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# Review of the application of CRISPR/Cas9 in axon regeneration

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**Abstract.** Neurological mechanistic or pathologic damage is often accompanied by neuronal loss. Although this loss is universally considered as irreversible, nervous system possessing a series of compensatory self-repair mechanisms after injury, one is axon regeneration. However, they are restricted by alterations in the microenvironment and multiple suppressors. Therefore, enhancing axonal regenerative capacity to improve plasticity after central nervous system injury has become a critical issue. Previous studies have shown high feasibility and application value of CRISPR/Cas9 in promoting neuronal axon regeneration. This article aims at evaluating the applicability and effectiveness of CRISPR/Cas9 and its derivative method, CRISPR activation system, when it comes to treating central nervous system injuries. Three main routes were reviewed here, including large-scale screening of related genes in vivo, promotion of endogenous neurotrophic factor expression and induction of reprogramming glial cells into neurons. Besides, considering the delay in protein knockdown with the Cas9 system and other shortcomings, its potential application such as improving the local microenvironment post-injury should not be neglected.

**Keywords:** CRISPR/Cas9, Neural Injury, Axon Regeneration.

## 1. Introduction

Neural injury induces a wide range of physiological changes. Though slightly differ between the central nervous system (CNS) and the peripheral nervous system (PNS), these changes include Wallerian degeneration of damaged cell bodies, immune cell-mediated inflammatory responses, and the formation of glial scars predominantly led by astrocytes. While permanent neuronal loss happens in most cases, nervous system possesses various post-injury repair mechanisms which can be seen as a compensatory mechanism, including axon regeneration. However, they are restricted by alterations in the microenvironment post-injury and multiple inhibitory factors. For instance, while isolating the injury site helps to prevent surrounding tissues from being affected, suppressors such as chondroitin sulfate proteoglycans (CSPGs) secreted by astrocytes and Nogo-A (also known as Reticulon 4) by oligodendrocytes significantly impede axonal elongation and collateral sprouting of nearby neurons. Efforts have been made to promote axonal regenerative capacity; however, most were found little or no effect in practical use. Thus, improving plasticity after CNS injury has become a critical issue.

CRISPR, also known as Clustered Regularly Inter-Spaced Palindromic Repeats, is a well-known method for editing genes that is based on bacteria's adaptive immune system. Consisting of CRISPR and CRISPR-associated (Cas) nucleases, it is well established for engineering application. Cas9 are

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directed to target DNA through designed spacer sequences of sgRNA, in order to cleave or edit specific DNA loci, thereby selectively up-regulating or down-regulating gene expression. Previous researches have shown high feasibility and application value of this technology in promoting neuronal axon regeneration. This article focuses on various application routes of CRISPR/Cas9 and its derived technologies in neural injury repair and provides prospects for future research.

## 2. Application of CRISPR/Cas9 in Promoting Axon Regeneration:

### *2.1. Large-scale screening of related genes in vivo*

Optic Nerve Crush (ONC) typically occurs after retinal ganglion cells (RGC) sustain mechanical injury. Jane A. Lindborg et al. used shRNA to inhibit and screen the expression of 392 related genes, identifying 40 genes that enhanced RGC survival and axon regeneration. For validation, these genes were subsequently assessed by independent CRISPR/Cas9 gene knockout experiments. Only 28 genes were confirmed with the limitaion of axon regeneration, showing a lack of precise matching between two methods [1]. Feng Tian et al. utilized a CRISPR/Cas9-based genome-wide screening to examine 1893 transcription factors (TFs) in vivo, and surprisingly, identified only 10 negative regulators affecting RGC survival and axon regeneration after ONC which are mainly implicated in epigenetic regulation and members of the BHLH family. Their results also indicate an independent regulating circuit for neuronal survival and axon regeneration, as genes involved in two processes have almost no overlap [2].

Besides, for spinal cord injuries (SCI) , another classic mechanical injury happened in central nervous system, as neurogenesis in the adult spinal cord is impossible to occur spontaneously, methods are necessary to solve this limitation. In mammals, prolonged inflammation after SCI is considered to be a serious factors hindering recovery. In zebrafish, the downregulation of pro-inflammatory cytokines quickly happened, resulting in the regeneration of axonal connections across the injury site. The regulation process is controlled by decreasing the quantity of anti-regenerative neutrophils and the release of pro-inflammatory proteins induced by injury mediated by blood-derived macrophages. Marcus Keatinge et al. conducted a rapid synthetic RNA Oligo CRISPR guide RNAs (sCrRNA) design and pre-selection process for in vivo phenotypic screening. 4 generated mutants (tgfb1a, tgfb3, tnfa, sparc) were observed to have an inflammation phenotype post-injury [3].

