Combinatorial Therapies After Spinal Cord Injury: How Can Biomaterials Help?

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Traumatic spinal cord injury (SCI) results in an immediate loss of motor and sensory function below the injury site and is associated with a poor prognosis. The inhibitory environment that develops in response to the injury is mainly due to local expression of inhibitory factors, scarring and the formation of cystic cavitations, all of which limit the regenerative capacity of endogenous or transplanted cells.

Strategies that demonstrate promising results induce a change in the microenvironment at- and around the lesion site to promote endogenous cell repair, including axonal regeneration or the integration of transplanted cells. To date, many of these strategies target only a single aspect of SCI; however, the multifaceted nature of SCI suggests that combinatorial strategies will likely be more effective.

Biomaterials are a key component of combinatorial strategies, as they have the potential to deliver drugs locally over a prolonged period of time and aid in cell survival, integration and differentiation. Here we summarize the advantages and limitations of widely used strategies to promote recovery after injury and highlight recent research where biomaterials aided combinatorial strategies to overcome some of the barriers of spinal cord regeneration.

1. Introduction

The many different causes of spinal cord injury (SCI) – sports-related injuries, falls, violence and motor vehicle accidents – already highlight that there is no common SCI and that it is unlikely that there will be one single strategy that promotes functional recovery in all cases.[1,2] Individuals with SCI experience disabilities that range from loss of sensation to partial to complete paralysis, depending on the level, type and severity of injury.

Although there is some endogenous repair after central nervous system (CNS) injury, it is incomplete. Many tested strategies are neuroprotective – they aim to prevent secondary cell death and minimize the extent of the injury or enhance the plasticity of spared circuits (see **Figure 1** for an overview) and have shown promising results.[3] These strategies, however, do not promote tissue repair or the restoration of severed axonal connections.[4] Furthermore, most strategies to date try to overcome only one of the obstacles to regeneration. For example, they only target one inhibitory substance or only aim to promote axonal regeneration. While a lot has been learned from these studies in terms of their individual potential, the multifaceted nature of SCI has limited their efficacy and it is unlikely that any one strategy alone can overcome all the barriers of regeneration and will therefore be limited in their ability to enhance functional recovery. A combinatorial approach that targets multiple aspects of the injury will likely be more effective. Biomaterials can aid in cell and drug delivery by both promoting cell survival, integration and differentiation, and providing a sustained local release of biomolecules without the need of catheters or repeated injections.[5]

It is beyond the scope of this review to analyze all the numerous therapeutic approaches for SCI; however, excellent reviews exist that focus on e.g. remyelination,[7] biologics,[8] neuronal relays,[9] biomaterials[5,10] or stem cells.[11,12] The aim of this article is to highlight recent advances in biomaterial design for cell and drug delivery and give an overview of biomaterial research that combine multiple drugs or cells, or drugs and cells to promote regeneration after SCI with a focus on neural cells.

2. Pathophysiology and Endogenous Repair

Due to the limited capacity of the CNS to regenerate tissue and axons lost due to injury, SCI is particularly devastating. Because of the intricate organization of the spinal cord transmitting both sensory and motor signals, SCI leads to a wide

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range of functional deficits below the level of injury. In this section we will present some of the inhibitory aspects of SCI (see **Table 1** for an overview) and highlight endogenous repair mechanisms.

Traumatic human SCI is a heterogeneous disorder and the characteristics of the lesion depend on the type of injury, severity and time after injury. Nonetheless, four main types of injury have been identified: (I) compression injury, (II) contusion injury, (III) laceration injury, (IV) solid core lesions.[16,17] Regardless of the type of injury, the physical insult disrupts the highly organized cytoarchitecture of the spinal cord, damaging numerous motor and sensory pathways, and causing necrotic cell death at the lesion site.[18] Such primary degenerative events are followed by a cascade of secondary degenerative events, including bleeding, ischemia, edema, inflammation, free-radical production, excitotoxicity, apoptosis, demyelination of spared axons, scarring and cystic cavitation, all of which contribute to further tissue loss.[16,19]

The limited regenerative capacity after SCI is partly due to an imbalance of local axon growth-promoting and growth-inhibitory molecules. This includes the relatively poor expression of growth factors and guidance cues at the lesion site, as well as the increased presence of inhibitory molecules such as chondroitin sulfate proteoglycan (CSPG) and myelin associated inhibitors (MAI), which will be discussed in more detail below as there are main targets of current intervention therapies.

2.1. Chondroitin Sulfate Proteoglycan

CSPGs are proteoglycans consisting of a core protein and glycosaminoglycan (GAG) side chains. The number and sulfation of GAG chains determines the specific type of CSPG, such as aggrecan, versican, neurocan and brevican.[20] While most are inhibitory to regeneration, the extent of their inhibition depends on the type of CSPG. Their potent inhibitory nature has been demonstrated both *in vitro*,[21,22] where axons preferentially grew on growth promoting substances such as laminin and avoided areas rich in CSPGs, and in vivo,[23,24] where transplanted neurons extended long axons until they reached tissue with high levels of CSPGs. Various cell types express CSPGS and contribute to its deposition around the lesion site, including microglia, macrophages, pericytes, and fibroblasts.[25,26]

Although it is well established that CSPGs form an inhibitory substrate, their receptors were only identified recently. The leukocyte common antigen-related phosphatase (LAR), the protein tyrosine phosphatase PTPσ, and the Nogo receptors NgR1 and NgR3 were identified as receptors for CSPGs.[27] At the intracellular level, downstream activation of the Rho/ROCK pathway, phosphorylation of EGFR, and inhibition of Akt and Erk1/2 phosphorylation have been implicated in CSPG mediated growth cone collapse.[28,29]

In addition, CSPGs are a major component of perineuronal nets (PNNs), which are aggregates of extracellular matrix (ECM) molecules that surround neuronal cell bodies and neurites to stabilize neuronal networks.[30] However, following SCI, CSPGs in PNNs can prevent axonal sprouting and synapse formation, effectively restricting neuroplasticity.

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2.2. Myelin Associated Inhibitors (MAIs)

The myelin sheaths of oligodendrocytes contain a number of potent axon growth-inhibitory molecules, which are released, as debris, following the degenerative events caused by the injury. MAIs include Nogo-A,[31,32] myelin-associated glycoprotein (MAG),[33] oligodendrocyte-myelin glycoprotein (OMgp),[34] and some semaphorins[35] and ephrins.[36]

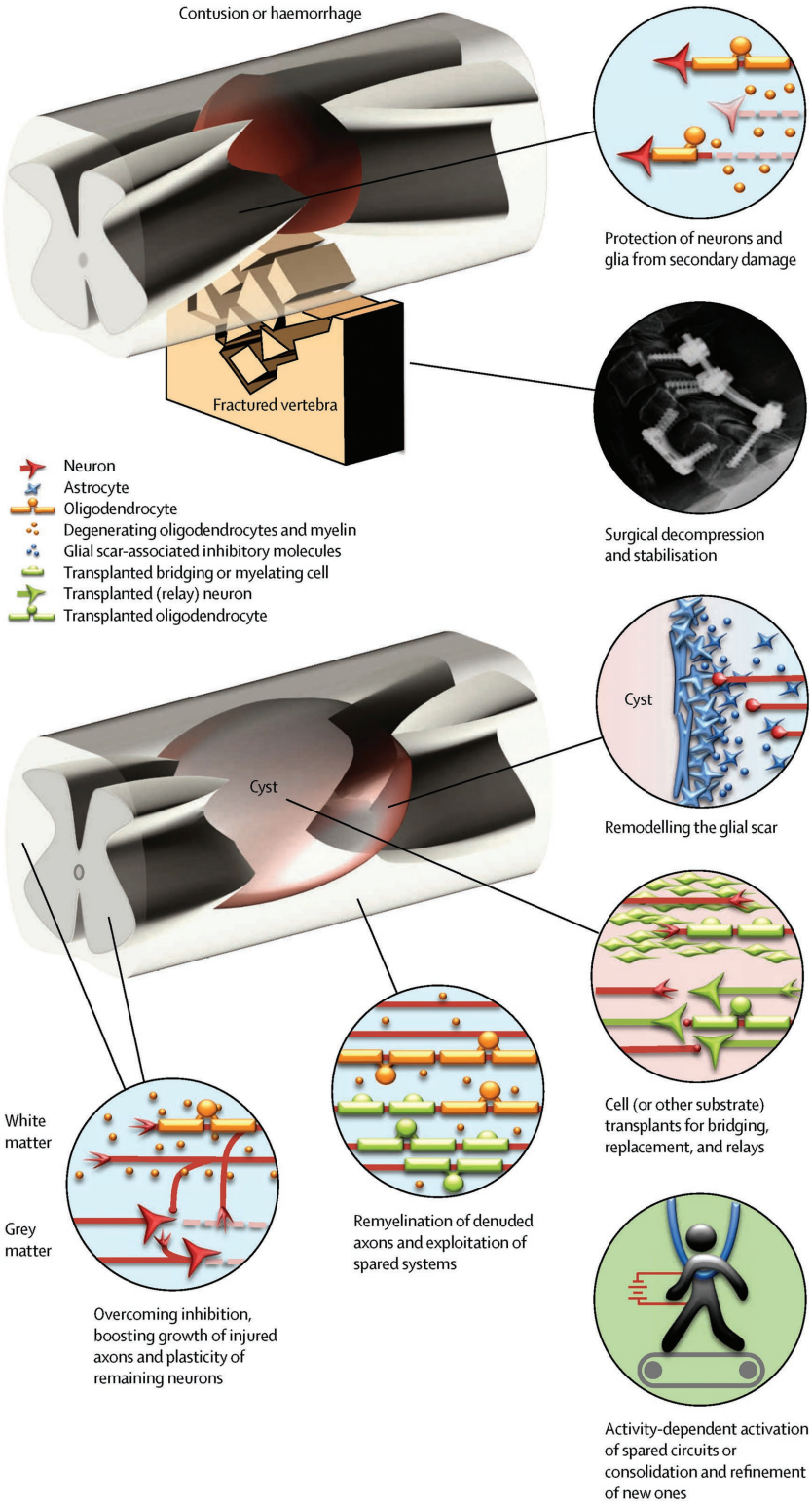

Figure 1. Experimental strategies to stop injury progression and promote repair of the spinal cord. Reproduced with permission.[6] Copyright 2014, Elsevier.

