For example, by providing permissive environment and structural guidance, biomaterial scaffolds can significantly facilitate supraspinal axon regeneration. Meanwhile, manipulating the growth-related gene expression in CNS enables the regenerating axons to grow robustly beyond the lesion site, which subsequently allows for the synaptic contact with either the transplanted stem cells or the downstream propriospinal/motor neurons. Therefore, a combinatorial therapeutic approach may promote maximal axon regrowth and functional recovery after SCI (Fig. 1).

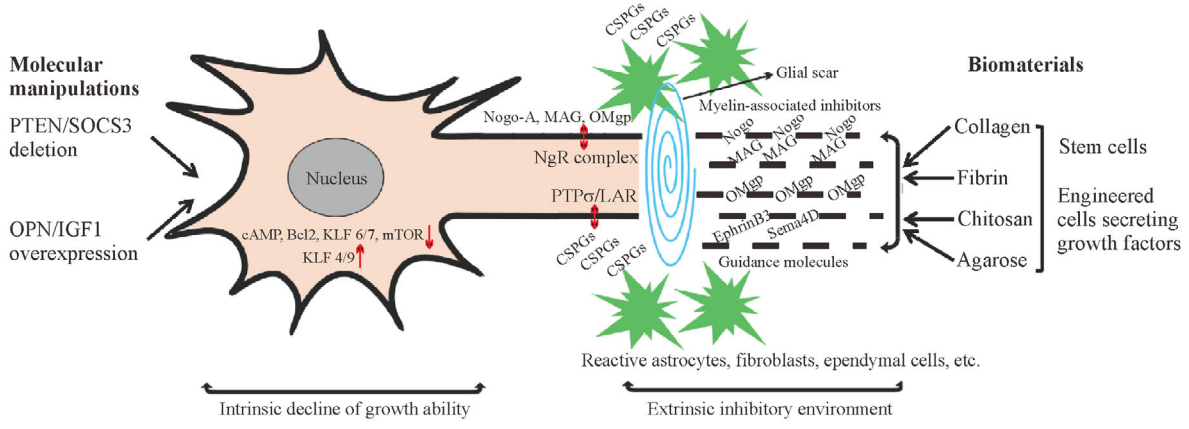

Fig. 1 Combinational strategies to enhance axon regrowth and functional recovery after SCI. CNS neurons switch from robust growth state to relatively static state during development, marked by expressional changes of key molecules and signaling pathways. After CNS injury, extrinsically, myelin-associated inhibitors (Nogo-A, MAG, OMgp), CSPGs, and glia scar/cavity create a high-growth impermissive environment. Successful axon regeneration is therefore impeded by intrinsic and extrinsic factors. By employing a biomaterial matrix that bridges the lesion site, providing growth-permissive environment by stem cells, or engineering the cell transplantation, the enhancement of axon regeneration induced by manipulation of cell autonomous growth capacity can synergistically boost and achieve significant functional recovery.

Recent findings have also shown that the combination of NSC transplantation (to bridge the extrinsic barrier) and the addition of a cocktail of growth factors (to enhance intrinsic growth capacities) enable long-distance axon regrowth in severe cases of post-SCI [30,55,56].

The concept of combinatorial therapy is not only restricted to the integration of biomaterial scaffolds with molecular manipulation when promoting CNS regenerative capacity, but it also improves these approaches. For example, the administration of chondroitinase (ChABC) that digest CSPGs, a major inhibitory molecule concentrated in the lesion site [12], or small molecules that prevent the binding of CSPGs to their receptors, has been proven to improve functional recovery at post-SCI [57,58]. Therefore, biomaterial scaffolds and cell transplantation, further combined with strategies on CSPGs-mediated inhibition, may enhance neutralization or even convert the inhibitory environment into one that is growth-permissive. Applying the same strategy through molecular or genetic approaches to reactivate CNS growth capability achieves significant functional recovery when combined with enhanced neural activity by chemogenetic stimulation [59].

In conclusion, due to the complexity of SCI itself, the multifaceted and combinatorial approaches should aim to achieve maximal axon regeneration by simultaneously enhancing the growth capacity of intrinsic CNS and creating axon growth-permissive micro-environments. The future direction is to primarily focus on the translatability of these approaches to benefit patients suffering from SCI.

Acknowledgements

This work was supported by the National Major Project of Research and Development (No. 2017YFA0104701), the National Key Basic Research Program of China (No. 2014CB542202), the National Natural Science Foundation of China (No. 31730031), and Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Compliance with ethics guidelines

Bin Yu and Xiaosong Gu declare that they have no conflict of interest or financial conflicts to disclose. This manuscript is a review article and does not involve a research protocol requiring approval by a relevant institutional review board or ethics committee.

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