Additionally, CRISPR/Cas9 was also combined with High-Content Screening (HCS) technology for gene function studies. HSC, with its capacity to process and analyze the signal intensity of multiple specific proteins in hundreds of cells in batch, is extensively employed due to its integration of automated analysis and diverse image analysis techniques. This is particularly useful for assessing cellular proliferation, including the regenerative growth of axons in neuronal cells. Unlike using RNA interference (RNAi), CRISPR/Cas9 achieves permanent genomic alterations, making it suitable for long-term studies of specific gene functions and enabling simultaneous targeting of multiple genes for multiplex knockouts or modifications.

Ben L. Callif et al. evaluated the applicability of CRISPR-based genome editing for primary neuron phenotype screening. Using multiwell transfection of CRISPR and sgRNA to knock out *NeuN* and *Pten* in neurons, axon outgrowth can be easily detected by automated screening platform [4].

### *2.2. Promotion of endogenous neurotrophic factor expression*

As a derivative technology of conventional CRISPR-Cas9, CRISPR activation (CRISPRa) is designed to upregulate the expression level of specific genes without changing its DNA sequence. CRISPRa employs a modified, catalytically inactive Cas9 (dCas9) fused with transcriptional activators. This dCas9-activator complex is guided to a target gene's promoter region by a custom-designed guide RNA (gRNA). Once bound, the complex enhances transcription, effectively increasing the gene's expression levels. Therefore, it can be used to precisely target silenced chromatin loci to promote the transcription of downstream neurotrophic factors [5].

Katherine E. Savell et al. utilized CRISPRa to target a single transcriptional promoter within the Brain-Derived Neurotrophic Factor (BDNF), achieving highly specific up-regulation of *Bdnf* variants

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transcription to study the gene's function in neurons. BDNF is one of the most important neurotrophins. It has a complex regulation role in diverse processes, including neuronal differentiation and synaptic development. Its structure comprises of nine non-coding exons (I-IXa) at the 5' end while one coding exon (IX) at the 3' end (IX). An upstream promoter region is present in every non-coding exon to initiate the transcription of different *Bdnf* variant. The production of various transcripts occurs after transcription when one or more specific non-coding 5' exon combines with the 3' coding exon. Their study demonstrated that CRISPRa could be used for high-precision targeting one variant of *Bdnf* and promoting its expression and examining downsteam gene expression profiles in the central nervous system [6].

Additionally, for ocular diseases such as glaucoma, ciliary neurotrophic factor (CNTF) is a promising therapeutic option because of its function in promoting axon outgrowth. However, while the expression of CNTF in RGCs delivered by adeno-associated virus leads to strong regeneration, recombinant CNTF (rCNTF) was found little or no effect. By using CRISPR to deplete the cognate receptor for C-C motif chemokine ligand 5 (CCL5) in RGCs, Lili Xie et al. found that CCL5 elevation, which leads to activation of immune system, is necessary for the effectiveness of CNTF gene therapy [7].

### *2.3. Induction of reprogramming glial cells into neurons*

It's universally admitted that, the adult CNS has almost no regenerative capacity, therefore unable to compromise lost neurons and induce functional recovery. A natural approach is to transform the glial cells nearby into specific neurons to reestablish synaptic connections and replace the function of lost neurons. Several studies have shown that endogenous overexpression of specific TFs is able to directly convert astrocytes into functional neurons.

For example, Haibo Zhou et al. successfully converted Müller cells into RGCs via CasRx-mediated *Ptbp1* gene knockout. As a result of transformation, partially restored visual responses and a vision-dependent behavior were observed in the central visual pathway suggesting alleviated disease symptoms associated with RGC [8]. Their results show the capability of a successfully designed CRISPR system to convert glial cells into various types of neurons in brain regions that experience neuronal loss, validating the plausibility of replenishing the required cell types with cell-transplantation based approaches.