Nogo-A has two inhibitory domains: amino-Nogo and Nogo-66, which is a 66 amino acid sequence. Although Nogo-A, MAG and OMgp are structurally distinct, they induce inhibition of neurite outgrowth and growth cone collapse by acting through the same receptor, Nogo receptor 1 (Ngr1). Binding of MAIs with NgR1 and its co-receptors (e.g. $P75^{NTR}$, LINGO-1)[37] activates the Rho/Rock signaling pathway, leading to the destabilization of the actin cytoskeleton, growth cone collapse and inhibition of neurite outgrowth.[38–40] Another, less studied, mechanism that results in retraction of growth cones is through a rise in intracellular calcium following activation of NgR1 and its co-receptors, which can activate epidermal growth factor receptor (EGFR)[29] or protein kinase C (PKC).[40–42] In healthy animals, MAIs stabilize existing connections and suppress plasticity. Therefore, some of the observed recovery following neutralization of MAIs is thought to be due to compensatory sprouting.[43]

2.3. Glial and Mesenchymal Scarring

Besides the above mentioned molecular barriers to axonal regeneration, reactive astrocytes, glial progenitors, microglia, macrophages, fibroblasts, and invading Schwann cells (SCs) participate in the formation of a physical barrier to tissue and axonal repair.[1,3] While the scar and the fluid filled cavity it encloses are not permissive to axonal growth and tissue regeneration at later stages, the scarring process is important to restrict injury progression at initial stages. To this end, recent studies have demonstrated that inhibition of scar formation worsens the outcome. For example, astrocytes, which are at least partly derived from endogenous neural stem cells (NSCs), seal off the injury site to prevent leakage of toxic substances and reduce damage caused by the secondary injury.[25,44]

2.4. Endogenous Repair Mechanisms

Besides the formation of the glial scar, which can be seen as an endogenous repair mechanism, new myelinating cells are formed, which contribute to remyelination of spared axons.[45,46]

However, little or no neurogenesis has been observed in the injured spinal cord.[44,47,48] CNS neurons rather lose some of their regenerative capacity during development, as they down-regulate receptors for trophic factors (e.g. tyrosine kinase receptors, Trks) while up-regulating receptors for growth inhibitory molecules (e.g. NgR complex, LAR and PTPσ), which mediate growth

Table 1. Overview of the degenerative events after spinal cord injury.[13–15]

Primary Injury	Secondary Injury		
Mechanical insult	Inflammation, disturbed blood vascular system		
Immediate *(<2 hours)*	Acute *(2 hours – 3 days)*	Intermediate *(3 days – weeks)*	Chronic *(weeks to month/years)*
	Neural cells		
– Necrosis – Axonal damage	– Oligodendrocyte apoptosis – Demyelination – Neurite growth-inhibitory factors – Neuronal apoptosis – Glutamate excitotoxicity – Axonal swelling – Astrocyte apoptosis – Ionic dysregulation	– Oligodendrocyte apoptosis – Demyelination – Astrocyte activity and scar formation – Cyst and syrinx formation – Schwann cell infiltration	– Wallerian degeneration – Demyelination – (Persistence of spared, demyelinated axons) – Scar mutation – Cavity formation – Syrinx formation – Schwannosis
	Inflammatory system		
– Microglia activity	– Microglia activity – Neutrophil infiltration – Release of cytokines – Free radical production – Lipid peroxidation	– Microglia activity – Monocyte, macrophage and lymphocyte infiltration/activity	– Microglia activity – Monocyte, macrophages and lymphocytes activity
	Blood vascular system		
– Oedema – Ischemia – Thrombosis – Gray matter haemorrhage – System events (system shock, spinal shock, hypotension, hypoxia)	– Oedema – Hemorrhage – Energy failure and decreased ATP – Nitrous oxide excess – Conduction block – Blood-spinal cord barrier (BSB) permeability	– Initiation of neovascularisation – BSB repair and oedema reduction	

cone collapse.[27,49–51] Nonetheless, early work on SCI has demonstrated that they maintain an intrinsic ability for axonal outgrowth.[52] This axonal sprouting can span the lesion site, but only few axons regenerate over long distances back to their original targets.[3,53–56] Local sprouting, in the spinal cord or the brain, and the formation of new connections can contribute to the limited compensatory recovery that is observed after injury.[3,57,58]

3. Biomaterial Design Consideration

Recent advances in materials science have led to innovative biomaterials for use in treatment strategies aimed at promoting functional tissue repair following SCI. The goal of these biomaterial-based therapies is to restore the anatomical structure and function of damaged tissue by combining the topographical cues of the material with cells and/or bioactive molecules. To achieve this goal, some general issues need to be kept in mind when considering the choice and design of biomaterials intended to promote functional repair following SCI: (1) Biodegradability, (2) biocompatibility, (3) cytocompatibility, (4) physical properties, and (5) topographical cues. In order to provide a growth-promoting environment that allows tissue and axonal regeneration, the biochemical, chemical, and physical properties of the scaffold must be designed in a way that ensures proper presentation of guidance cues to allow substrate remodeling and axons to cross the lesion site. Ideally, the regenerated axons grow further distal, towards functionally relevant targets.

Hollow conduits represent one of the simplest physical guidance cues to bridge a gap created after e.g. transection or resection type injuries. However, they have to be flexible, gas permeable, and not irritate or mechanically damage the adjacent spinal cord.[59–61] An additional advantage of these tubes is that they can easily be loaded with additional elements, including bioactive molecules, cells, drugs, and oriented microstructures. However, these (or any 3D) scaffolds have to be implanted, potentially limiting their use to laceration type injuries, as they can potentially damage spared axons present after contusion/compression type injuries.

Hydrogels, on the other hand, have the advantage that they can be injected and readily adopt to irregular lesion conformations, facilitating minimally invasive surgery. Hydrogels are usually non-toxic and their high water content allows for cell migration and molecule diffusion out of the scaffold. In addition, their mechanical properties can be modified to match those of the spinal cord, while still providing a physical structure. Furthermore, they can help prevent cell dispersion after injection by providing a physical scaffold to embedded cells.[5,62]

Both types of scaffold, 3D scaffolds and hydrogels, can be modified with therapeutic molecules to promote cell survival during transplantation or to provide a local, sustained release of drugs to the injured spinal cord. **Figure 2** gives an overview of the conceptually different ways that have been explored to promote recovery after SCI using biomaterials.

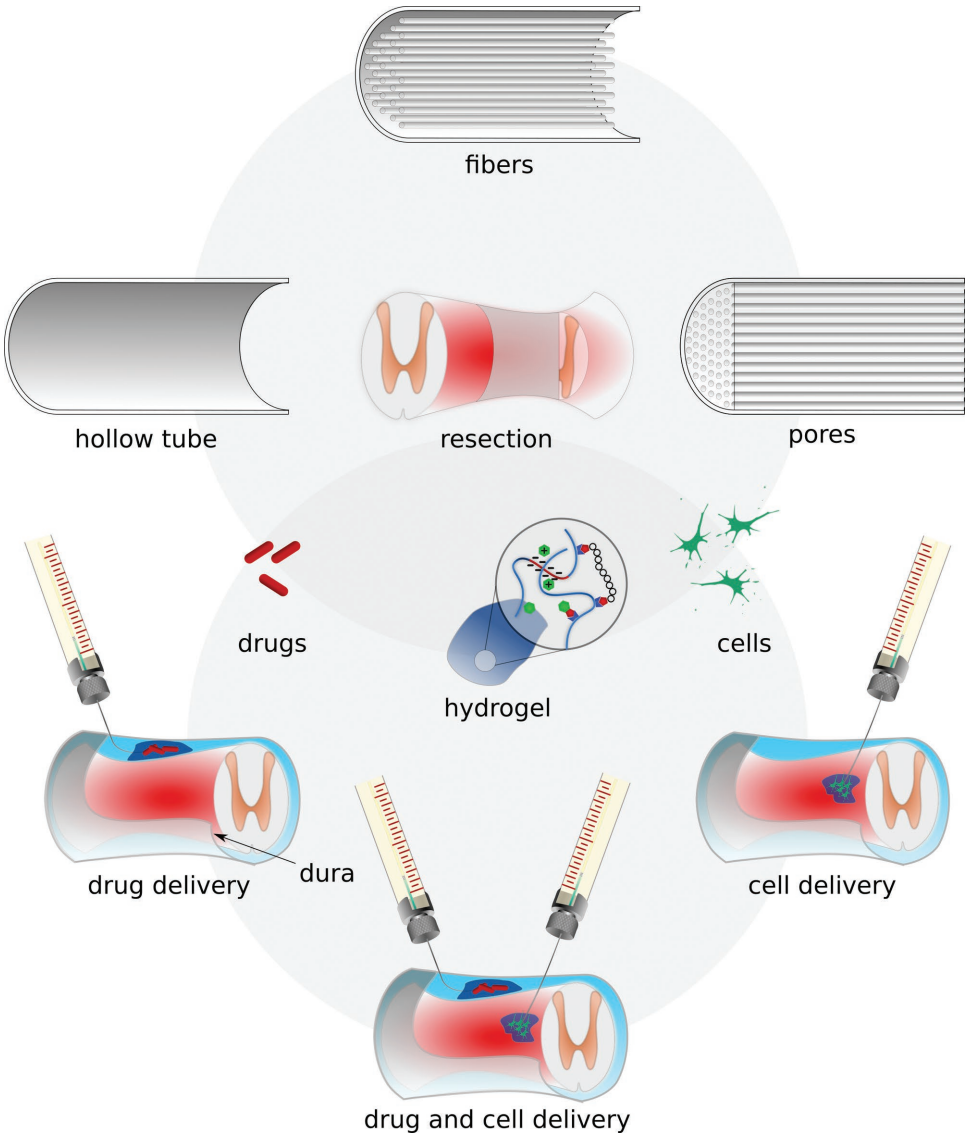

Figure 2. Schematic of possible biomaterial designs. Tubular scaffolds can provide the basis for resection or full transection type injuries. They can be filled with fibers or pores to provide a guidance structure for cells and processes. In addition they can be combined with hydrogels, drugs and cells. Hydrogels are more versatile and can be used in different ways and in different injury types. Stiffer hydrogels can be implanted, similar to scaffolds, while soft hydrogels can be injected in a minimally invasive manner. Furthermore, they can be modified in various ways to deliver drugs or present adhesive peptides. They can deliver drugs after intrathecal injection (between the dura and the spinal cord) or cells after intraspinal injection, either individually or as part of a combinatorial strategy.

