Gene Delivery Strategies to Promote Spinal Cord Repair

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ABSTRACT: Gene therapies hold great promise for the treatment of many neurodegenerative disorders and traumatic injuries in the central nervous system. However, development of effective methods to deliver such therapies in a controlled manner to the spinal cord is a necessity for their translation to the clinic. Although essential progress has been made to improve efficiency of transgene delivery and reduce the immunogenicity of genetic vectors, there is still much work to be done to achieve clinical strategies capable of reversing neurodegeneration and mediating tissue regeneration. In particular, strategies to achieve localized, robust expression of therapeutic transgenes by target cell types, at controlled levels over defined time periods, will be necessary to fully regenerate functional spinal cord tissues. This review summarizes the progress over the last decade toward the development of effective gene therapies in the spinal cord, including identification of appropriate target genes, improvements to design of genetic vectors, advances in delivery methods, and strategies for delivery of multiple transgenes with synergistic actions. The potential of biomaterials to mediate gene delivery while simultaneously providing inductive scaffolding to facilitate tissue regeneration is also discussed.

KEYWORDS: gene therapy, spinal cord, neurodegeneration

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Spinal Cord Degeneration and Barriers to Regeneration

Spinal cord degeneration is often devastating, resulting in chronic neuropathic pain, partial or complete paralysis, and even death. Traumatic spinal cord injury (SCI) is characterized by partial or complete paralysis and loss of sensation below the injury, and is estimated to affect over 6 million people worldwide, with about 273,000 affected persons and 12,000 new cases per year in the United States. While the cost of the injury can vary depending on location and degree of injury, a tetraplegia (injury at the cervical vertebrae, C1–C4) is estimated to cost over $1 million in medical expenses in the first year after SCI, while $180,000 in yearly expenses thereafter. Several neurodegenerative diseases also result in substantial loss of spinal cord tissue function and significant monetary burden, including amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and spinal muscular atrophy (SMA). In addition, persons living with loss of spinal cord function report a much lower quality of life due to physical limitations and pain. While some improvements have been made in living standards, care, and treatments designed to halt degeneration and repair the spinal cord, there is still no cure or even adequate treatment to partially restore spinal cord function lost to any of these clinical pathologies.

SCI triggers a complicated cascade of destructive events, each of which presents a unique therapeutic target. As such, experts generally agree that combinatorial strategies offering mechanisms to ameliorate multiple barriers to regeneration simultaneously will be necessary to completely restore function after SCI. Individual barriers to repair after SCI include similar mechanisms to those that manifest in neurodegenerative disorders affecting spinal cord. In particular, hyper-inflammation is a contributing factor in the majority of these disorders. In MS and SCI, this overactive immune response causes oligodendrocyte death and demyelination of axons. In ALS, SMA, and SCI, degeneration of motor neurons occurs. Thus, although much of the gene therapy research discussed in this review focuses on models of SCI, the concepts are applicable to a wide range of neurodegenerative disorders.

The actual process of injury in the spinal cord is a complicated sequence that can be generalized as a primary injury (the direct loss of axon connectivity and myelination as a result of contusion, laceration, or other physical injuries) and the more complex secondary injury. In the minutes immediately following the injury, peripheral blood cells, cytokines, and tissue debris accumulate near the lesion and contribute to a neurotoxic milieu. The soft tissue of the spinal cord swells against
the vertebrae and restricts blood flow to the damaged area, leading to immediate ischemia and cell death. Over the next few days to weeks, this inflammatory environment leads to apoptosis of specialized central nervous system (CNS) cells, including neurons and oligodendrocytes, which ultimately causes progressive loss of spinal function.\textsuperscript{3,10} Eventually, the hallmark characteristics of secondary injury take shape, including loss of local vasculature and degeneration of surrounding myelinated axons and interneurons.\textsuperscript{8,12} A fluid-filled cyst lined with reactive astrocytes, called the glial scar, is left in place at the SCI lesion. The scar serves to reseal the blood–brain barrier and contain the damaged area.\textsuperscript{12} However, the glial scar typically extends beyond the margins of the original injury and presents an impassable barrier to the growth of new and regenerating axons.

Despite these barriers, the spinal cord retains an innate ability to regenerate and remodel, and recovery of function after spinal cord trauma is likely possible if appropriate treatments are provided. Currently, the standard clinical treatments for SCI aim to reduce inflammation at the acute stage of injury. These include administration of steroids,\textsuperscript{1,8} anti-inflammatory\textsuperscript{13} and systemic hypothermia.\textsuperscript{14} A number of therapeutic strategies aimed at addressing additional barriers to regeneration and chronic SCI have been explored experimentally. Many of these strategies have focused on delivering various growth factors to provide protection against apoptosis and encourage regeneration of axons and vasculature.\textsuperscript{15–19} Others have focused on biomaterial-based treatments that seek to replace the inhibitory environment that develops after injury with extracellular matrix cues that permit migration of axons and other cells.\textsuperscript{20,21} In addition, cell-based treatments, which range from mesenchymal stem cells to astrocytes to neural stem cells, have shown promise as mediators of spinal cord repair.\textsuperscript{2,4,5,22} Perhaps the most robust repair has been observed when cells, biomaterials, and/or growth factors were combined into a single therapy.\textsuperscript{2–6} Recently, robust clinical benefits have been achieved when epidural stimulation and physical therapy were combined to reactivate dormant neural circuits and perhaps induce plasticity of spared neural circuits.\textsuperscript{23}

### Potential Therapeutic Genes for Spinal Cord Repair

Several pathways affect the ability of axons to functionally regenerate in the spinal cord, and precise genetic regulation of these pathways at various points in the signaling cascade has proven to be a valuable tool. Genetic manipulation has dramatically increased the field’s basic understanding of the mechanisms preventing regeneration and how to manipulate the intrinsic regenerative programs that are typically silenced in adult tissues. This research has led to the identification of biomolecular targets that have the potential to induce robust clinical benefits. Parallel work has developed fundamental techniques for direct delivery of gene therapies to the CNS. Gene therapies provide the flexibility for transgene expression to be sustained at controlled levels over long periods of times, theoretically throughout a patient’s lifetime, or restricted to only short time periods, depending on the clinical goal. In contrast, proteins have relatively short half-lives, requiring that they be continually replenished to achieve therapeutic bioactivity over long time periods, typically using osmotic mini-pumps in the spinal cord.

Gene delivery has many advantages over other therapeutic strategies. One distinct advantage of gene, over protein, delivery is that multiple therapeutic genes can be delivered in tandem from the same delivery systems, which include vectors and biomaterials, without having to optimize the system separately. In the case of protein delivery, the system would need to be adjusted, often significantly, for each new protein evaluated to maintain its bioactivity and characterize its bioavailability once administered. Gene delivery provides the opportunity to design therapies with excellent flexibility, where transgene expression can be 1) localized to specific cell types or tissues, 2) delivered with transient or sustained temporal profiles, and 3) selected to either up- or downregulate targets on multiple levels of biological systems, including secreted diffusible or extracellular matrix factors, cell surface receptors, cytosolic signaling molecules, and transcription factors. RNA interference (RNAi), which includes small interfering RNA (siRNA) and microRNA (miRNA), is commonly used to reduce or knock out expression of specific genes.\textsuperscript{24} Effects of various RNAi have been investigated extensively in the context of spinal cord repair\textsuperscript{25–29} and is an important tool for studying SCI. While siRNA is usually highly specific for a particular gene and completely knocks out its expression, miRNA is more promiscuous and can regulate hundreds of mRNA sequences, resulting in various levels of inhibition. In most cases, techniques and considerations for DNA and RNA delivery can be considered interchangeable.

