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Inducible CRISPR/Cas9 Systems in the Treatment of Neurodegenerative Diseases

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ABSTRACT

Inducible clustered, regularly interspaced, short, palindromic repeats with the CRISPR-associated protein 9 (CRISPR/Cas9) system are a legitimate avenue for treating Parkinson's and Huntington's diseases. The CRISPR/Cas9 system, introduced in 2012, is a breakthrough gene-editing mechanism that can be used to modify genomes. The CRISPR/Cas9 system has been used to treat neurodegenerative diseases such as Parkinson's and Huntington's. Until now, a majority of CRISPR/Cas9 approaches have been geared towards treating the symptoms of these diseases rather than the causes themselves. Drug inducible CRISPR/Cas9 systems provide more avenues for novel treatments of Parkinson's and Huntington's diseases that target the genetic causes of these diseases rather than the symptoms. Although there are many limitations to CRISPR, such as delivery methods and target specificity, many improvements are being implemented to increase the efficiency and efficacy of the CRISPR/Cas9 system.

CRISPR/Cas9 Systems are Continually Advancing

The history of gene editing began in the 1970s, when researchers produced the first transgenic mice. However, it proved impossible to perform a targeted insertion into a cell's genome using their method (Kozovska et al., 2021). Since then, scientists have collaborated in a worldwide effort to develop efficient and practical gene targeting and editing systems. The first gene targeting system, zinc finger nucleases (ZNFs), was introduced in 2002 (Lino et al., 2018). However, inaccuracy, complexity, and lack of specificity of ZNFs often resulted in undesirable genetic modifications (Kozovska et al., 2021). The second gene targeting system, transcription activator-like effector nucleases (TALENs), was introduced in 2010. TALENs performed differently than ZNFs as it was more straightforward in its design and less complex in its execution (Kozovska et al., 2021). Finally, clustered, regularly interspaced, short, palindromic repeats with the CRISPR-associated protein 9 (CRISPR/Cas9) was introduced in 2012 (Kozovska et al., 2021). The CRISPR/Cas9 system was a breakthrough technology due to an increase in design simplicity, efficiency, and accuracy in gene targeting and editing capabilities.

Mechanism and Function of the CRISPR/Cas9 System

Gene therapy is the process by which specific genes or genomes are introduced to a cell to achieve a specific result (Bulcha et al., 2021). Gene editing is the process that allows for the direct editing of a cell's DNA (Kozovska et al., 2021). The CRISPR/Cas9 system is a newer technology that has advanced the field of gene editing at an astounding pace. For example, genetically engineered mouse models usually took scientists a year or two to generate. With CRISPR, scientists can create genetic mouse models within months (NCI, 2020). The CRISPR/Cas9 system essentially provides a mechanism by which errors in a genome can be induced in order to insert, delete, overexpress, or inhibit specific genes within the genome (Redman et al., 2016). The CRISPR/Cas9 system alleviates some of the

concerns regarding accuracy and efficacy of the gene editing mechanisms of TALENs and ZFNs (Kozovska et al., 2021).

The general mechanism of the CRISPR/Cas9 system involves a guide RNA (sgRNA), a CRISPR-associated protein 9 (Cas9), and a short sequence in the target gene called the protospacer-adjacent motif (PAM) (Carroll, 2016). The sgRNA directs the Cas9 protein to a target gene, and the Cas9 protein causes a double-stranded DNA break. Due to this double-stranded DNA breakage, the existing DNA can be removed or new DNA can be introduced (Carroll, 2016). However, the PAM must be present in order for Cas9 binding to take place. The genetic modifications are limited to the target site and highlights the accuracy and specificity of the system. Despite the advancement in limiting off-target effects, caution still needs to be taken. If Cas9 expression is not controlled by careful target selection and cautious design, the CRISPR/Cas9 system can be limited by off-target effects and immune system responses (Lundin et al., 2020). The most important aspect to the accuracy of the CRISPR/Cas9 system is the design of the sgRNA. Rational design of the sgRNA will refine target DNA site selection and reduce off-target effects further (Lino et al., 2018). Designing highly specific sgRNA that only match to a certain sequence of bases can minimize the chance of the CRISPR/Cas9 mis-targeting to prevent off-target effects as well (Rahman et al., 2019).