In the following paragraphs, we introduce some aspects that can be modified to promote cell-substrate interactions of either host or grafted cells with transplanted biomaterials. While some of them have already been used in in vivo studies, either on their own or in combination with a single drug or cell type, only few were used in combination with multiple drugs or cells.

3.1. Biomaterial Types

Biomaterials can be derived from natural or synthetic polymers, which can be further separated into degradable or non-degradable materials.[59,63] Natural polymers are easily harvested and their physical, mechanical, and biological properties are well studied. They are biodegradable and contain signals for cell adhesion, but often lose bioactivity during sterilization. Unfortunately, reproducibility using natural polymers is often low, due to variability in source material and processing methods.[63,64] Fast biodegradation and low mechanical strength are other potential disadvantages; however, they can be overcome by cross-linking techniques. Synthetic biomaterials are easy to sterilize and their key parameters, such as porosity, architecture, stiffness, and degradation rate, can be finely tuned to the desired application. Disadvantages include poor biocompatibility due to the lack of recognition factors, which can be overcome by functionalization with adhesive peptide sequences

and/or growth factors.[63] See the section 3.6. to biomaterials for more advantages/disadvantages of natural and synthetic materials.

3.2. Mechanical Cues

Stiffness and surface topography are important factors for the design of effective biomaterials as they can influence cell survival, fate, differentiation, migration, integration, and orientation. For example, gel stiffness has been shown to affect the differentiation profile of rat neural stem cells (NSCs).[65] Softer (< 1 kPa) methacrylamide chitosan hydrogels promoted the neuronal and astrocytic differentiation of NSCs whereas stiffer hydrogels (> 7 kPa) promoted the differentiation into oligodendrocytes. The highest rate of proliferation was observed on gels with an intermediate stiffness (3.5 kPa). This demonstrates that the cell fate is influenced by small differences in mechanical properties. However, different cell types respond differently to changes in their microenvironment, embryonic stem cells (ESCs) for example demonstrated an increase in viability when cultured on stiff poly(acrylamide) gels.[66] Further research is required to develop more effective biomaterials for tissue repair.

3.3. Topographical Cues

The native ECM provides structural support in the form of fibers, ridges, and pores and is constantly remodeled to provide cues for cellular organization and cell-cell interactions.[67,68] Chen et al. demonstrated that the size and shape of ECM-coated 2D biomaterial substrates influences the fate and function of adherent cells.[69–71] For example, small islands of ECM molecules led to apoptosis of seeded cells while larger islands promoted their proliferation. Others could demonstrate that oriented fibers or grooves and aligned ECM molecules can guide the growth of neural cells and their processes.[72–75] This is particularly interesting for biomaterials intended to promote the repair of highly organized tissue, such as white matter tracts of the spinal cord.[76] The fiber or groove diameter also influences the orientation of process outgrowth, with small-diameter fibers inducing greater oriented process growth compared to larger fibers.[77,78] This became especially apparent with electrospun nanofibers, which clearly promoted oriented outgrowth and migration of axons, Schwann cells and astrocytes in vitro.[74,75] However, although some 3D nanofiber scaffolds have been developed,[79] their application after SCI remains sparse.[80] Nonetheless, several different oriented 3D scaffolds have been developed and tested after SCI, and have been shown to promote oriented repair. For example, Tsai et al. demonstrated improved tissue regeneration after implantation of poly(2-hydroxyethyl methacrylate-co-methylmethacrylate) (HEMA) hydrogel channels.[81] A multi-channeled scaffold with tunable properties (i.e. channel diameter, wall porosity) comprised of poly(D,L-lactic-co-glycolic acid) (PLGA) also promoted axonal regeneration after implantation into the transected spinal cord when seeded with Schwann cells.[82]

3.4. Cell-Substrate Interactions

The ECM is comprised of proteoglycans such as CSPGs, glycosaminoglycans, for instance hyaluronan (HA), and proteins, including laminin, collagen and fibronectin.[20] Cell surface receptors recognize these ECM proteins and influence cell differentiation, migration, and proliferation. For example, integrins bind to fibronectin to promote cell adhesion and viability.[83] While the uninjured adult CNS contains limited fibronectin,[20,84] it plays an important role in the developing CNS and promotes axonal regeneration of adult neurons.[85] The discovery of the fibronectin-derived short synthetic peptide, arginine-glycine-aspartic acid (RGD), which promotes cell adhesion and viability, allowed for easier modification of biomaterials compared to using the full protein.[86,87] Many other ECM-derived synthetic peptides have been investigated since the discovery of RGD, including the laminin-derived peptides isoleucine-lysine-valine-alanine-valine (IKVAV) and tyrosine-isoleucine-glycine-serine-arginine (YIGSR).[88,89] IKVAVA and YIGSR are able to promote neurite outgrowth and cell adhesion, respectively. The neural cell adhesion molecule (NCAM)-derived amino acid sequence, EVYVVAENQQGKSKA, acts similar and can increase neuronal survival and neurite outgrowth.[90]

While taking advantage of the of key cell-ECM interactions using these cell-adhesive peptides, it was discovered that the peptide conformation and presentation is critical to its binding with its integrin receptor. To this end, longer peptide chains or cyclic peptides demonstrated better bioactivity than their short counterparts.[91–93] Similarly, specific coupling chemistry leads to controlled biomolecule orientation and better bioactivity compared to simple adsorption and/or non-specific conjugation of the peptides.[94] For example, RGD functionalized elastin-mimetic polypeptide hydrogels and YIGSR and IKVAV functionalized dextran hydrogels promoted neurite outgrowth from dorsal root ganglia.[95,96]

Similarly, growth factors have to be conjugated to biomaterials at specific sites for cellular recognition. This can be achieved with biotin and streptavidin, which have a high affinity to each other. For example, agarose and hyaluronan/methylcellulose (HAMC) hydrogels were modified with streptavidin to allow conjugation of biotin-platelet-derived growth factor (PDGF-AA), which promoted oligodendroglial differentiation or rat NSPCs.[97,98] Similarly, neuronal differentiation was induced by conjugating biotin-interferon-γ to streptavidin-modified chitosan hydrogels.[99] Interestingly, differentiation by immobilized factors was similar or better compared to soluble factors, demonstrating the potential of functionalized biomaterials to influence cell fate in transplantation studies.

The interactions between immobilized growth factors and cell adhesion molecules has been reviewed elsewhere,[5] but recent research indicates that combining multiple growth factors or growth factors and adhesive peptides on one backbone improves signaling and bioactivity and is an important consideration for future studies. As an example, greater NSPCs differentiation into oligodendrocytes was observed when PDGF-A and GRGDS were both conjugated to the same polymer backbone compared to controls of each alone on separate backbones.[98]

3.5. Drug Release

Therapeutic molecules, such as growth factors, proteins, and small molecules have been pursued to promote neurogenesis, plasticity, axonal regeneration, degradation or removal of inhibitory substances, and neuroprotection following delivery to the injured spinal cord. For example, growth factors such as neurotrophic factor 3 (NT-3) and glial cell-derived neurotrophic factor (GDNF) act neuroprotective and promote axonal outgrowth following SCI.[100,101] Most importantly, NT-3 has been shown to increase corticospinal tract (CST) axonal growth/sprouting and promote functional recovery.[102–106] However, therapeutics that are injected systemically often require high doses to reach effective concentrations at the injury due to their limited diffusion across the blood-spinal cord barrier.[107] This can lead to off-target distribution and systemic cytotoxicity.[108] Local injections, for example into the intrathecal space, result in higher concentrations immediately after injection, but therapeutics are cleared rapidly by the cerebrospinal fluid flow.[109] The drug release from biomaterials can be fine tuned to allow for a continuous, local release, minimizing the amount of injections.[110] To this end, while therapeutic molecules are often encapsulated in polymeric particles for delivery, a recent study demonstrated controlled release without encapsulation by taking advantage of the electrostatic interactions between PLGA nanoparticles and growth factors, potentially overcoming problems such as low loading, poor encapsulation efficiency, and loss of protein activity.[111] In contrast to degradation-controlled release formulations, affinity based release systems take advantage of reversible interactions between therapeutic proteins and a binding partner to slow the diffusive release.[112] For example, using an SH3/SH3-binding peptide affinity system, Vulic et al. demonstrated controlled release of FGF-2 and Pakulska et al. demonstrated controlled release of chondroitinase ABC (ChABC) from hydrogels.[113–115]

3.6. Immune Response to Biomaterials

The normal spinal cord is isolated from circulating immune cells by the blood-spinal cord barrier; however, after injury many resident cells, such as astrocytes, microglia, oligodendrocytes and, to some extent, neurons respond and release inflammatory cytokines, which aid in the recruitment of circulating immune cells.[116,117] While all materials will initiate a host response when implanted, such as encapsulation by fibrotic tissue, the extent depends on chemical composition, mechanical and physical properties, including shape, size and porosity.[118,119] The immune response is additionally affected by the degradation products of biodegradable materials and morphological/surface changes thereof. While the host response to biomaterial implantation in the CNS is not well understood, some general observations made in other organs with respect to natural and synthetic materials also apply to the CNS.