For spinal cord injuries, Meiling Zhou et al. utilized the CRISPRa system to activate *Ngn2* and *Isl1* endogenously, thus reprogramming mouse spinal cord astrocytes and mouse embryonic fibroblasts (MEFs) into motor neurons (MN) [5], *Ngn2* and *Isl1* are one of examined combinations in their study with different functions. The expression of Ngn2 in neurons is determined by its interaction with other factors, whereas *Isl1* are responsible for MN specification [9, 10]. Over 70% of spinal cord astrocytes were able to be reprogrammed into iMNs, whereas those from MEFs was about 64%, relatively higher than a previous study which were trying to create MNs by promoting the expression of seven transcription factors (*Ascl1*, *Brn2*, *Myt1l*, *Lhx3*, *Hb9*, *Isl1*, and *Ngn2*) in fibroblasts [11].

However, as operational process is fraught with significant risks and uncertainties, and the guidance signals for inducing glial cell conversion into specific neurons were not yet fully explored, issues such as low in vivo conversion efficiency and insufficient purity remain, posing significant barriers to clinical application.

## 3. Discussion

Irreversible neuronal loss is a characteristic of neurological diseases such as glaucoma and neurodegenerative diseases like Alzheimer's disease and Parkinson's disease. Many of their regulatory mechanisms remain unclear. The advent of CRISPR technology offers additional options for traditional signaling pathway research and disease treatment.

However, the application of CRRISPR/Cas9 also presents several notable issues: Firstly, experiments by Ben L. Callif et al. on primary cortical neurons indicate that, compared to shRNA, there is a delay in protein knockdown with the Cas9 system. This cannot be resolved by using sgRNAs that target multiple exonic regions of a single gene [4]. Combined use of CRRISPR/Cas9 and high-throughput/high-content

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screening implies a lag for meaningful decrease of target protein expression levels and observable phenotypic results. Secondly, during practical application, there are additional challenges and risks due to limitations of specific delivery methods, such as the inability to transfect target cells because of the tropism of viral vectors, and the potential for insertional mutations [12]. Moreover, for protocols involving the endogenous induction of cell reprogramming, sustained high expression following successful conversion may disrupt the local microenvironment and interfere with normal neural circuit. Given that, building a controllable regulation system and turning off after axon regeneration may be a plausible solution [13].

Nevertheless, it is worth noting that the CRISPR system has additional advantages in protocols involving the overexpression of neurotrophic factors to trigger downstream biological functions. Neurotrophic factors such as BDNF, LIF, and IGF-1 often act through different but partially overlapping regulatory networks. Given the differential expression of single receptors in various neuronal cells [14], overexpression of a single neurotrophic factor may be inefficient. The CRISPR/Cas9 system can easily increase the number of genes to be activated by simply adding additional sgRNA cassettes (approximately 156 bp of cDNA per sgRNA), significantly enhancing regulatory efficiency. In view of the above reasons, this system also holds potential for improving the local microenvironment and symptoms associated with neuropathy after injuries.

## 4. Conclusion

Overall, this article summarized three main application of CRISPR/Cas9 and CRISPR activation system in order to restore lost neuronal functions and promote recovery in neural injuries. The utilization of CRISPR/Cas9 for massive scale gene screening has identified key regulators that influence neuronal survival and axon regeneration, offering potential targets for therapeutic intervention. Its derivatives, CRISPRa system has demonstrated its capability in promoting the expression of neurotrophic factors, thereby enhancing neuronal growth and survival. It is considered to be particularly efficient and convenient when it is used to endogenous up-regulating the expression of multiple genes, as only additional sgRNA cassettes needs to be added to target the DNA sequence. Moreover, innovative approaches have been made to reprogram glial cells into functional neurons by using CRISPRa, which presents a revolutionary strategy for addressing the challenge of neuronal loss in the central nervous system.

It's not hard to see that the ability of Cas nucleases to precisely edit genes has provided a robust platform for investigating the mechanisms of axon regeneration, neurotrophic factor expression, and glial cell reprogramming. The exploration of CRISPR/Cas9 technology and its derivatives in neural injury repair undoubtedly holds significant promise for advancing understanding and treatment of nervous system injuries.

However, while significant progress has been made, several technical and ethical issues still remain. Future research should pay more attention on refining these techniques, improving delivery methods, and understanding the long-term effects of CRISPR-mediated gene editing in vivo. By addressing these challenges, CRISPR/Cas9 technology could pave the way for developing effective therapies for neural injuries, ultimately improving outcomes for patients with CNS and PNS damage.

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