To promote spinal cord repair, researchers have explored gene delivery as a mechanism to reduce post-injury pain and inflammation.\textsuperscript{3,10} provide a supportive vascular environment,\textsuperscript{16,18,30–32} decrease the glial scar,\textsuperscript{29,33–36} increase cell survival, increase axonal regeneration and plasticity,\textsuperscript{17,27,28,37–50} modulate the immune response,\textsuperscript{51–53} and increase recruitment, differentiation, and/or engraftment of endogenous or transplanted stem/progenitor cells (Fig. 1, Table 1).\textsuperscript{32,45,54–60} The role of gene therapy at the acute stage of injury has been somewhat limited because of the time delay between administration of genetic vectors and expression of delivered genes.\textsuperscript{39,60–63} However, after SCI, neutrophils and macrophages that cross the broken blood–brain barrier and accumulate at the injury site persist well into the chronic phase, remaining over the course of months to years.\textsuperscript{9,51,52,64,65} This nonresolving inflammation continues to induce apoptosis of surrounding neurons and oligodendrocytes.

Genetic therapies designed to reduce inflammation have explored the effects of overexpression of anti-inflammatory factors (for a detailed review, see Gensel et al.\textsuperscript{25}). Overexpression of factors that promote resident macrophages to adopt the
M2, or “resolving” phenotype, which include interleukin-10 (IL-10),53,66 or, conversely, downregulation of factors that promote the M1 phenotype present in active inflammation, may be a particularly effective way to modulate the immune response after SCI and in MS.9,52

In addition to immunomodulatory approaches, tissue-sparing and cell survival can be increased by increased blood vessel growth to reduce hypoxic stress. Increased tissue-sparing has been reported with delivery of angiogenic factors, including vascular endothelial growth factor (VEGF)16,18,31,32 and basic fibroblast growth factor (bFGF).31,67 Factors providing protection against hypoxia, such as hypoxia inducible factor 1 alpha (HIF1α),32,68 also may be beneficial. Upregulation of diffusible neurotrophic and anti-apoptotic factors, or downregulation of apoptotic intracellular pathways, has also been shown to have positive effects of cell survival after SCI. Overexpression of several secreted neurotrophins, most commonly neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF),17,33,40,42,44–46,48–50,54,57 has been demonstrated to be beneficial. Similarly, neurotrophic signaling can be increased by overexpression of the cell receptors for neural survival factors, such as retinoic acid receptor beta (RARβ)41 and trk receptors.42,69 Concurrent overexpression of neurotrophins and their receptors has been reported to further augment axon regeneration and functional recovery after SCI.58,69 Alternative strategies have directly targeted antiapoptotic pathways, most commonly by inducing overexpression of Bcl-2.70,71

In order to regenerate function after SCI, the spared axons must navigate through or around the lesion area to reach their synaptic targets. Although adult spinal cord neurons and oligodendrocytes do have the intrinsic ability to regenerate, these programs are typically dormant after development.22 Several strategies have been designed with the goal of activating intrinsic programs for axon plasticity and remodeling of the inhibitory extracellular environment that surrounds axons after SCI. For example, researchers have reported increased axonal plasticity through genetic augmentation of the same neurotrophic factors that enhance neuronal sparing, including nerve growth factor (NGF),15,19,72 BDNF,33,44,45,49,50 and NT-3.17,40,46,48–50,57,73 Production of spatial gradients of neurotrophic factors provides chemotactic guidance to further promote axonal sprouting40,45,46,48,74, however, defined gradients have been challenging to create in vivo. Others have used genetic vectors to overexpress regenerative cell adhesion molecules, such as L1,75,76 the membrane-crossing mimetic peptide,29,77 or the plasticity-associated polysialylated neural cell adhesion molecule (PSA-NCAM),78 each of which interacts with the extracellular matrix to mediate axonal migration. Likewise, vectors that induce overexpression of permissive matrix proteins may also be beneficial.20,21

Inhibition of Rho kinase (ROCK), a signaling molecule that is activated by the myelin-derived inhibitors in the SCI environment, has also been demonstrated as a method to increase axonal plasticity.79–81 Several small-molecule ROCK inhibitors have been evaluated for their effects on SCI repair, in particular a compound known as Y-27632. Genetic vectors offer an alternative method for ROCK inhibition by direct manipulation of host biology, a strategy that is likely to be better sustained over time and more specific to ROCK, as many small-molecule inhibitors also block other signaling molecules. Manipulation of the glial scar, which is composed of chondroitin sulfate proteoglycans that inhibit axon migration, can further minimize barriers to axonal regeneration.82,83 Approaches to reduce the glial scar include therapies designed to downregulate astrocyte reactivity/astrogliosis, and chondroitin sulfate expression,25,36,84 overexpression of chondroitinase enzyme to degrade existing scar,14,35,85,86 or upregulation of extracellular matrix components that promote axon migration.20,21,77 Each of these approaches has been shown to induce modest improvements in function in rodent models of SCI.