Natural materials offer a number of advantages over synthetic materials: they have a native ligand landscape, inherent bioactivity, and undergo natural remodeling, thereby avoiding aspects of the foreign body response associated with many synthetic polymers.[120] Disadvantages of natural biomaterials include potential immunogenicity, biologic variability among sources, and sometimes a more complex host response.[121] For example, Gal epitopes are expressed on cells of non-primate mammals, preventing transplantation of xenografts to humans, as we produce a natural antibody to this epitope.[120] Decellularized materials is comprised of the native ECM, which is highly conserved across mammalian species, allowing safe implantation of xenogeneic and allogeneic material. The ECM of the spinal cord is mainly comprised of glycosaminoglycans (hyaluronic acid) and proteoglycans. Other components include laminin, netrin-1, nidogen, reelin, tenascins, as well as growth factors, such as FGF-2 and EGF. Degradation of ECM proteins can result in the generation of bioactive peptides that influence both infiltrating and resident cells. While decellularization reduces the materials' immunogenicity, it is associated with a loss of the scaffold's biomechanical properties.[121] Chemical cross-linking is commonly used to prevent degradation of decellularized material, yet it also reduces the release of growth factors and bioactive peptides.[122] Degradation is a key characteristic for the success of ECM-derived materials, as it allows host cells to remodel it. When the material cannot degrade, e.g., due to processing with cross-linking agents, the material is more likely subject to a chronic inflammatory response, resulting in scar tissue and/or encapsulation.[122]

Synthetic biomaterials offer certain advantages over natural materials: they have fewer impurities, pathogens or contaminants, lower batch-to-batch variability, and more reproducible mechanical and physical properties. Disadvantages are that synthetic polymeric materials can contain unreacted monomer, initiator fragments, oligomers, stabilizers, and other additives that are potentially toxic to cells and tissues, eliciting an immune response. In addition, macrophages may undergo apoptosis, releasing toxic, and damaged waste products that further induce an immune response.[123] Non-degradable materials are encapsulated in fibrous tissue over time, limiting their usefulness. The use of biodegradable polymers, such as poly(lactic acid) (PLA), will produce an early inflammatory response; however, the inflammation may subside upon degradation of the material. Therefore, biodegradable polymers can limit chronic effects.

Some of the coupling agents used to bind proteins or peptides to either synthetic or natural polymers can also elicit an immune response. For example, the bacterial protein streptavidin has a strong binding affinity for biotin and it is commonly used due to its thermal and chemical stability. However, it is highly immunogenic, which limits its clinical use. Attempts to overcome the immunogenicity while maintaining its core function include site-directed mutagenesis.[124,125] Cell-material hybrids elicit an adaptive immune reaction that further influences the host response to the material used.[126]

These studies demonstrate that a better understand of cell-substrate, and cell-cell interactions within biomaterials is needed to develop novel biomaterial- and cell-based therapeutics strategies for functional repair. In the following we will highlight some biomaterial-based treatment strategies involving combinations of multiple drugs and/or cells.

4. Combinations Focusing on Cell Delivery

Biomaterials can aid in cell-based delivery strategies in various ways: they can either promote the survival, differentiation, and integration of grafted cells directly or target the host tissue to promote axonal regeneration, protect host neural cells, or neutralize inhibitory substances to enhance the effect of co-delivered cells. While design parameters for biomaterials have been described above, this section will first discuss some considerations for choosing a certain cell type and then highlight a few strategies combining different cell types or cells with drug delivery.

Donor cells may replace lost glial cells and neurons, contribute to the re-establishment of new functional local circuits and remyelinate spared axons. In addition, they can provide an avenue for continuous growth factor delivery, which can alter the environment, making it more conducive for regeneration. To this end, cells produce a wide variety of growth promoting molecules, including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), nerve growth factor (NGF), and neurotrophin 3 (NT-3), and ECM proteins like laminin, fibronectin, and collagen I/III and IV.[127–129]

Unfortunately, the injured adult spinal cord is a poor microenvironment for cell survival, neuronal differentiation, and maturation. Therefore, major challenges for cell-mediated repair after SCI include: controlling the survival, integration and differentiation of transplanted cells. Combinatorial strategies, which positively influence these variables, are an area of intense research. Combinations that target the host rather than the cell graft ideally target an aspect of the injury that is not already influenced by the transplant to maximise the effect of the combinatorial strategy.

Early examples of tissue transplantation include grafting of peripheral nerves or fetal tissue, which are able to promote some degree of axonal regeneration.[130–132] This is partly due to the permissive environment presented by e.g. NSCs, Schwann cells or olfactory ensheathing cells (OECs), which express neurotrophic factors and a growth-supporting ECM.[133–135] Studies focusing on highly enriched population of Schwann cells or OECs demonstrated that they were able to promote axonal regeneration following injury.[136–138] Interestingly, the need for combinatorial strategies became quickly apparent. Although CNS fibers regenerated into the grafts, they failed to re-enter the host spinal cord.[136,139] In addition, the corticospinal tract (CST), which is responsible for the majority of voluntary movement, failed to regenerate and enter into Schwann cell or peripheral nerve grafts.[140–143] Consequently, combination therapies have been evaluated. Furthermore, the optimal cell type and cell source has yet to be identified. The potential of any cell type to protect or repair an injury should be validated by using alternative cell types as controls. To select a certain cell type for a clinical trial without investigating alternatives might be short-sighted. If one cell type becomes the 'gold standard' without being compared against other cell types, then potentially better cells might not be further investigated.

4.1. Cell Source

Autologous grafts are attractive since they should not require immunosuppression; however, while Schwann cells or OECs can be harvested from the patient, they require invasive surgery and result in donor side morbidity. In addition, the number of cells harvested may be insufficient for direct transplantation, requiring expansion in vitro in defined culture conditions.[144,145]

Fetal- or adult-derived neural tissue was initially used to harvest NSCs, while more recent research focused on the generation of NSCs from embryonic- or induced pluripotent stem cells (ESC, iPS).[146–149] Pluripotent stem cells enable the generation of purified populations of specific cell types, such as oligodendrocytes, astrocytes or neuronal subtypes, for transplantation. However, full differentiation of pluripotent cells into a specific cell type is time and labour intensive and direct conversion may prove a valuable alternative, if cells need to be transplanted shortly after injury.[150] However, directly converted cells do not lose their ageing signatures and, similar to autologous grafts, cells have to be differentiated in sufficient numbers from the start. In addition, reprogramming poses many problems, including apoptosis, cell senescence, insertional mutagenesis, inefficiency, uncontrolled silencing of transgenes, residual expression and re-activation of reprogramming factors, and strong immunogenicity.[151] Therefore, not all reported cell reprogramming technologies will prove useful for SCI, but some may be useful for combinatorial treatment strategies.

4.2. Cell Specificity

Recent research points towards a regional specificity of neural cells, which occurs early in development and appears to be conserved among vertebrates.[152,153] Interestingly, Tuszynski's group found that NSCs promoted greater axonal regeneration after SCI when they are derived from or differentiated into cells of the spinal cord compared to cells from other CNS regions.[154] Similarly, transplantation of human forebrain GABAergic neurons and their progenitors, but not of spinal GABAergic cells, into the striatum of quinolinic acid-lesioned mice overcame the motor deficits.[155] In contrast, other studies have demonstrated that forebrain cells can promote functional recovery when grafted into the spinal cord after injury. Human ESC-derived forebrain GABAergic neurons integrated into the rat spinal cord and reduced pain after injury, and a direct comparison between fetal rat forebrain and spinal cord NSC grafts demonstrated that both promoted locomotor recovery.[156,157] Further studies are needed to determine the extent of specificity needed for successful regeneration.

4.3. Cell Survival

The vast amount of cell death after transplantation is a critical challenge for cell-based therapies, and only a low percentage of grafted cells survive in studies using immunocompetent rodents, even with immune suppression.[158] Good survival is critical to evaluate the potential of the grafted cells and dead

cells may worsen the outcome.[159] Immunodeficient mice or rats usually result in better survival of transplanted cells and may therefore be a good alternative to immunosuppressive drugs, which have to be either injected daily or delivered via minipumps.[158] Various other ways were tested to achieve better survival, with mixed results. For example, ESCs overexpressing the anti-apoptotic protein BCL2 formed tumour-like structures, accompanied by increased morbidity and mortality.[160] In contrast, mouse ESCs, engineered to express the cells adhesion molecule L1, survived longer and spread further than non-transfected cells.[161] Transient expression of proteins might reduce the risk of negative side effects. Biomaterials may be an alternative to genetically engineered cells by providing cell adhesive molecules to prevent anoikis- programmed cell death due to a lack of adhesion to an extracellular matrix,[162] and sustained growth factor delivery to promote survival, integration and differentiation. In particular, hydrogels have been shown to promote cell survival after injection into the eye, brain, and spinal cord.[163–165]

4.4. Combinatorial Therapies Including Cell Delivery

In lesions where a gap is created, transplanted cells often wash away, which can be avoided using biomaterials. To this end, fibrin glue was used to keep Schwann cells within polyacrylonitrile/polyvinylchloride (PAN/PVC) polymer guidance channels, which were combined with neurotrophic factors to promote axonal regeneration and neuroprotection after complete transection.[139,141,142] Fibrin glue was also used to hold the nerve endings of transplanted peripheral nerves in place, which, together with acidic fibroblast growth factor (aFGF), improved functional recovery.[166] Ferguson et al. used minced peripheral nerve tissue and transplanted it with a gelling collagen matrix. In combination with injection of growth factors to the lesion site this led to sprouting of the CST.[100]

To deliver drugs for a prolonged period of time, many earlier studies simply soaked gelfoam with growth factors and placed them close to the injury site.[166–174] The combination of fetal spinal cord tissue with NT-3 or BDNF laden gelfoam prevented neuronal loss.[175] Gelfoam soaked with NT-3, GDNF, IGF, bFGF, TGFbeta, or CNTF in combination with peripheral nerves promoted functional recovery, axonal regeneration and neuronal survival;[166,168–170,172,173] with BDNF,[167] TGF-beta,[169] and CNTF,[170,173] having greater beneficial effects in some studies (**Table 2**). In regards to the corticospinal tract, the main descending fibre tract for voluntary movement, NT-3 has been most often cited as growth promoting.[102,176] Others used osmotic minipumps to deliver growth factors locally. Pearse et al. combined Rolipram with Schwann cells and cyclic AMP, and observed greater myelination, axonal sparing and increased functional recovery.[177] Using Schwann cells expressing the bifunctional molecule, D15A, which mimics the actions of both NT-3 and BDNF, in combination with Rolipram, led to an increase in myelination, axonal ingrowth and functional recovery; however, Rolipram seems to be mainly responsible for these beneficial effects rather than D15A.[178]

While these studies demonstrate some beneficial effects, drug and cell delivery via injection or gelfoam are non-ideal, due to an uneven, short-lived release of the delivered drug. Although osmotic pumps enable sustained and local delivery, they are invasive, and prone to failure and infections. As an alternative, biomaterials can provide sustained release and protect growth factors or other drugs from degradation, potentially avoiding repeated injections.