Inducible CRISPR/Cas9 Systems Allow for Temporal Control of Gene Editing

Due to its efficiency, simplicity, and scalability, the CRISPR/Cas9 system has been readily adopted for a variety of genetic screens. For example, the CRISPR/Cas9 system is used for genomic-wide loss-of-function screens in proliferation, drug resistance, viral infection, metabolism, and metastasis (Sun et al., 2019). However, Cas9 and its sgRNA are constantly expressed. This limits the application of the CRISPR/Cas9 system to instances in which the genome editing process does not need to be precisely controlled temporally (Sun et al. 2019). An inducible CRISPR/Cas9 system which allows temporal control of the genome editing process enhances the CRISPR/Cas9 system and widens its range of applications. Inducible systems may be useful for treating numerous diseases, including neurodegenerative diseases that have limited treatments/cures.

Two systems of inducible CRISPR/Cas9 systems that have been developed are the light-inducible CRISPR/Cas9 systems and the emerging drug inducible CRISPR/Cas9 systems (Zhang et al., 2019). Drug inducible CRISPR/Cas9 systems may be more initially translatable into therapies for neurodegenerative diseases than light inducible systems due to the difficulties associated with utilizing light-emitting devices in the human brain. The drug inducible CRISPR/Cas9 system can be once again divided into two categories depending on which level chemical control occurs. In the first category, chemical control occurs in the transcription of Cas9 and sgRNA. In the second category, chemical control occurs at the protein level, including chemically induced proximity systems, intein splicing systems, 4-Hydroxytamoxifen-Estrogen Receptor based nuclear localization systems, allosterically regulated Cas9 systems, and destabilizing domain mediated protein degradation systems (Zhang et al., 2019).

Symptoms and Treatments of Parkinson's and Huntington's Diseases

Neurodegenerative diseases are characterized by the loss of function of one or more types of nerve cells in the brain or peripheral nervous system. The risk of neurodegenerative disease increases with age due to the natural weakening of the nervous system over time (Singh et al., 2006). As a result, neurodegenerative diseases take effect in a quicker and more severe manner. Due to a lack of effective treatment mechanisms for certain neurodegenerative diseases, the CRISPR/Cas9 system is generating significant momentum as a potential approach to neurodegenerative diseases, specifically Parkinson's and Huntington's diseases (Karimian et al., 2020).

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. PD is often associated with a low level of dopamine in a patient's body and leads to significant difficulties in movement such as tremors, slowed movement, rigid muscles, loss of movement, and speech impediments (Singh et al.,

2006). To this day, PD remains incurable despite the vast amount of research poured into it dating back to 1817, the year it was first documented by Dr. James Parkinson (Singh et al., 2006). Current therapies can be used to manage the symptoms, but they have limitations as no current therapy can stop or reverse the effects of PD. The most widespread treatment of PD is levodopa. Levodopa is the precursor to dopamine, and once administered in a patient's body, will be turned into dopamine. This treatment temporarily alleviates the concern of low dopamine levels. However, levodopa is associated with many negative side effects such as the "on-off" phenomena, dyskinesias, and levodopa resistance (Singh et al., 2006). The "on-off" phenomenon refers to the continually shortening duration of levodopa effectiveness which usually occurs in patients after 4-6 years of therapy. The side-effect of this phenomenon is periods of severe akinesia. Levodopa is also highly likely to stop working after a certain period of use (Singh et al., 2006).

Huntington's disease (HD) is a neurodegenerative disease caused by CAG repeat expansions in the huntingtin (HTT) gene. The CAG repeats produce an abnormal HTT protein which is longer than the normal protein. The CAG repeats result in the addition of glutamine residues to the HTT protein (Karimian et al., 2020). People with greater than 39 CAG repeats are certain to develop Huntington's disease. Huntington is also a hereditary and an autosomal dominant disorder, meaning that a child only needs to receive one copy of the atypical gene from the parents to develop the disease (McColgan et al., 2017; Mayo Clinic, 2022). The symptoms of Huntington's disease include bradykinesia, dystonia, balance, gait disturbance, and physical weakness. Chorea is one of the most prominent symptoms of HD and is treated with tetrabenazine. Psychiatric symptoms of HD such as depression, anxiety, OCD, and irritability can be treated with therapy or specific drugs depending on the symptom (McColgan et al., 2017). Currently, the symptoms of Huntington's disease are treated rather than the disease itself. These are several therapeutic options available to treat the symptoms of HD, but there is no certain cure for it yet (Karimian et al., 2020).