Gene therapies can also be designed to target either endogenous or transplanted stem/progenitor cells and direct their
| THERAPEUTIC TARGETS                        | GENETIC MANIPULATION                        | DELIVERY STRATEGIES | VECTOR           | DETAILS                             | CITATION |
|------------------------------------------|---------------------------------------------|---------------------|------------------|-------------------------------------|----------|
| Neuron and oligodendrocyte survival      | Apoptin (to induce apoptosis)               | Injection into cord | polyplex         | L-arginine grafted polyamidoamine   | Pennant 2014 [166] |
|                                          | Bcl-2                                        | Injection into cord | adenovirus       |                                     | Yukawa 2002 [71] |
|                                          | BDNF, FGF-2, NT-3                           | Biomaterials        | plasmid          | Gene-activated matrix               | Berry 2001 [38]  |
|                                          | BDNF, NT-3                                  | Transplantation of genetically modified cells | adeno-associated virus | Schwann Cells in PAN-PVC tube | Blits 2003 [39]  |
|                                          | BDNF, CNTF, GDNF, NT-3, others             | Transplantation of genetically modified cells | lentivirus       | Transduced neural progenitor cells. | Blits 2005 [54]  |
|                                          | NT-3                                        | Transplantation of genetically modified cells | lentivirus       | Transduced neural progenitor cells. | Kusano 2010 [57] |
|                                          | BDNF                                        | Retrograde transduction from peripheral system | adenovirus       | Injection into sternomastoid muscles | Nakajima 2010 [33]; Uchida 2010 [144]; Nakajima 2014 [145] |
|                                          | BDNF, NT-3                                  | Transplantation of genetically modified cells | retrovirus       | Transduced glial progenitor cells.  | Cao 2005 [55]   |
|                                          | BDNF, NT-3                                  | Transplantation of genetically modified cells | retrovirus       | Transduced fibroblasts              | Tobias 2003 [153] |
|                                          | BDNF                                        | Transplantation of genetically modified cells | adenovirus       | Transduced mesenchymal stem cells.  | Sasaki 2009 [44] |
|                                          | BDNF                                        | Injection into cord | adeno-associated virus |                                     | Ruitenber 2004 [147] |
|                                          | BDNF                                        | Biomaterials, injection into. the cord | polyplex         | pol(γN'N-(2-aminoethyl)-2-aminoethyl)aspartamide) | Hayakawa 2014 [124] |
|                                          | VEGF, FGF-2                                 | Biomaterials        | plasmid          | PLG bridge implants                 | De Laporte 2011 [31] |
|                                          | Hypoxia-inducible factor 1 alpha (HIF-1α)   | Injection into cord | adenovirus       |                                     | Chen 2013 [69]   |
|                                          | Survival motor neuron (SMN)                 | System through blood-brain barrier | adeno-associated virus | Intravenous bolus injection | Foust 2010 [111] |
|                                          | Survival motor neuron (SMN)                 | Retrograde transduction from peripheral system | adeno-associated virus | Injection into gastrocnemius muscles | Benkhelif-Ziyyat 2014 [112] |
| Axon guidance, plasticity and remyelination | BDNF, GDNF, CNTF                           | Retrograde transduction from peripheral system | adenovirus       | Facial muscle injection             | Baumgartner 1997 [15] |
|                                          | BDNF, (TET-inducible promoter)              | Transplantation of genetically modified cells | retrovirus       | Transduced fibroblasts              | Blesch 2007 [42] |
|                                          | BDNF, NT-3                                  | Biomaterials        | lentivirus       | PLG bridge implants                 | Tuinstra 2012 [49] |
|                                          | BDNF, NT-3                                  | Transplantation of genetically modified cells | lentivirus       | Transduced Schwann cells seeded onto PLG scaffolds | Hurtado 2006 [155] |
|                                          | BDNF                                        | Injection into cord, transplantation of genetically modified cells | lentivirus       | Injection rostral to lesion, concurrent progenitor cell transplant | Bonner 2010 [45] |
|                                          | NGF, Semaphorin-A                           | Injection into cord | adenovirus       | Rostral to lesion                   | Cameron 2006 [73] |
| Gene Delivery Method | Technique Description | Vector | Additional Information |
|----------------------|-----------------------|--------|------------------------|
| NT-3                | Injection into cord, transplantation of genetically modified cells | lentivirus | Injection rostral to lesion, concurrent marrow stromal cell transplant | Taylor 2006 [40] |
| NT-3 (TET-inducible) | Injection into cord, transplantation of genetically modified cells | lentivirus | Injection rostral to lesion, concurrent marrow stromal cell transplant | Hou 2012 [48] |
| Cadherin-1 (RNAi)   | Injection into cord | lentivirus | RNAi | Qi 2014 [28] |
| Neuronal calcium sensor-1 | Injection into cord | lentivirus | Injection into sensorimotor cortex | Yip 2010 [37] |
| NGF | Biomaterials | plasmid | Fibronectin-patterned, PLG bridge implants | De Laporte 2010 [30] |
| NGF | Transplantation of genetically modified cells | retrovirus | Transduced fibroblasts | Grill 1997 [37] |
| NT-3 | Biomaterials Transplantation of genetically modified cells | lentivirus | Templated agarose scaffolds with bone marrow stromal cells | Gros 2010 [46] |
| NT-3 | Biomaterials | polyplex | 2-(dimethylamino) ethyl methacrylate, complexed onto collagen-based scaffolds | Yao 2013 [166] |
| NT-3 | Retrograde transduction from peripheral system | adenovirus | Via sciatic nerve | Zhou, 2003 [17] |
| Retinoic acid receptor β2 | Injection into cord | lentivirus | | Yip, 2006 [14] |
| trkB | Injection into cord | lentivirus | | Hollis 2009 [43] |
| trkB (siRNA) | System through blood-brain barrier | adenovirus | Intrapleural injection | Mantilla 2013 [70] |
| trkC, NT-3 | Transplantation of genetically modified cells | adenovirus | Transplant of NT-3-Schwann cells and trkC-neural stem cells, seeded in Gelfoam | Wang 2011 [58] |
| Immune cell modulation | Chondroitinase ABC | Injection into cord | lentivirus | Bartus 2014 [35] |
| IL-10 | Biomaterials | plasmid | Silica nanoparticle "protocells" | Dengler 2013 [53] |
| Wnt3a, Wnt5a | Injection into cord | Adeno-associated virus | | Rodriguez 2014 [59] |
| Glial scar formation/extracellular matrix | Chondroitinase ABC | Injection to cord | lentivirus | Zhao 2011 [34] |
| GFAP and Vimentin (siRNA) | Biomaterials, injection into the cord | adenovirus | Injection in atelocollagen carrier | Toyooka 2011 [25] |
| GFAP and Vimentin (siRNA) | Physical methods, biomaterials | plasmid | Atelocollagen carrier, photomechanical wave | Ando 2012 [26] |
| GFAP and Vimentin (shRNA) | Injection into cord | lentivirus | | Desclaux 2015 [36] |
| L1 (TET inducible) | Transplantation of genetically modified cells | cell line | Transplant of genetically modified neural progenitor cells | Yoo 2014 [61] |
| NG2(shRNA) and NT-3 | Injection into cord | lentivirus | | Donnelly 2012 [84] |

(Continued)
### Table 1. (Continued)

| DELIVERY STRATEGIES | GENETIC MANIPULATION | VECTOR | CITATION |
|----------------------|-----------------------|---------|----------|
| Injection into cord  | Receptor PTPr (RNA)   | lentivirus | Zhou 2014 [29] |
| Injection into cord  | Transplantation of genetically modified cells | lentivirus | Lipavá, 2001 [76] |
| Injection into cord  | Transplantation of genetically modified cells | lentivirus | Can 2013 [27] |
| Injection into cord  | Transplantation of genetically modified cells | lentivirus | Papastefanaki 2007 [78] |
| Injection into cord  | Transplantation of genetically modified cells | lentivirus | Thomas, Seidlits 2014 [60] |
| Injection into cord  | Transplantation of genetically modified cells | lentivirus | Cao 2010 [56] |
| Injection into cord  | Transplantation of genetically modified cells | lentivirus | Pluronic-127 encapsulation, implanted in cord | Wu 2013 [186] |
| Injection into cord  | Transplantation of genetically modified cells | lentivirus | Chitosan/heparin modified PLg bridge implants | Thomas, Seidlits 2014 [60] |
| Injection into cord  | Transplantation of genetically modified cells | lentivirus | Cao 2010 [56] |

**ThERaPEUTIC TaRGETS**

- Direct differentiation into mature, specialized CNS cells, including interneurons, motor neurons, and oligodendrocytes which are lost in ALS and MS, respectively, and after SCI. Both endogenous and transplanted neural stem/progenitor cells have the capacity to differentiate into neurons and oligodendrocytes that functionally engraft into the spinal cord. 87–90 Although endogenous stem/progenitor cells accumulate and proliferate at lesion sites after an injury, the process of differentiation is highly inefficient. Genetic vectors have been explored to increase survival of these cells, alter the local microenvironment to permit differentiation, and trigger intrinsic development programs to direct differentiation down a specific lineage (Fig. 2). 54,59,60,90,91 Gene therapies with intrinsic cell targets, such as transcription factors, may be particularly useful to essentially “reprogram” endogenous adult stem/progenitor cells to regain their ability to develop into new functional spinal cord tissue.