The physical blend of HA and methyl cellulose (HAMC, first described by Gupta et al.[188]) forms an injectable hydrogel, that was used to deliver rat brain NSCs after a clip compression injury. HAMC was modified with PDGF-A to improve survival and differentiation into oligodendrocytes. While only a limited number of surviving cells was found, the hydrogel promoted the survival of host neurons and oligodendrocytes associated with better functional recovery on the ladder walk.[187] Further modification with RGD (HAMC-RGD-PDGF[98]) promoted the survival, integration and differentiation of human pluripotent stem cell-derived oligodendrocyte progenitor cells. While the study was hampered by over-proliferation of the cells, it demonstrated that the modified HAMC hydrogel reduces tumor formation by promoting differentiation in vivo. Control animals receiving cells without hydrogel demonstrated extensive tumor formation and a decline in motor function.[164]

Using a similar strategy, mouse ESC-derived NSPC-seeded fibrin scaffolds were transplanted sub-acutely following a hemisection injury. The scaffolds contained heparin-binding peptides, which bind heparin and then heparin binding proteins (i.e., PDGF-AA and NT-3).[180,181] The fibrin gel promoted the survival of the transplanted cells and co-delivery with growth factors further improved cell viability and differentiation into neurons at 2 weeks. However, the combination of cells and drug delivery led to tumor formation by week 8, demonstrating the need for transplantation of well defined, pure cell populations.[181] Interestingly, the combination therapy, without the heparin binding system, performed best at later time points in terms of cell survival, neuronal differentiation and behavioural recovery.[181]

Tuszynski's lab used fibrin to deliver cells with a cocktail of growth factors, which helped retain rat embryonic NSCs, human embryonic and human induced pluripotent stem cell-derived NSCs at the injection site after transection type injuries (**Figure 3**).[154,165,179] The grafts extended long axons throughout the CNS and promoted regeneration of the CST, both of which formed new synaptic connections. The regeneration of the CST required direct contact with the graft, suggesting a ligand-receptor interaction rather than growth by diffusible factors. Both human and rat NSCs had a similar effect on axonal regeneration and functional recovery.[154,165] Interestingly, the study investigating human iPS-derived NSCs failed to demonstrate any functional beneficial effects, which might be due to collagen deposits at the lesion site in some of the animals, which prevented axonal growth.[179] As mentioned earlier, differentiation into spinal cord rather than forebrain NSCs promoted the greatest amount of regeneration, indicating that regional specificity might be an important factor to increase the beneficial effects of transplanted cells.[154]

Table 2. Combinatorial strategies focused on cell delivery.

Growth factor/ molecule	Cell/tissue	Biomaterial	Injury model/survival times	Comment	Citation
IGF, bFGF, or TGF-beta, at 4 weeks	rat peripheral nerve at 35 days	gelfoam for drug delivery	C3 hemisection 3 mm 9 weeks chronic injury	All growth factors performed better than PBS (all groups received peripheral nerves), the greatest increase in axonal sprouting was observed with TGFbeta.	[169]
CNTF or bFGF at 4 or, 8 weeks	rat peripheral nerve at 35, 63 days	gelfoam for drug delivery	C3 hemisection 3 mm 9 weeks chronic injury	Treatment with bFGF 8 weeks after injury was less effective compared with treatment 4 weeks after injury. CNTF was equally effective at both time points.	[170]
NT-3 or BDNF	rat fetal spinal cord tissue	gelfoam for drug delivery	T6 hemisection 1 and 4 weeks	The combination of either neurotrophic factor with the fetal tissue had the least amount of neuronal loss, with BDNF demonstrating better morphological preservation.	[175]
NT-3, BDNF, or CNTF	rat peripheral nerve	gelfoam for drug delivery	C2/3 dorsal hemisection, chronic 9 week survival	GF were needed to promote axonal growth into the PN graft, with CNTF demonstrating the greatest effect	[173]
GDNF	rat peripheral nerve graft	gelfoam for drug delivery	C3 hemisection Acute and chronic 1 and 4 weeks	GDNF acted neuroprotective and promoted axonal regeneration into the peripheral nerve graft	[168]
	rat Schwann cells (P4, sciatic nerve) rat NSCs (newborn, hippocampus)	gelfoam for cell delivery	T9/10 lateral hemisection 1, 2, 3 and 4 weeks	Schwann cells improved NSCs survival, and differentiation into neurons	[174]
	TrkC overexpressing rat NSC (hippocampus, newborn) rat NT-3 overexpressing Schwann cells	gelfoam for cell delivery	T10 transection 9–10 weeks	Better differentiation of NSCs into neurons with combination, which also lead to greater axonal regeneration and functional recovery.	[172]
NT-3, BDNF	rat Schwann cells	matrigel, PAN/PVC copolymer channels 60:40 for cell delivery	T8 transection	Combined strategy with growth factor needed for supraspinal axonal regeneration into the tube	[141,142]
IN-1, encapsulated hybridoma cell graft or infusion of supernatant aFGF fibrin glue	human Schwann cells	PAN/PVC guidance channels with matrigel for Schwann cell delivery Millipore capsules for hybridoma cell delivery	T8 transection 5 weeks	Schwann cells + IN-1 supernatant support sprouting Schwann cells plus aFGF-fibrin glue support regeneration of some fibers and reduced die-back.	[139]
IN-1 mouse hybridoma cell transplants either encapsulated or as tumour	Rat embryonic spinal cord tissue (E14-16) Rat pons P0	gelfoam, collagen, glass fibres, laminin coated Millipore filter, carbon fibres, Kevlar fibres, ECM from human placenta	low thoracic, bilateral dorsal transection (2/3 of thickness) 3–4 weeks	Embryonic tissue promoted CST regrowth, but the combination with IN-1 was needed for caudal elongation. Biomaterials failed to serve as bridging material.	[171]
rolipram, cAMP	rat Schwann cells	minipump for drug delivery	T8 moderate contusion injury 2 and 8 weeks	The combination of Rolipram and cAMP had the greatest effect on cAMP levels, axonal sparing, myelination, and demonstrated improved locomotor function.	[177]
Rolipram	rat Schwann cells, non-modified or expressing D15A (bifunctional molecule NT-3 and BDNF)	minipump/matrigel	T8, contusion injury, 25 mm drop, MASCIS 13 weeks	D15A-cells + rolipram usually performed best, but rolipram seemed more important than D15A	[178]
BDNF, NT-3 and GDNF into cavity	minced rat peripheral nerve	gelling collagen matrix	T10 dorsal hemisection Chronic 25 weeks	Combination therapy led to sustained regeneration of the CST	[100]

Table 2. Continued.

Growth factor/ molecule	Cell/tissue	Biomaterial	Injury model/survival times	Comment	Citation
aFGF	rat peripheral nerve graft	fibrin glue to fix peripheral nerves gelfoam to deliver aFGF	T8 transection (5 mm removed)/ 1 year	The combination led to the most improved functional recovery.	[166]
BDNF, NT-3, PDGF-AA, IGF-1, EGF, bFGF, aFGF, GDNF, HGF, calpain inhibitor MDL28170	E14 rat NSC Human ESC-derived NSC 566RSC, HNES7	fibrin matrix for cell and drug delivery	T3 complete transaction 2 mm (C5 hemisection for human cells), 7 and 9 weeks	The fibrin matrix promoted the survival of grafted cells. Extensive axonal outgrowth rostral and caudal from the transplanted cells. The combination promoted functional recovery. Human and rat performed similar (histologically) indicating translational relevance	[165]
BDNF, NT-3, PDGF-AA, IGF-1, EGF, bFGF, aFGF, GDNF, HGF, calpain inhibitor MDL28170	Human induced PSC-derived NSCs 86 y old male	fibrin matrix for cell and drug delivery	C5 hemisection 3 month survival	Fibrin matrix promoted the survival of grafted cells. Extensive outgrowth from grafted cells, but without functional recovery. Few cells were positive for more mature marker	[179]
NT-3, PDGF	mouse ESC-derived NSCs	Fibrin/heparin for cell and drug delivery.	Dorsal hemisection T9 2, 4 & 8 weeks survival	Heparin binding system delivered the growth factors over an extended period of time. However, many cells overproliferated in this group. Combined therapy without heparin had more differentiated cells, and demonstrated the greatest functional recovery.	[180,181]
hypothermia (33-34C)	NgR silenced NSC, Schwann cells	PLGA scaffold for cell delivery	T9 hemisection 8 weeks	More surviving cells and improved functional recovery with combinatorial therapy.	[182]
	rat NSC (fetal spinal cord) rat Schwann cells (sciatic nerve)	Orientated PLGA scaffold for cell delivery	T9 lateral hemisection, 3 mm 24 weeks	Co-transplantation of the cells with PLGA had greater beneficial effects compared to NSC with PLGA	[183]
dbcAMP for pre-differentiation or delivered with microspheres	adult rat brain NSCs	Chitosan channel/ fibrin for cell delivery PLGA microspheres for drug delivery	T8 transection 2 and 6 weeks	dbcAMP pretreated NSCs survived best and promoted the greatest differentiation into neurons. Improved functional recovery with chitosan channels and pre-differentiated cells.	[184]
NgR(310)ecto-FC (NOGO-66 receptor) bFGF, EGF, PDGF via osmotic pump	rat brain NSCs	laminin coated chitosan channels for cell delivery osmotic pump for drug delivery	T8 transaction, 2mm 12–14 weeks	Greater survival of NSCs with GF; NgR had no effect on survival. More oligodendrocytes/myelination, and sprouting with NgR. Synergistic effects of cells, NgR, GF on bridging, not on axonal regeneration. No significant functional improvement.	[185]
	rat NSC (P1 SVZ) rat endothelial cells (ECs, fat pad) 1:10 ratio	PEG/PLL hydrogel for cell delivery orientated PLGA scaffold for axonal guidance	T9/10 lateral hemisection, 4 mm 8 weeks	The combination of NSCs and ECs had more blood vessels than ECs alone, with an partly established BSB barrier. The combination also promoted the greatest axonal regeneration; however no behavioural improvements were found.	[186]
PDGF	Adult rat brain NSCs	HAMC for cell and drug delivery	T2 clip compression, 26g 2 and 9 weeks	The combination acted neuroprotective and more (host)oligodendrocytes were found rostral to the lesion. Improved functional recovery on latter walk	[187]
PDGF RGD	Human iPS-derived oligodendrocyte precursor cells	HAMC for cell and drug delivery	T2 clip compression 26g 2 and 9 weeks	HAMC-RGD-PDGF promoted cell survival, differentiation and prevented tumour formation. No functional recovery due to overproliferation.	[164]