Table 1. Table of Parkinson's and Huntington's diseases, the suspected pathologies, current treatments, and potential CRISPR/Cas9 treatments.

Disease	Suspected Pathology	Treatment	Potential Treatments
Parkinson's	Lewy bodies, SNCA gene, α-synuclein, and uncontrolled dopaminergic cell apoptosis (Karimian et al., 2020)	Levodopa (decreased effectiveness with 4-6 years of use) (Singh et al., 2006)	CRISPR/Cas9-mediated temporal control of PARKIN down-regulation in humans (Rahman et al., 2019)
Huntington's	Abnormal HTT protein due to CAG repeat expansions in the HTT gene (Karimian et al., 2020)	Cognitive, behavioral, or psychodynamic therapy and specific drugs to address symptoms (such as tetrabenazine) (McColgan et al., 2017)	CRISPR/Cas9-mediated inactivation of mutant HTT expression in humans (Karimian et al., 2020)

CRISPR/Cas9 Systems can Treat Parkinson's and Huntington's Diseases

The usual pathology of PD is characterized by misfolded proteins called Lewy bodies and their main component α-synuclein. It has been postulated that the SCNA gene, which encodes α-synuclein, is significantly related to PD (Karimian et al., 2020). When Lewy bodies are formed in the substantia nigra area, a critical region in the brain dedicated to the production of dopamine, dopaminergic cells face apoptosis. When dopaminergic cells in the substantia nigra undergo apoptosis, no dopamine is produced, and the basal ganglia receives no signal to perform its functions.

This phenomenon eventually leads to disuse atrophy in the corpus striatum (Lee et al., 2019). Several new stem cell studies utilize neural stem cells, induced pluripotent stem cell-derived dopaminergic neurons, and mesenchymal stem cells to produce therapeutic effects. Although these PD treatments demonstrate better recovery rates than L-Dopa, there are still many improvements to be made (Lee et al., 2019).

Other studies have found that activated microglial cells contribute to PD. Microglial cells synthesize and secrete advanced glycation end product-albumin (AGE-albumin). AGE-albumin is a possible inducer of neuronal death through an increase in the AGE receptor (RAGE). PD brain damage is associated with inflammation through microglial over activation. Therefore, an abundance of activated microglial cells producing AGE-albumin induces increased levels of neuronal RAGE-albumin, resulting in dopamine neuronal death (Bayarsaikhan et al., 2016). In response to the identification of AGE-albumin from activated microglial cells as a main cause of PD, a potential treatment method, inhibition of AGE-albumin with a soluble Receptor for AGEs (sRAGE) using Umbilical Cord Blood-Derived Mesenchymal Stem cells (UCB-MSC), was developed (Lee et al., 2019). The sRAGE secreting UCB-MSC was generated by the CRISPR/Cas9 system. The inhibition of AGE-albumin using sRAGE was found to protect neuronal death in PD mice, providing a promising direction for this treatment (Lee et al., 2019).

Autophagy is the cellular recycling process that delivers cells to lysosomes for destruction (Thomas et al., 2013). Mitophagy, the process in which mitochondria are directed to autophagy, is controlled by the PARKIN protein through the PINK1/PARKIN pathway in mitochondria. The PARKIN protein triggers the buildup of S65-phosphorylated ubiquitin (pUb). The buildup of pUb triggers mitophagy (Rahman et al., 2019). In PD patients, the mitophagic process is altered, suggesting a key role in the prognosis of PD. The CRISPR/Cas9 system has been used to edit the regulation of the PARKIN protein. In situations where the PARKIN protein is down regulated using the Thanatos-associated protein 11 (THAP), levels of pUb significantly decrease, slowing down or stopping the progression of mitophagy (Rahman et al., 2019). An inducible CRISPR/Cas9 system may be of use in this particular instance as excess down regulation of the PARKIN protein is detrimental to the brain. Temporal control of PARKIN down regulation could provide a mechanism that prevents unnecessary mitophagy, but does not alter normal and routine mitophagy. The correct intervals and instances at which the PARKIN protein needs to be down regulated could be identified through repeated testing and trials.

Since the cause of Huntington's disease has been identified as an abnormal huntingtin (HTT) protein due to CAG repeat expansions in the HTT gene, the CRISPR/Cas9 technique focuses on the inhibition of the abnormal HTT protein (Karimian et al., 2020). It has been determined that CRISPR/Cas9-mediated inactivation of mutant HTT (mHTT) expression in the striatum of mHTT-expressing mice effectively reduces the production of mHTT (Karimian et al., 2020).