Genetic vectors can be chosen to perturb intracellular signaling pathways at a variety of points in the signaling cascade. Effects of this perturbation can be quite broad, if targeted upstream, or relatively narrow, if directed at more downstream branches. For example, the intracellular signaling molecule cyclic adenosine monophosphate (cAMP) has been widely used as a target for SCI repair. 92,93 Elevated cAMP levels have been associated with increased axon regeneration and decreased myelin inhibition. 94 Researchers have evaluated the manipulation of cAMP pathways at several levels, including extracellular factors, cell surface receptors, cAMP protein itself, and its downstream transcription factor targets. Extrinsic neurotrophins, such as NT-3 and BDNF, have been shown to increase intracellular cAMP, while extrinsic myelin inhibition factors, such as Nogo, downregulate cAMP levels. 94 Alternatively, overexpression of the cell surface receptor for BDNF, trkB, stimulates axon plasticity, while Nogo and LINGO-1 receptors inhibit axon regeneration. 43,94 Finally, transcription factor targets of cAMP, including the cAMP response element binding protein (CREB) 69 and activating transcription factor 3 (ATF3), 95,96 have been upregulated to gain similar pro-regenerative benefits. The ability to apply similar genetic techniques to manipulate biological processes at multiple levels substantially augments researchers’ ability to study and understand the underlying biochemical mechanisms and presents the opportunity to develop therapies that target multiple levels of signaling simultaneously to gain synergistic benefits.

**Types of Vectors**

Genetic vectors can be broadly classified as viral and nonviral carriers. Viruses are at the forefront of gene delivery research, as they have evolved over millions of years to deliver their payload to targeted cells, and therefore can be highly efficient and versatile gene delivery mechanisms. Viral vectors can lead to direct synthesis of pro-regenerative factors for long-term, localized availability in the spinal cord. Furthermore, researchers
have exploited highly evolved mechanisms that viruses have developed to cross the blood–brain barrier and target specific cell types.\textsuperscript{36,97,98} Viral vectors commonly investigated for gene therapies include herpes simplex virus (HSV), retrovirus, lentivirus, adenovirus, and adeno-associated virus (AAV).

In general, most viral vectors exhibit highly efficient gene transduction to cells in the CNS. However, the time delay between vector administration and the onset of transgene expression is dependent on the type of viral vector. Expression of transgenes delivered by HSV or adenovirus appears relatively quickly, within 24 hours after administration.\textsuperscript{39,61} Typically, transgene expression is apparent 48 hours after infection with lentivirus;\textsuperscript{61} however, this time may be closer to 5 days in the spinal cord.\textsuperscript{60,61} AAV requires the longest delay, with 2 weeks separating administration of vectors and the onset of transgene expression.\textsuperscript{39} Retrovirus carries RNA that is reverse-transcribed after infection and the resulting DNA is integrated into the host genome. Classic retroviral vectors can only infect actively dividing cells, which severely limits their utility to transduce neurons and oligodendrocytes in the CNS. As a solution, researchers have engineered self-inactivating lentiviral vectors from classical retrovirus, which are safer and efficiently transduce nondividing cells.\textsuperscript{99,100} AAV and adenovirus are also highly effective transducers of nondividing cells. Researchers have focused on lentivirus and AAV to establish long-term gene expression from nondividing cells in the CNS.\textsuperscript{41,101,102}

Different viral vectors have tropism to different cells. For example, lentivirus selectively transduces astrocytes and immune cells in the injured spinal cord, rather than neurons, while AAV can exhibit a range of cellular tropisms in the CNS, depending on the vector serotype.\textsuperscript{36,61,103–106} Host tropism, vector stability, and host immune response can be altered during a process known as virus pseudotyping. In this process, hybrid viruses are created by combining and packaging envelope vectors from various parent viruses. A great deal of research has been done to optimize spinal cord–targeted transduction efficiency with pseudotypes, mainly with AAV.\textsuperscript{107,108} AAV pseudotype determines the types of cell infected and the extent of vector diffusion from the site of delivery.\textsuperscript{106,107,109,110} AAV9 has been found to be particularly effective at targeting motor neurons in the spinal cord, independent of the delivery method, and thus is promising for treatment of diseases with underlying motor neuron dysfunction, including SMA and ALS.\textsuperscript{97,102,106,107,109,111,112} In addition to AAV, a modified lentiviral vector has been recently reported to enhance tropism for motor neurons.\textsuperscript{113}

With the exception of adenovirus, where the DNA remains episomal, the majority of viral vectors integrate delivered DNA into the host genome. Thus, adenovirus is most suitable when only transient expression is desired. Although integration into the host genome allows for long-term transgene expression, it carries a risk of complications from insertional mutagenesis, such as cancer. Loss of stable, long-term
expression with integrating vectors is directly correlated with immune-induced gene silencing in vivo. HSV, adenovirus, and retrovirus all elicit strong immune responses and a high probability of gene silencing, which have both limited their clinical utility. Alternatively, AAV and lentivirus elicit a minimal immune response, which allows them to avoid gene silencing and establish stable, long-term transgene expression in vivo. Despite the many benefits of viral delivery, there are some key drawbacks. Gene silencing by methylation, attenuation of gene expression by interferon-\(\gamma\) and tumor necrosis factor-\(\alpha\), insertion mutagenesis, and host immune response limit their safety and efficacy. Many researchers have focused on engineering novel viral-based vectors that avoid these issues. For example, one group has used the GlyAla repeat from Epstein–Barr nuclear antigen 1, a sequence known to transfer “immune-stealth” properties in vivo. This modification increased transgene expression in the spinal cord from between 2 and 4 weeks to over 4 months. Even with improvements in viral design, these shortcomings have hindered the clinical utility of viral vectors. For more information on viral vectors in the CNS, see the review previously published by Lentz et al.

To avoid the risks associated with viral vectors, many researchers are developing nonviral vectors for gene delivery. Nonviral techniques to introduce genetic information require protection of DNA or RNA from degradation until delivery and mechanisms for efficient delivery of genes into target cells. Compared to their viral counterparts, nonviral gene delivery methods are significantly less efficient; however, many researchers have focused on improving vector stability and cell internalization of delivered genes. As with viral vectors, designing strategies to avoid detection by the immune system has also been a major goal. Generally, nonviral delivery strategies seek to complex nucleic acids with various polymers, biomolecules, nanoparticles, lipid vesicles, and other materials that serve to protect naked plasmid and carry it across cell membranes so the nucleic acid payload is released in the cytosol (Fig. 3A). One of the first methods developed for nonviral gene delivery employed cationic nanoparticles, most commonly made of calcium phosphate. Despite many improvements to vectors over calcium phosphate nanoparticles, nonviral delivery to the CNS, and in particular the injured spinal cord, has been more difficult than delivery to peripheral tissues due to inefficient crossing of the blood–brain barrier and vector instability when exposed to the highly inflammatory environment present after injury. For comprehensive reviews of nonviral gene delivery in the CNS, refer to reviews by Yao et al. and Pérez-Martínez et al.