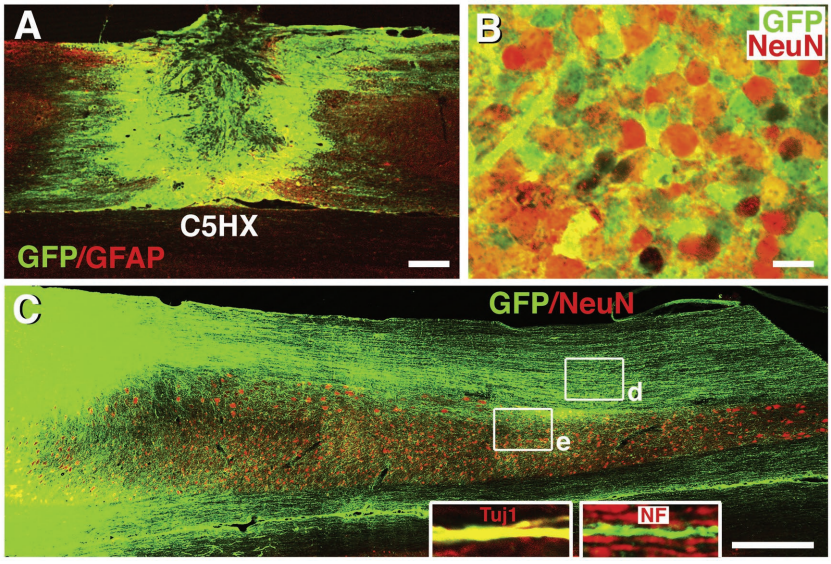

Figure 3. Survival, differentiation, and growth of human iPSC-derived neural stem cells in sites of spinal cord injury. A) GFP-labeled human iPSC-derived neural stem cells were grafted into sites of C5 hemisection spinal cord injury. Horizontal section immunolabeled for GFP and GFAP indicates that implants survive well and distribute through the lesion cavity. B) The majority of cells within the graft were NeuN-positive, indicating neuronal differentiation. C–E) Very large numbers of GFP-labeled axons extend caudally into the host spinal cord (D) white matter and (E) gray matter. Insets in (C) indicate that axons co-localize with Tuj1 but not neurofilament (NF). Scale bar indicates 350 mm in (A), 10 mm in (B), 600 mm in (C). Reproduced with permission.[179] Copyright 2014, CellPress.

4.5. Cellular Guidance

Cellular grafts, either in solution or in hydrogels, lack orientation along the longitudinal axis of the spinal cord, which results in random axonal growth that is unable to cross larger lesions and prevent reconnection of axons with their original target. Biomaterial scaffolds can guide regenerating axons either from grafted or from host neurons.[59–61] Although many orientated structures were developed for repair of the injured spinal cord, not many were tested in combinatorial strategies using more than one other component.

Simple tubes or channels were used to deliver Schwann cells to the transected spinal cord, but growth factors (i.e. NT-3 and BDNF) were needed to promote the regeneration of supraspinal axons.[136,142] Schwab's group tested a diverse set of biomaterials in combination with IN-1 mouse hybridoma cells and rat embryonic spinal cord tissue to support axonal outgrowth across the lesion site, including gelfoam, collagen, glass fibres, laminin coated Millipore filter, carbon fibres, Kevlar fibres, and ECM from human placenta, but none of them proved to be useful.[171]

Two short studies investigated orientated PLGA scaffolds in combination with Schwann cells and NSCs. They could demonstrate that Schwann cells promoted the survival of co-seeded NSCs while NgR-silenced NSCs and Schwann cells further combined with mild hypothermia led to functional recovery.[183,182] Guo et al. investigated the effect of Nogo-66 receptor protein delivery with a minipump in combination with NSC-seeded chitosan channels.[185] While the Nogo-66 receptor protein did not affect cell survival, it enhanced axonal regeneration. Additional delivery of a growth factor cocktail of bFGF, EGF and PDGF promoted cell survival. The combination of Nogo-66 receptor protein, NSPCs, and growth factor cocktail had synergistic effect on the formation of the tissue bridging the spinal cord, but not on functional regeneration.[185] Interestingly, pre-treating NSCs with dbcAMP and transplanting them within fibrin gel filled chitosan channels led to an increased survival of NSCs, neuronal differentiation and improved functional recovery.[184] To, at least partially, account for the difference in gray and white matter, Rauch et al. developed a combination of oriented PLGA and cell-seeded hydrogels (**Figure 4**). Co-seeding NSCs and endothelial cells improved the vasculature and promoted the repair of the blood-spinal cord barrier. The combination also promoted the greatest axonal regeneration; however, no behavioural improvements were observed.[186]

4.6. Disadvantages of Cell Transplantation

Potential adverse effects can derive from various sources. For example, Hofstetter et al. demonstrated that the uncontrolled differentiation of transplanted NSCs into astrocytes lead to allodynia. Suppression of astrocytic differentiation prevented allodynia and improved locomotor function.[189] Even functional connections made by regenerating axons can have adverse effects, leading to spasticity or pain, effectively worsening the outcome.[165] This further highlights the complexity of the neural circuitry and the care that has to be taken during any attempt at reconstruction. Adverse outcomes can result from inappropriate synaptic connections, and improvements in functional outcomes might require restriction of synaptic connections to specific subsets of cells or sub-regions of dendritic architecture.[190] In addition, axonal regeneration may require specific rehabilitation strategies to enable formation of appropriate connections.[191,192] In light of this, rigorous testing for changes in nociception should take place in animal models of SCI before moving to the human patient. This is especially important as many patients with SCI rate neurologic pain as one of the worst consequences of SCI.[193]

Additionally, cells derived from pluripotent stem cells may overproliferate or form tumors. As such, different strategies have been pursued to block teratoma formation: suicide genes,[194,195] cell sorting to increase purity,[196] immunodepletion,[197] cytotoxic antibodies[198] and selective ablation of pluripotent cells with small molecules.[199] These strategies have demonstrated partial success, but guiding cell fate and increasing cell purity before and/or after transplantation are important factors to success.

5. Combinations Focusing on CSPGs

CSPGs are detrimental to axonal and tissue regeneration. In this section we describe ways to overcome their inhibitory