Challenges of CRISPR/Cas9 systems in Humans

A main concern with the CRISPR/Cas9 system is off-target effects. Off-target effects include genome toxicity, carcinogenesis, genome instability, gene functional disruptions, and epigenetic alterations (Karimian et al., 2020). Since genomic changes induced using the CRISPR/Cas9 system are permanent, the off-target effects need to be identified and prevented. However, there are several methods and techniques to detect off-target effects. Digested Genome Sequencing (Digenome-seq) can be used to identify off-target effects on Cas9 and is based on DNA cleavage. Digenome-seq identifies off-target effects using an RNA/DNA bulge and is extremely sensitive (Manghwar et al., 2020). CIRCLE-seq, DISCOVER-Seq, GOTI, and VIVO are also powerful approaches to evaluate off-target effects induced by CRISPR/Cas9 which work through powerful sequencing technology in vivo and in vitro (Manghwar et al., 2020). Promising endeavors being developed to reduce off-target effects are rational sgRNA design, comparative transcriptome analysis, screening after Cas9 treatment, and target site specificity (Manghwar et al., 2020). Extensive studies have been conducted to determine rules and design parameters in order to design more specific CRISPR/Cas9 systems (Wang et al., 2018). Comparative transcriptome analysis and screenings reveal differences in gene expressions, allowing for more detailed analyses of the design of specific CRISPR/Cas9 systems (Buchanan-Wollaston et al., 2005).

Another complication with the CRISPR/Cas9 system is the target DNA site selection and sgRNA. Limitations on the target DNA site selection and design specification of sgRNA are significant roadblocks to CRISPR/Cas9 usage and reduce the simplicity of the process (Lino et al., 2018).

Another important limitation of CRISPR is the delivery mechanism, especially in the case of a drug inducible system. Each delivery mechanism is suited to a particular application and has its weaknesses. Microinjection uses a needle to inject DNA plasmids, Cas9, sgRNA, and a protein directly to a target site in a cell. The main advantage of microinjection is the guaranteed delivery into the cell of interest. But, microinjection is often time-consuming and limited to *in vitro* as well (Lino et al., 2018). Electroporation utilizes electrical currents to open pores in the cellular membranes of cells suspended in a buffer solution. Content that is extremely small, such as DNA plasmids, Cas9, and sgRNA, are then able to flow into the cell through these pores. Electroporation is a well-known and well-tested technique that delivers cargo directly to the cell population. Despite its advantages, electroporation is also generally limited to *in vitro* rather than *in vivo* (Lino et al., 2018). Other delivery vehicles used with the CRISPR/Cas9 system include adeno-associated virus, adenovirus, lentivirus, and lipid nanoparticles (Lino et al., 2018).

CRISPR/Cas9 systems are also held back by the length of the research process. Research begins with *in vitro* trials and transitions to *in vivo* trials in species such as mice. It is here that issues with delivery mechanisms arise. Many delivery mechanisms are suited specifically for *in vitro* and new methods must be adapted to deliver the system. Eventually, *in vivo* in the form of human clinical trials are established. The clinical trials are conducted in a series of "phases."

Finally, inducible CRISPR/Cas9 systems have some crucial limitations. Many light-inducible and drug-inducible systems are viable in theory, but are extremely difficult to apply in the human body. Lighting up or stimulating a cell in a petri dish or in a test tube is significantly different from doing the same to a cell in the human body. Lights may not be able to reach certain places in the human body. Drugs may get filtered as they pass through the bloodstream and might have a difficult time crossing the blood-brain barrier to effectively achieve the desired result.

Conclusion

The CRISPR/Cas9 system is a promising tool in the treatment of neurodegenerative diseases. Through careful research and testing, the CRISPR/Cas9 system has been deemed a viable treatment for the symptoms of Parkinson's and Huntington's diseases. However, inducible systems remain a relatively novel concept regarding the prevention of neurodegenerative diseases. Shortcomings such as delivery mechanisms, target site specificity, and off-target effects are all limitations for the implementation of CRISPR/Cas9 treatments. Drug inducible CRISPR/Cas9 systems may not combat all of these concerns, but provide the vital advantage of temporal control. As advancements in the field of genetic editing are continually made, drug inducible CRISPR/Cas9 systems may become a premier option for the prevention and cure of Parkinson's and Huntington's diseases.

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