Non-integrating vectors may be able to achieve higher levels of expression per transfected cell at early times after vector administration, because multiple plasmid copies encoding the therapeutic transgene can be incorporated into a single cell. However, episomal nucleic acids are eventually degraded (Fig. 3B,C), while integrating vectors, such as AAV or lentivirus, can achieve steady levels of transgene expression over periods of weeks to years (Fig. 4D,E). This type of long-term expression is often desired for treatment of SCI and chronic neurodegeneration, where, although many regenerative factors are naturally overexpressed as an immediate response to insult, their levels are quickly depleted before the slow process of repair can occur. For example, when lentivirus encoding sonic hedgehog (SHH) was delivered to recruit endogenous neural progenitors to the lesion in an acute model of mouse SCI, both controls and experimental groups exhibited high numbers of Sox2+ neural progenitors 1 week after injury (Fig. 2). In contrast, by 8 weeks after injury, the groups with sustained SHH overexpression had significantly more progenitors and more newly myelinated axons near the lesion. On the other hand, indefinite continuation of growth factor expression is probably not desirable. For example, although some neurotrophic factors may induce axonal plasticity and sprouting, eventually these new connections need to stabilize to be functionally mature. In these cases, adenovirus may be more appropriate to achieve transient gene expression; however, its use may require immune suppression.

Ideal vectors would combine the high delivery efficiency of viral vectors, low immunogenicity of AAV and lentivirus, and transient expression provided by non-integrating vectors. For this reason, many researchers have been working to engineer non-integrating versions of lentivirus, which have the delivery efficiency of viral vectors but cause only transient transgene expression.

### Controlling Transgene Expression Through Vector Design

Promoters and enhancers can be manipulated in a number of ways to gain better control over transgene expression in vivo. The choice of the promoter greatly affects the levels and duration of expression and provides control to restrict expression to specific cell types. Absolute levels of transgene expression can be controlled by the incorporation of promoters whose basal levels of expression vary. The constitutive promoter cytomegalovirus (CMV), which has been widely used for spinal cord applications, is selectively silenced in neurons but not glia, and thus has potential as a glial-targeted promoter in spinal cord injuries. Dual promoter plasmids could be used to restrict transgene expression to cells that require coexpression of multiple protein markers for identification, including microglia and many other immune cells. Dual promoters have been used to simultaneously monitor differentiation- and knockdown-specific genes in mesenchymal stem cells. Similarly, they would be a powerful tool to study cell changes during spinal cord degeneration and regeneration.

Promoters can also impart temporal control of transgene expression through the use of inducible promoters, such as TET (tetracycline)-inducible systems, where doxycycline administration can be used to induce or prevent transgene expression.
The TET-inducible system was used to induce overexpression of the cell adhesion molecule L1 by transduced human neural stem cells transplanted into mice after SCI. Activation of L1 expression caused the animals to gain motor function. Alternatively, conditionally expressed promoters can be used to activate genes only under the appropriate circumstances, for example, delivering genes selectively to cells in a hypoxic state or within an inducible Cre-recombinase knock-out or knock-in animal. Other inducible expression systems, such as the cumate switch, are also actively being explored for the CNS gene therapies.

For a comprehensive review of inducible systems for CNS gene therapies, refer to Naidoo et al.

Technologies for Delivery of Gene Therapies to the Spinal Cord

There are many different ways to deliver therapeutic genes to the injured spinal cord. Cells can be transduced in vitro prior to implantation near the injury, where the transplanted cells then secrete proteins encoded by the delivered transgenes to aid regeneration. Naked or nano-complexed plasmids can be administered via injection either systemically or directly to the spinal cord. Alternatively, viral vectors can be effectively delivered to the spinal cord via retrograde transduction from the peripheral tissue sites. Finally, vectors can be delivered from biomaterial carriers to contribute other benefits such as increased vector survival or increased local retention.

Direct injection, systemic delivery, and retrograde transduction. Direct injection of vectors into the spinal cord is a relatively simple, yet invasive, approach to achieve localized transgene expression. While administration via peripheral arteries is no doubt less invasive, the impermeable blood–brain barrier prevents most systemically delivered therapies from reaching CNS targets and often leads to transgene expression in off-target, immune-associated organs such as the spleen. Notably, some AAV pseudotypes have been shown to effectively cross the blood–brain barrier and transduce cells in the spinal cord when injected systemically. Many viral vectors, including HSV, AAV, lentivirus, and adenovirus, can be delivered into the CNS via retrograde transport from axons at peripheral neuromuscular junctions. Although this process is more complicated and invasive than intravenous administration, it is still a safer and more efficient alternative than direct injection into the spinal cord or brain. Furthermore, retrograde transduction is a viable strategy to enhance transgene expression by motor neurons and holds particular promise for treatment of disorders involving motor neuron degeneration, including ALS, SCI, and SMA. Effective transgene expression has also been demonstrated by anterograde transduction after intracerebral injection. Since region-specific injections into the brain are more difficult and invasive than into the spinal cord, their clinical utility is limited to applications where highly localized targeting in the brain is desired.
such as in Parkinson's disease. However, viral antegrade tract tracing is a valuable tool for analysis of axon regeneration in SCI research studies.\textsuperscript{148,149}

To address this issue with other vector types and further improve viral-mediated gene delivery, several groups have explored novel strategies to increase the probability that systemically administered gene therapies effectively cross the blood–brain barrier. One group formed nanoparticles from poly-L-lysine dendrigrafts and then linked the nanoparticles to plasmid encoding an anti-apoptotic transgene.\textsuperscript{150} When administered intravenously, particle accumulation and transfection were increased in the brain. Furthermore, transgene expression induced neuroprotection after ischemic reperfusion injury, leading to decreased apoptosis and reduced infarct area. Another technique utilizes mannitol, a hyperosmotic sugar alcohol known to make the blood–brain barrier permeable, to carry vectors into the CNS.\textsuperscript{102,151,152} In a separate study, proteins derived from the rabies virus and known to target cells of the CNS were conjugated onto a copolymer of polyethyleneimine (PEI) and mannitol and used to selectively deliver genes across the blood–brain barrier from peripheral tissues.\textsuperscript{153}

Transplantation of genetically engineered cells. A simple method to deliver therapeutic factors involves \textit{in vitro} transduction of various cell types, including progenitors, prior to implantation into the spinal cord.\textsuperscript{7,48,54–57,67,127,154–156} In theory, autologous cells could be removed from a host, transduced \textit{ex vivo}, and reintroduced to prevent immune rejection. Furthermore, transplantation of transduced cells avoids potential complications from direct infection of host cells \textit{in vivo}. Transplantation of rat fibroblasts genetically modified to secrete NGF into rats with SCI was reported to increase axonal outgrowth.\textsuperscript{4,37} Notably, this study demonstrated regeneration in the chronic stage of SCI, which is more challenging than in the acute phase. For detailed reviews of strategies for transplantation of genetically engineered cells in SCI models, see Blesch et al.\textsuperscript{4} and Tang et al.\textsuperscript{156} This strategy is most suitable to deliver transgenes for secreted factors, which can diffuse into the surrounding tissue and affect host cells. However, a major drawback is the limited cell survival during the implantation or injection procedures as well as in the inflammatory environment once implanted into the lesion.\textsuperscript{91,157}