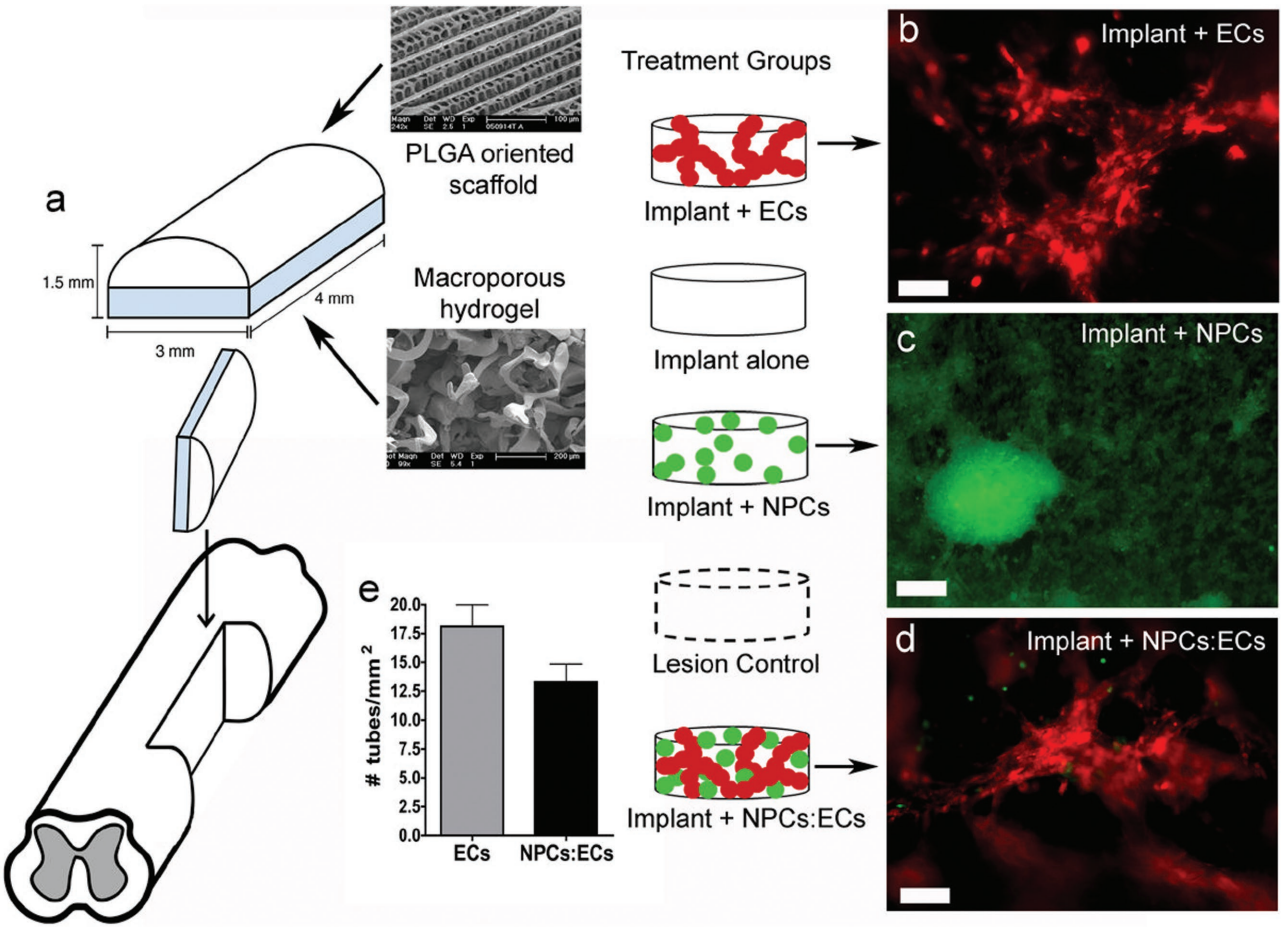

Figure 4. Implant design: a) Two component implant includes an outer PLGA oriented scaffold and an inner PEG/PLL macroporous hydrogel. b) Live image of DiI-labeled rat endothelial cells (ECs, red) cultured on a macroporous gel for one day. c) Live image of GFP-positive rat NSCs (green) cultured on a macroporous gel for seven days. d) Live image of DiI-labeled rat ECs (red) and GFP-positive rat NSCs (green) cultured on a macroporous gel for one day. Scale bar in (b–d) represents 100 µm. e) The number of EC tubes in the implant plus ECs and implant plus NSCs:ECs groups implanted into the hemisected spinal cord. Reproduced with permission.[186]

action and highlight some combinatorial strategies involving chondroitinase ABC (ChABC), which degrades the CSPGs of the glial scar.

Multiple ways to overcome the inhibitory effect of CSPGs have been investigated, but the bacterial enzyme ChABC is the most extensively studied method to date. ChABC promotes axonal sprouting/regeneration by degrading the inhibitory GAG side chains of CSPGs at the lesion site and perineuronal nets (PNNs),[200] and its effect has been well established (reviewed in[201]). Other means of overcoming CSPG inhibition include blocking the axonal receptors of CSPGs, which have been identified recently, as described above. For example, systemic delivery of leukocyte common antigen-related phosphatase (LAR)-targeting peptides promoted significant growth of descending serotonergic fibers and improved locomotor function.[202] Similarly, receptor PTP σ double knockouts promoted growth of sensory and CST axons,[203,204] and blocking of PTPσ with a peptide mimetic of the receptor domain restored serotonergic innervations below the level of spinal cord injury, and facilitated motor recovery.[205]

Although the beneficial effects of ChABC are well established, many details are not fully understood, such as the optimal time window for its delivery. CSPGs reach peak levels two weeks after injury and remain upregulated for over a month after injury.[206,207] Although this could suggest that long-term delivery is necessary, delivery of the enzyme is complicated by the instability of ChABC at body temperature[208,209] In addition, it has also been argued that turnover of ECM molecules is slow and sustained delivery might not be necessary.[210] Nonetheless, biomaterials have been used to achieve sustained local delivery of active ChABC. For example, delivery from a hydrogel-microtube scaffold system enhanced ChABC thermal stability and pro-longed its enzyme activity, leading to functional recovery.[211] Pakulska et al. reported the release of bioactive ChABC from a physically and chemically crosslinked methylcellulose hydrogel through a reversible, affinity-based mechanism.[114,115] Furthermore, release from highly concentrated fibrin gels led to a significantly higher concentration of ChABC in the spinal cord compared to intraspinal injections for up to 3 weeks.[212]

Interestingly, functional recovery following ChABC treatment has been inconsistent when used in more traumatic and clinically relevant spinal contusion injuries, in comparison to hemisection or transection injuries.[213,214] To achieve functional recovery in these clinically relevant SCI models, it may be necessary to deliver ChABC in combination with other therapeutic means to promote regeneration. Blocking the inhibitory action of CSPGs in combination therapies may have several advantages. It can increase axon regeneration into scaffolds or allow them to re-enter the cord at the distal end, and improve migration and integration of transplanted cells.[206,215]

5.1. ChABC + Neurotrophic Factors

The combination of ChABC and neurotrophins aims to remove the inhibitory effect of CSPGs and promote axonal growth over the now more permissive environment. To this end, Lee et al. developed trehalose-ChABC and NT-3 loaded lipid microtubes that were embedded in agarose gels and implanted after a dorsal hemisection at the T10 vertebral level (**Table 3**).[211] The combination treatment resulted in improved axonal sprouting and motor recovery. Similarly, ChABC- and NGF–loaded alginate microspheres, which were incorporated into polydioxane electrospun fibres, led to improved locomotor function after implantation into a complete T9/T10 transection injury.[216] The implantation of a poly(ε-caprolactone) scaffold loaded with NT-3 overexpressing NSCs in combination with ChABC (via an osmotic pump) into T-7/8 hemisection models of SCI resulted in rostro-caudal migration of transplanted cells, axonal growth into the scaffold, and locomotor functionality in both the ipsilateral and contralateral hindlimbs.[217] Osmotic minipump delivery of ChABC has been combined with intraspinally injected OECs and a semipermeable PAN/PVC guidance channel containing Schwann cells embedded in media/Matrigel connecting the rostral and caudal stumps following a T8 complete transection injury. The combination treatments resulted in significant locomotor recovery, as well as increased numbers of myelinated axons and seroteonergic fibres in the Schwann cell bridge.[218] Although the combination of ChABC and neurotrophic factors provided a more permissive environment, only limited sprouting occurred, rather than long distance axonal regeneration. This indicates that either the optimal combinations have not been found yet or that further components, such as cells, are required. These studies indicate that the combination of ChABC and cells is useful to promote functional recovery after severe SCI. Future studies using methods other than osmotic pumps to deliver ChABC will be of interest.

5.2. Disadvantages of ChABC Delivery

There are three subfamilies of chondroitinases: chondroitinase ABC, chondroitinase AC, and chondroitinase B. Chondroitinase ABC is most commonly used and has the broadest substrate specificity as it degrades chondroitin sulphate, dermatan sulphate and hyaluronan.[225,226] A potential issue with using chondroitinase ABC is its lack of specificity as it not only degrades inhibitory CSPGs such as brevican and aggrecan, but also growth-promoting CSPGs such as CSPG4 and CSPG5, all of which are upregulated post-SCI.[227] Delayed injection of growth promoting substrates after ChABC treatment may circumvent this problem and could explain the often observed beneficial effects of co-treatment of ChABC with cells, which often express ECM molecules known to promote axonal outgrowth. Enhanced plasticity caused by ChABC treatment could also have adverse effects, such as spasticity or pain, and specific rehabilitation strategies may be required to enable formation of appropriate connections.[191,192]

One of the biggest challenges with ChABC is its delivery because it is a very fragile protein. It needs to delivered locally for a sustained period of time, and until recently, there has not been a way to achieve this.[114,115]

6. Combinations with Anti Myelin Associated Inhibitors

Myelin associated inhibitors (MAIs) have been demonstrated to be potent inhibitors to axonal growth. The development of an antibody against NOGO-A was one the first approaches described to neutralize the inhibitory environment at the lesion site. While this strategy proved to be useful in promoting the regeneration of severed axons, functional recovery was not always observed. Due to the potent axonal growth promoting properties of anti-MAI, they may be key to a successful combinatorial strategy. In this section we first describe ways to overcome their inhibitory action and then highlight some combinatorial strategies involving anti-MAI.

Targeting MAIs aims to remove their inhibitory influence and create a more growth-promoting environment, either by blocking the inhibitors themselves, their receptors, or their downstream pathways. While most often used to promote the regeneration of host axons, targeting MAIs could equally be beneficial for transplantation of neuronal cell populations to help them regenerate axons and make new connections with host neurons.

The first immunological tool that was used to neutralize the MAI Nogo-A, was the monoclonal antibody IN-1,[228] which demonstrated potency in vitro and in vivo, and resulted in long distance axon regeneration in the injured adult rat spinal cord.[57,229,230] Further antibodies aiming to neutralize Nogo-A include IN-1, 11C7, and 7B12, all of which have been shown to reduce myelin inhibition and enhance axonal sprouting and outgrowth, often associated with improved locomotor function (Reviewed in[231]). Rather than only blocking the inhibition from Nogo-A, a more comprehensive strategy is to block the NgR1 receptor through which all of the MAIs act, or to block intracellular Rho signaling pathways which eventually cause cytoskeleton destabilization and growth inhibition. For example, a specific antagonist of NOGO-66 action at NgR1 is NEP1-40 (NOGO extracellular peptide, residues 1 to 40), which has been shown to increase locomotor recovery after rodent spinal cord injury.[232] Strategies to disrupt the Rho/Rock signaling pathway include the pyridine derivative Y-27632 which inhibits ROCK,[233] Clostridium botulinum-derived Rho antagonist (C3 ribosyltransferase),[234] and VX-210 (formerly Cethrin).[235] While not all of these have been used in combinatorial strategies, they certainly offer the possibility to be a key ingredient for future

Table 3. Combinatorial strategies focused on anti-inhibitory molecules.