Physical delivery methods. Several physical methods have been demonstrated to enhance DNA delivery, including pressure waves, electrical gradients, magnetic fields, and ultrasound. Although these techniques increase delivery efficiency, they are often associated with increased collateral damage to the surrounding tissue.\textsuperscript{158} However, new technological advancements are continually lowering this risk. Microbubbles generated from ultrasound transducers have been used to increase the transfection efficiency of plasmid DNA, without affecting plasmid quality or otherwise harming laboratory animals.\textsuperscript{159} Photomechanical waves, also known as laser-induced stress waves, can increase the permeability of cell membranes \textit{in vivo} and have been used to deliver RNAi vectors into rat spinal cords after injury.\textsuperscript{160} Electroporation, a technique that generates an electric field to drive diffusion of nucleic acids across the cell membrane, can increase transfection efficiency 100–1000-fold compared to delivery of naked plasmids and has been demonstrated to successfully deliver transgene to mouse spinal cords.\textsuperscript{160,161} A similar technique, magnetofection, uses oscillating magnetic fields to deliver genes into cells of interest using magnetic nanoparticles linked to the plasmids.\textsuperscript{159,162–164} Magnetofection has the additional benefit of localized or directed anatomical delivery of plasmids \textit{in vivo}.\textsuperscript{164} Furthermore, transplanted cells can be tracked via internalized magnetic particles, which remain after transfection in vitro.\textsuperscript{125,162,164,165} Magnetofection has also been used in conjunction with PEI complexes containing luciferase for delivery to rat spinal cords.\textsuperscript{163} In this study, transmembrane passage and nuclear localization were further enhanced by complexation of magnetic particles and plasmids with the TAT peptide, a cationic sequence derived from HIV-1 TAT protein that mediates viral entrance into the cell.

Polyplexes and nanocarriers for nonviral delivery. Polyplexes and nanocarriers have been used extensively to enhance the stability and transfection efficiency of nonviral vectors. Polyplexes consist of cationic polymers electrostatically complexed with naked plasmids. Complexation protects plasmids from nucleases and other destructive elements present \textit{in vivo}. In addition, the electrostatic interaction between the cationic polymer and the anionic cell membrane enhances cell uptake. The major advantage of polyplexes over viral vectors is the former’s ability to select cationic polymers that do not elicit an immune response. Many materials can be used to make polyplexes, but PEI carriers are the most widely used carriers due to their efficient vector delivery and buffering environment.\textsuperscript{124,126} Other base materials used to create nanoparticle polyplexes for nucleic acid delivery to the CNS have include inorganic nanoparticles (eg, silica), natural polymers (eg, chitosan), PEI, liposomes, dendrimers, and carbon-based nanoparticles. These materials are reviewed in detail by Pérez-Martínez et al.\textsuperscript{125} and Yin et al.\textsuperscript{126} One group reported the use of polyplexes formed by a knotted single-chain polymer of cyclized 2-(dimethylamino) ethyl methacrylate.\textsuperscript{166} Delivering plasmids encoding for NT-3 overexpression from multichannel collagen scaffolds, these polyplexes were able to increase axon regeneration when compared to polyplex-delivered luciferase. A similar starburst cationic polymer, L-arginine-grafted polyamidoamine (PAM-RG4), has been used to successfully deliver vectors encoding genes that induce tumor cell apoptosis to spinal cord tumor.\textsuperscript{167}

Polyplexes and most nanocarriers are highly tunable and can be altered chemically to incorporate targeting peptides, stabilizing copolymers, therapeutic drugs, and other desired features. Designer polyplexes have been used to increase stability, increasing cell binding and internalization, or directing gene delivery to a specific cell type. For example, targeted delivery
to the CNS has been facilitated by conjugating a 12-mer peptide, Tet1 from tetanus toxin, onto PEI stabilized with polyethylene glycol (PEG). Tet1 added a mechanism for targeted transfection of neurons. Recently, Dengler et al. demonstrated the use of charged amorphous silica nanoparticles, called "protocells", as carriers for DNA delivery. These particles are highly porous and can be coated with liposomes that deliver packets of therapeutic payload. There are many advantages to protocell delivery, including high surface area, low toxicity, and a highly modifiable surface. When directly applied to rat spinal cord, protocells effectively delivered transgene encoding for overexpression of IL-10, which successfully suppressed inflammation and reduced neuropathic pain after SCI. Alternatively, novel protein nanovectors have been developed and optimized for delivery to injured brain tissue. Although not yet investigated in the spinal cord, protein-based carriers are attractive because they can be manipulated to incorporate biomolecules that target delivered genes to specific cell types or provide addition cues to promote tissue regeneration.

**Gene delivery from biocompatible materials.** While direct or systemic injection effectually delivers nucleic acids to the spinal cord, biomaterials can achieve improved efficacy and localized expression of therapeutic transgenes. Vector stability can be increased by immobilization of vectors via physical entrapment within biomaterial pores and/or incorporating sites that noncovalently interact with the vectors. Vector immobilization also acts to spatially confine transduction to cells that physically interact with vectors on the biomaterial carrier. Biomaterial scaffolds can be modified to control the numbers and types of cells with which they interact, which ultimately determines the levels of transgene expression (for details, see reviews by Shea et al., Hydrogel scaffolds. Macroporous, biodegradable poly(lactide-co-glycolide) (PLG) scaffolds presenting a longitudinal, multichannel architecture have been extensively investigated for their ability to mediate highly localized transgene expression after SCI (Fig. 4A-C). Although PLG bridge scaffolds have been shown to effectively deliver both viral and nonviral vectors, viral vectors resulted in sustained levels of transgene expression over at least 2 months and positively affected tissue regeneration in a mouse model of SCI (Fig. 4D, E). The number of active lentiviral particles immobilized onto scaffolds prior to implantation into the injured spinal cord increased when the scaffolds were modified with phosphatidylserine, hydroxyapatite, or successive layers of chitosan and heparin. Of these, the chitosan/heparin modification resulted in the greatest benefit to transgene expression levels and scaffold integration with the host spinal cord tissue.

Spatial patterns of transgene expression have been employed to provide directional orientation to cellular processes or to create a tissue interface. The spatial patterning of gene therapy vectors on biomaterial scaffolds has been achieved using antibodies, biotin–avidin binding, and nonspecific interactions, to spatially control gene delivery both in vitro and in vivo. The patterned expression of soluble factors results in localized gradients surrounding transgene-expressing cells, which may mimic those observed during developmental and repair processes. Spatial patterning of vectors onto multichannel PLG bridges improved directed growth of axons along vector-patterned channels for distances up to 1.5 mm beyond the pattern. Despite promising results, a key problem with prefabricated bridges for spinal cord regeneration is ensuring an appropriate fit within the lesion cavity, without damaging or removing surrounding spared tissue. In neurodegenerative diseases and in most cases of SCI, which involve contusions, removal of additional tissue would be devastating to patients and is not an option. One potential alternative is to form bridges in situ using injectable materials designed to conform to the contour of the native tissue. Due the availability of aqueous, biocompatible precursors and cross-linking chemistries, hydrogels make excellent injectable materials for CNS applications, as recently reviewed by Pakulcka et al. Hydrogel vehicles for gene delivery have also been reviewed recently. In addition to gene delivery systems, hydrogels can be designed to present a local microenvironment that mimics healthy spinal cord to provide resident cells with cues required to reestablish proper tissue function.
Hydrogels fabricated from pluronic F127, a synthetic, thermoresponsive triblock copolymer that transitions from a liquid at 4 °C to a cross-linked hydrogel around body temperature, have been used to effectively deliver lentiviral vectors to rat brain. Lentivirus was suspended in a liquid pluronic gel and the mixture delivered via intracranial injection. More recently, injectable pluronic gels were used to deliver lentiviral vectors encoding RNAi for Lingo-1 into injured rat spinal cords. Results showed that the combination of biomaterial and lentivirus reduced the viral concentration required for delivery (compared to delivery of lentivirus alone). Furthermore, lentiviral-mediated knockdown of Lingo-1 improved neurite outgrowth, synapse formation, and functional recovery. Injured animals treated with agarose scaffolds, researchers injected lentivirus encoding NT-3 into spinal cords rostral to the implant, creating a neurotrophic factor gradient. Via chemotaxis, NT-3 gradients significantly increased the number of axons exiting scaffolds. Gene-activated matrices (GAMs), a mixture of biodegradable matrix and plasmid vectors, have been used to support robust cell infiltration and subsequent transfection by nonviral vectors. Although most GAMs reported are composed of PLG copolymers, those investigated for CNS applications are typically made of poly-D-lysine–plasmid polyplexes mixed with a collagen hydrogel-like paste. In an optic nerve injury model, GAMs improved local vector retention, increased neuronal survival, and reduced scar formation, local and systemic toxicity, and secondary injury.

Additional control over vector availability from hydrogels has been demonstrated in vitro by the incorporation of cationic moieties to increase vector complexation or cell-degradable sequences that allow infiltrating cells better access to vectors in hydrogels. Modification of PLG bridges with chitosan and heparin significantly increased the absolute levels of luciferase expression, compared to unmodified bridges or those modified with chitosan only, during the entire 59-day study. In an optic nerve injury model, GAMs improved local vector retention, increased neuronal survival, and reduced scar formation, local and systemic toxicity, and secondary injury. Additional control over vector availability from hydrogels has been demonstrated in vitro by the incorporation of cationic moieties to increase vector complexation or cell-degradable sequences that allow infiltrating cells better access to vectors in hydrogels. Modification of PLG bridges with chitosan and heparin significantly increased the absolute levels of luciferase expression, compared to unmodified bridges or those modified with chitosan only, during the entire 59-day study. In an optic nerve injury model, GAMs improved local vector retention, increased neuronal survival, and reduced scar formation, local and systemic toxicity, and secondary injury.
as electrostatic stabilization of genetic vectors. For example, fibronectin has been shown to increase the transduction efficiency of retroviral vectors in vitro\textsuperscript{21,194} and of plasmid polyplexes coated onto PLG bridges when implanted after SCI\textsuperscript{177}. Although not widely investigated as a gene carrier in SCI models, fibrin materials have also been shown to significantly improve transgene expression in vitro and in vivo\textsuperscript{195–197}.

As fibrin hydrogels have been reported to improve functional recovery when injected into the injured spinal cord\textsuperscript{198,199}, the addition of gene therapies to fibrin hydrogels is likely to contribute additional benefits to repair after SCI.

**Remaining Challenges, Emerging Opportunities, and Clinical Potential**

The number of genetic therapies entering clinical trials for CNS disorders has been on the rise over the past decade. The majority of these treatments deliver viral vectors carrying therapeutic genes either peripherally or directly into CNS by simple injection. Using this strategy, Phase I/II clinical trials are currently under way to test the safety and efficacy of genetic therapies for the treatment of SMA\textsuperscript{111,112}, Sanfilippo syndrome\textsuperscript{200}, and neuropathic pain\textsuperscript{201}. Although these strategies have shown modest success and demonstrate the immense promise of gene therapies to the spinal cord, full realization of the potential of genetic therapies will require the development of more efficient cell- and tissue-specific delivery systems with improved temporal and spatial control.

In particular, genetic therapies aimed at directing differentiation of endogenous or transplanted stem/progenitor cells, which have the potential to replace damaged neurons or oligodendrocytes, will require relatively tight temporal controls to create growth factor profiles that mimic those during progressive stages of fetal development. In addition, achievement of robust expression of multiple therapeutic genes with separate temporal controls will be necessary to adequately recapitulate development and fully differentiate new specialized CNS cells in vivo\textsuperscript{202}. Tandem delivery of multiple genes will also be required to achieve functional tissue regeneration after SCI, where each gene is chosen to address a specific barrier to regeneration, such as glial scar formation, demyelination, or hyperinflammation. Ideally, researchers aim to develop truly biomimetic strategies where local environmental cues trigger the expression of specific transgenes by specialized cell types and where this response is timed with events on the levels of tissue (inflammation, regeneration, etc.) and cells (differentiation, migration, etc.).

**Improving transgene expression levels: Relevance for concurrent delivery of multiple vectors.** A major challenge to combinatorial delivery of multiple genes to the spinal cord is the inability to achieve robust, bioactive levels of transgene expression. Although many studies have demonstrated therapeutic overexpression of a single transgene, simultaneous expression of two or more transgenes at therapeutic levels has proved difficult. Recent studies have demonstrated that the phenotypic response to gene delivery depends on the number of cells that express the transgene, their level of expression, and the duration of expression\textsuperscript{175,195}. After delivery, the majority of virus or complexed plasmid is inactivated and cleared by a maximum of 72 hours\textsuperscript{98}. This relatively short period of activity dictates that those cells that are able to physically encounter the vectors during this time will become transfected/transduced\textsuperscript{62,195,203}. Therapies capable of fully restoring functional tissue after SCI will likely require combinations of factors to be delivered, where each factor addresses a specific barrier to regeneration. However, practical realization of a treatment that delivers genetic vectors encoding multiple transgenes has been challenging, in large part because too few cells encounter and take up transgenes before the vectors are deactivated.