Anti-inhibitory molecule(s)	Combination therapeutic	Biomaterial	Injury model/survival times	Comments about combination group	Citation
Trehalose-ChABC	NT-3	lipid microtubes embedded in Agarose gel for both molecules	T10 Dorsal over hemisection, 6 weeks post-injury	Reduced CSPG levels, improved functional recovery and sprouting of serotonergic fibres.	[211]
ChABC	NGF	polydioxanone electrospun filaments for drug delivery of both molecules from alginate microspheres filaments fixed with fibrin gels	T9/T10 transection, 3 weeks post-injury	Improved functional recovery.	[216]
ChABC	NSCs or NT-3 overexpressing NSCs,	osmotic pump for drug delivery poly(ε-Caprolactone) scaffold for cell delivery	T7/8 hemisection, 9 weeks post-injury	Greater neuronal and oligodendroglial differentiation of NT-3 NSCs. Increased migration of transplanted cells, axonal growth into the scaffold. Greatest functional recovery with full combination.	[217]
ChABC	OECs (direct injection) Schwann cells	osmotic pump for drug delivery Matrigel filled PAN/PVC guidance channels for Schwann cell delivery	T8 transection, 8 weeks post- injury	Increased locomotor recovery, as well as increased numbers of myelinated axons and serotonergic fibres in the Schwann cell bridge	[218]
NEP1-40, ChABC	ESC-derived progenitor motor neurons (pMNs)	Fibrin scaffold for cell and drug delivery: PLGA microparticles for NEP1-40, lipid microtubes for ChABC, NT-3 and PDGF modified with a heparin binding domain, injected 2 weeks post-injury	T8 dorsal hemisection, 4 weeks post-injury	The combination of pMNs with sustained-delivery of anti-inhibitory molecules led to reduced cell survival and increased macrophage infiltration. Increased CSPGs levels in groups that received cells + AIMS	[219]
Ligand binding domain of the ephrinB3 and sema4D receptors, NEP1-40		Linear porous collagen scaffold for drug delivery: ephrinB3 and sema4D receptors modified with a collagen binding domain, physically absorbed NEP1-40	T10 transection, 12 weeks post-injury	Increased axonal regeneration into the lesion Enhanced locomotor recovery	[220]
Ligand binding domain of the ephrinB3 and sema4D receptors, NEP1-40	BDNF, NT-3 dibutryl cyclic AMP (cAMP)	Linear porous collagen scaffold for drug delivery: ephrinB3 and sema4D receptors, and BDNF and NT-3 modified with a collagen binding domain, physically absorbed NEP1-40, cAMP injections	T10 transection, 12 weeks post-injury	Reduced lesion size, increased axonal regeneration and angiogenesis. Enhanced locomotor recovery.	[221]
Anti-Nogo-A	NT-3	PLGA nanoparticles embedded in HAMC for delivery of both molecules	T1/2 clip compression, 8 weeks post-injury	Increase in axon density in both the single and combination treatment groups. Improved functional recovery only in the combination group.	[222]
Anti-NgR antibodies	VEGF and BDNF	Hyaluronan-based scaffold crosslinked with poly(l-lysine) with a longitudinal multi-tubular conformation for both molecules: Anti-NgR adsorbed, PLGA microspheres for VEGF and BDNF	T9/10 dorsal hemisection, 8 weeks post-injury	Improved locomotor recovery. Increased neurite outgrowth into the lesion, and enhanced angiogenesis.	[223]
Antibody 151IgG (inhibits EGFR signaling)	BDNF	Linear porous collagen binding scaffold for drug delivery: crosslinked with the antibody, BDNF modified with a collagen binding domain	T8/9 transection, 8 weeks post-injury	Increased axonal growth at the lesion site Decreased GFAP density Recovery of electrical transmission of synapses	[224]

studies. Nonetheless, there are some problems associated with the delivery of these agents, besides the drawbacks of systemic delivery or the use of osmotic minipumps. For example, IN-1 is an IgM antibody, which is not very stable at normal body temperature.[139] Biomaterials can help stabilize proteins and provide their prolonged bioactive release.[236]

6.1. Anti-MAI + Other Anti-Inhibitory Factors

While delivery of anti-MAIs removes inhibition by myelin debris, other molecules, such as CSPGs, may still prevent substantial axonal outgrowth. In an attempt to overcome inhibition from both sources, ChABC and NEP1-40 were co-delivered intrathecally using osmotic pumps, while animals also underwent treadmill training, which demonstrated a significant increase in functional recovery in comparison to single treatments (Table 3).[237]

In order to achieve similar levels of recovery through dual anti-inhibition without the use of minipumps, Wilems et al. developed a fibrin-based scaffold to deliver NEP1-40 from PLGA microparticles for 2 weeks, and ChABC from lipid microtubes for 1 week in vitro.[238] Interestingly, acute implantation of the delivery system (fibrin scaffold with empty microspheres and lipid microtubes) after T8 dorsal hemisection resulted in a significant decrease in axon growth; however, this effect was rescued in animals receiving ChABC and NEP1-40 laden fibrin scaffolds.

In a follow up study, the ChABC and NEP1-40 dual delivery system was used in combination with ESC-derived progenitor motor neurons (pMNs), and a cocktail of factors (ATIII peptide, heparin, NT-3 and PDGF-AA) two weeks after T8 dorsal hemisection.[219] Unexpectedly, the combination of MAIs and pMNs (with or without GFs) had a negative effect on both cell survival and macrophage invasion compared to animals receiving MAIs alone. This study demonstrates that although combinatorial strategies are promising, certain combinations may aggravate negative side-effects instead of increasing positive outcomes.

To provide an oriented substrate and promote axonal regeneration, Li et al. developed functionalized linear collagen scaffolds by physically absorbing NEP1-40 and immobilizing ephrinB3 (CBD-B1) and sema4D (CBD-A4) receptors using collagen binding domains.[220] The scaffolds were implanted following a T10 complete transection injury. The scaffold alone provided axonal guidance, but did not promote axonal regeneration to the same extent as the scaffold with either CBD-B1 alone, CBD-A4 alone, or with both receptors and NEP1-40, which demonstrated the greatest amount of axonal regeneration. Unfortunately, the scaffold with NEP1-40 alone was not tested. The authors state that locomotor recovery was highest with the combination; however the other treatment groups were not plotted, so it remains unclear if there was an added benefit of the combination or if a simpler combination was sufficient. In vitro, there was no added benefit of combining the two receptors compared to each alone.

6.2. Anti-MAI + Neurotrophic Factors

Similar to the combination of ChABC and neurotrophic factors, anti-MAI inhibitors and neurotrophic factors have often been delivered in combination to overcome inhibition and promote axonal growth. The aforementioned linear porous collagen binding scaffold with NEP1-40, EphrinB3 and sema4D receptors, was further modified with BDNF and NT-3, and delivered in combination with dibutryl cyclic AMP (dbcAMP) injections, which can activate regeneration-associated genes (Table 3).[221] Following implantation, animals receiving the full combinatorial treatment demonstrated a significantly smaller lesion size, increased axonal regeneration and angiogenesis, and enhanced locomotor recovery. Further additions to the treatment demonstrated increasingly beneficial effects in most investigated aspects, indicating that the added complexity was worth the effort. Compared to their previous study,[220,221] it seems important to target different aspects of the injury with each additional treatment.

Elliott Donaghue et al. developed a HAMC hydrogel/PLGA nanoparticle drug delivery system that released dispersed anti-Nogo-A over 10 days and encapsulated NT-3 over 58 days in vitro.[222] Although both single, and combination treatment led to an increase in axon density after acute injection into the intrathecal space of rats with a T2 compression injury, only the combination led to functional improvements.

In an interesting set of studies, a poly-L-lysine (PLL) crosslinked hyaluronan hydrogel was first modified with the nogo-66 receptor antibody, which promoted axonal regeneration and myelination after implantation into a lateral hemisection injury at T8/9.[239] In a subsequent study, the hydrogel was further modified with BDNF and VEGF laden microparticles, and designed to have a longitudinal multi-tubular conformation.[223] Following implantation into T9/T10 dorsal hemisection spinal cord injury, animals receiving the full combination treatment showed significantly greater axonal growth, angiogenesis, and functional recovery.

In an attempt to block the inhibitory action of both CSPGs and MAI, Han et al. targeted their downstream signaling pathway by inhibiting epidermal growth factor receptor (EGFR) activation with the antibody 151IgG. The antibody was conjugated to an orientated collagen scaffold that was further modified with BDNF through a collagen binding domain.[224] The triple functional biomaterial promoted axonal growth at the lesion site, electrophysiological recovery, and reduced glial scarring compared to all control groups after T8/9 transection.

6.3. Disadvantages of MAI Blockers

Many studies have already recognized that the monoclonal antibody IN-1 only blocks the effect of Nogo-A, but not of other MAIs, such as MAG and OMgp. This may explain why only a small proportion of the damaged fibers regenerate after treatment with this antibody.[240] Rather than only blocking the inhibition from Nogo-A, a more comprehensive strategy is to block the NgR1 receptor through which all of the MAIs act or to block intracellular Rho signaling pathways. However, there are still other less extensively studied MAIs such as semaphorin4D and ephrinB3, which do not act through NgR1 and still inhibit axonal regeneration.

There seems to be a vulnerable phase after activation of growth and plasticity in which forced activity can be harmful.

This was demonstrated after stroke, where animals receiving sequential treatment of anti-Nogo-A and functional training performed significantly better than animals receiving the treatments simultaneously.[241,242] The enhanced growth and plasticity can lead to the formation of a large number of new connections within and between different areas of the injured CNS, which may be weak and imprecise. Selecting and stabilizing meaningful connections while pruning malfunctional ones seems to work best after a certain period of time. This clearly demonstrated that not only the right combinations have to be found, but also the right timing between different treatments.

7. Conclusions and Future Perspective

Many combinatorial strategies have demonstrated greater beneficial effects than the delivery of their individual components. While some studies used innovative biomaterials to deliver drugs or cells, the majority relied on simple methods, such as gelfoam or osmotic minipumps. Future studies should implement novel biomaterials to better harvest the potential of drugs and cells. Addressing multiple aspects of tissue regeneration in a combinatorial strategy can maximize functional recovery and provide a robust effect that can be translated into the clinic. However, it is currently unknown which combination, concentration of biomolecules, and the time period for delivery will be the most beneficial. Interests, experiences, intentions, and strategies vary between researchers, which lead to a wide variety of therapeutic strategies. With the notable exception of the NIH "Facilities of Research-Spinal Cord Injury" project, that supported independent replication of published studies,[243–248] few in vivo studies are replicated in independent laboratories, perhaps hampering clinical translation. Although time consuming and academically not very rewarding, independent replication of promising results in the same and different animal models of disease is key to decreasing the number of failed trials. Even though the development and replication of combinatorial strategies can be especially difficult, requiring expertise in many different areas that not many laboratories or even universities have, more studies that only vary one aspect of the therapy while holding the other treatment(s) constant are necessary.

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