**Vector and plasmid design.** Substantial improvements in delivery efficacy have been made through the initial design of both encoding plasmids and delivery vectors. Gene therapy vectors have been improved by the addition of multiple functional groups to enhance transport to the target tissue, evade the host immune system, promote binding to the target cells, and facilitate intracellular trafficking\textsuperscript{100,133,169,170,204,205}. Another potential way to increase expression of multiple transgenes is the use of bicistronic plasmids, where two or more factors are encoded in the same vector under the control of the same promoter region\textsuperscript{134,206}. Theoretically, the number of cells expressing two transgenes from a bicistronic plasmid would be equivalent to numbers of cells that would take up vectors encoding a single transgene using comparable delivery methods. This technique has been used to induce simultaneous overexpression of NT-3 and BDNF to prevent apoptosis of rat spinal cord neurons\textsuperscript{50}. Bicistronic plasmids can also include a reporter gene, such as firefly luciferase, linked to expression of a therapeutic gene, which is incredibly useful for studying the underlying biology of neurodegeneration as well as development and characterization of new gene therapies. However, much work remains to be done to create systems for the simultaneous control over the timing of expression of multiple vectors.

**Biomaterial-based regulation of transgene expression.** The number of cells that take up vectors can be increased at localized sites in the spinal cord when vectors are delivered from biomaterial scaffolds designed to promote cell infiltration and migration\textsuperscript{62,115,173–175}. This is accomplished by modifying biomaterials with cell-adhesive molecules, interconnected macroporosity, and/or cell-degradable motifs to control the number and type of host cells that infiltrate the implant and take up therapeutic vectors in the process. Material design can also be used to retain infiltrating transduced cells, resulting in localized transgene expression. On the flip side, biomaterial characteristics can be altered to increase retention and preserve bioactivity of genetic vectors\textsuperscript{178,180,190,192,197}. For example, additional modification of scaffolds with moieties known to stabilize overexpressed therapeutic proteins, such as heparin which sequesters a number of growth factors, increases overall efficacy\textsuperscript{60,63}. Using scaffolds incorporating macroporosity optimized for maximum cell infiltration and immobilized...
heparin, one study demonstrated simultaneous expression of two transgenes in the spinal cord for at least 8 weeks (Fig. 4D, E). This multifaceted approach modulates cell infiltration into and host tissue integration with the scaffold while simultaneously increasing vector bioavailability and delivery efficiency. Furthermore, biomaterial-based systems that achieve efficient localized gene transfer are being developed to simultaneously present controlled microenvironments that promote wound healing or tissue regeneration.

Spatial and temporal control of transgene expression. Despite significant improvements to delivery methods and vectors that have increased transgene expression, cell-specific and temporal control of transgene expression at therapeutic levels still poses a significant challenge. Both these controls will be necessary to effectively reprogram endogenous or transplanted cells to replace lost neurons and oligodendrocytes, whose death causes many of the symptoms in CNS injury and neurodegenerative diseases. Although several vectors have been shown to restrict transgene expression to specific cell types, including neural progenitor/stem cells, the numbers of these cells that both receive therapeutic genes and express the transgene in vivo are often too low to achieve bioactive concentrations of encoded proteins in the spinal cord. It should be noted that reporter transgenes, such as firefly luciferase and fluorescent proteins, often can be detected at lower levels of expression than those required for bioactivity of many therapeutic proteins.

Spatially defined concentration gradients. After SCI, gradients of various neurotrophic factors significantly increase the distance traveled by regenerating axons. However, defined spatial gradients of transgenes have been challenging to create in vivo. Although researchers have reported increases in axonal outgrowth when gradients were created by direct injection of viral vectors adjacent to SCI lesions, this strategy yields highly undefined gradients. Thus, there is a need for new gene delivery technologies that can facilitate formation of controllable gradients of encoded transgene. One potential method is to pattern vectors onto biomaterials, which confines transgene expression to cells that physically encounter patterned vectors.

Inducible promoters. Another promising strategy for spatial and temporal control is the use of vectors that include inducible promoters. It has been recently demonstrated that therapeutic levels of NT-3 can be achieved after injection of lentiviral vectors encoding TET-inducible promoters into rodent spinal cords after injury. Despite promising results when cells transduced ex vivo with TET-inducible vectors were transplanted, it has been more challenging to achieve therapeutic levels of transgene expression with the TET promoter through direct transduction of host spinal cord cells in vivo. Recently, a new technology for spatial and temporal control of transgene expression using heat-inducible promoters was reported. In this strategy, plasmids containing therapeutic genes controlled by a heat-inducible promoter were conjugated to magnetic particles prior to delivery so that particles were confined to transfected cells. When a magnetic field was applied, the heat generated by magnetic particles within the field induced localized transgene expression. In theory, by combining inducible and cell-specific promoters, therapies could be designed to selectivity turn genes on or off in a single cell type at clinician-specified times and locations.

Integrating and nonintegrating vectors. Timing and duration of expression is particularly critical for in situ reprogramming of endogenous or transplanted stem cells and host inflammatory cells. For example, growth factors such as SHH and platelet-derived growth factor-AA (PDGF-AA) increase the numbers of endogenous progenitor cells available at the injury site and promote differentiation into oligodendrocyte progenitors, but both can also inhibit full maturation. For scientists to recreate developmental processes, temporally controlled delivery of multiple transgene vectors and/or soluble factors from scaffolds over the period of weeks or months will likely be required. For example, as viral vectors result in long-term expression at lower levels than nonviral vectors, simultaneous delivery of multiple transgenes—one via an integrating vector and another via a nonintegrating vector—could be used to gain temporal control. In acute SCI, one may want to deliver a nonintegrating vector with a short lag between administration and transgene expression chosen to downregulate the secondary injury cascade in tandem with an integrating vector with a longer time delay to transgene expression encoding for a factor to increase axon plasticity. It should be noted that, although expression of some transgenes may not be desired until the chronic phase of injury, it may still be necessary to deliver vectors during the acute phase before the glial scar forms and poses a barrier for vectors to access cells within lesions. In this case, vectors such as AAV, for which there is a significant time delay between administration and transgene expression, may be ideal. Few studies have investigated the efficacy of gene delivery in chronic stages of SCI and subsequent effects of transgene expression on regeneration. However, such studies will become necessary to develop therapies applicable to patients currently living with SCI. The majority of studies in chronic injury models to date have explored direct injection of viral vectors into the spinal cord or transplantation of ex vivo transduced cells. Although these studies have reported modest benefits when delivered vectors encoded for neurotrophic factors, strategies for repair in the chronic phase after SCI pose definite challenges. For example, it may be necessary to provide combinatorial treatments that aim to degrade the well-established glial scar and provide neurotrophic factors.

Conclusions
A myriad of gene candidates could be manipulated with spatial and temporal controls to create an environment that induces spinal cord regeneration. While progress has been made to enhance the efficiency and precision of gene delivery
systems, technological advancements depend on improved understanding of the underlying biology. Advantageously, genetic engineering provides a means for better understanding of the role of specific genes in degeneration and repair while exploring the utility of perturbing the same genes to enhance tissue repair. Furthermore, advanced tissue regeneration strategies will likely require combining gene delivery with other therapeutic strategies, including delivery of pharmaceuticals, proteins, cells, and biomaterial scaffolds. In particular, delivery of genetic vectors from biomaterial scaffolds provides a unique opportunity to capitalize on the potential synergy between the biomaterial design and gene delivery. In theory, control over cell adhesion, cell migration, gene expression, and availability of delivered soluble factors can be combined in a single scaffold that mimics the complex microenvironment present during in vivo tissue development and promotes spinal cord regeneration in adults.

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