Applications of CRISPR/Cas9 for Gene Editing in Hereditary Movement Disorders

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ABSTRACT

Gene therapy is a potential therapeutic strategy for treating hereditary movement disorders, including hereditary ataxia, dystonia, Huntington's disease, and Parkinson's disease. Genome editing is a type of genetic engineering in which DNA is inserted, deleted or replaced in the genome using modified nucleases. Recently, clustered regularly interspaced short palindromic repeat/CRISPR associated protein 9 (CRISPR/Cas9) has been used as an essential tool in biotechnology. Cas9 is an RNA-guided DNA endonuclease enzyme that was originally associated with the adaptive immune system of Streptococcus pyogenes and is now being utilized as a genome editing tool to induce double strand breaks in DNA. CRISPR/Cas9 has advantages in terms of clinical applicability over other genome editing technologies such as zinc-finger nucleases and transcription activator-like effector nucleases because of easy in vivo delivery. Here, we review and discuss the applicability of CRISPR/Cas9 to preclinical studies or gene therapy in hereditary movement disorders.

Key Words
CRISPR/Cas9; gene editing; gene therapy; hereditary movement disorders.
INTRODUCTION

In the early 21st century, the Human Genome Project was successfully completed, revealing the entire sequence of the human genome. This success has accelerated the rate of genomic research, which addresses the function of genes and their resultant translated proteins. Over the last decade, due to the advances in next-generation sequencing, a rapidly increasing number of pathogenic variants and mutations has been discovered. Additionally, over last 5 years, genomic engineering technologies (that is, the modification of the genome at precise, predetermined loci) have achieved huge technical improvements that are now being utilized as valuable tools in preclinical research that may eventually give aid to patients suffering from intractable diseases.

An increasing number of genetic mutations that cause hereditary movement disorders presenting with ataxia, dystonia, parkinsonism, chorea or spastic paraparesis have been identified. Although various pathogenic mechanisms such as protein aggregation, mitochondrial dysfunction, oxidative stress, apoptosis and autophagy have been identified from these genes, disease-modifying treatments for neurodegenerative disorders or hereditary movement disorders are lacking. Novel chemical drugs, stem cell therapies, and gene therapies have been suggested as promising new therapies for these disorders. The currently available drugs for these disorders are for symptomatic treatment; however, they fail to cure the disease or reverse disease progression. Although the theory behind stem cell therapies is promising, there are still many technical obstacles to be solved. Moreover, a large amount of data from preclinical studies and clinical trials as well as data about safety are needed before the broad application of these therapies to patients. Gene therapies using genome editing technologies are another potentially powerful therapeutic strategy for the disease-modifying treatment of hereditary movement disorders or neurodegenerative disorders. Here, we discuss the applicability of the newest genome engineering method, the clustered regularly interspaced short palindromic repeat/CRISPR associated protein 9 (CRISPR/Cas9) system, to hereditary movement disorders.

GENE THERAPY METHODS: GENE SILENCING AND GENE EDITING

Gene therapy refers to the introduction of defined genetic material to specific target cells or tissues of a patient for the final purpose of curing or altering particular disease symptoms. This has long fascinated clinicians and scientists because it has the potential to ultimately cure a disease. Gene therapy can be classified into two categories: gene silencing and gene editing (Table 1). Gene silencing is a general term used to describe the suppression of gene expression. RNA interference, antisense oligonucleotides and microRNAs are all gene silencing technologies and were the ‘gold standards’ for the knockdown of genes and studying gene function in vitro and in vivo for many years. Double-stranded RNA (dsRNA) is a key molecule in gene silencing; dsRNAs are processed into small interfering RNAs (siRNAs) by the endonuclease Dicer, and these siRNAs are loaded into the RNA-induced silencing complex complex that pairs with the messenger RNA (mRNA) through base-pairing, causing the mRNA to be subsequently degraded.

Gene editing was developed to improve the limitations of gene silencing. Genome editing inserts, deletes or replaces target DNA sequences in the genome using engineered nucleases such as zinc-finger nucleases (ZFNs), tran-

| Table 1. Gene silencing vs. gene editing |
|----------------------------------------|
| **Gene silencing** | **Gene editing** |
| Approach | RNAi, ASO, miRNA | ZFNs, TALENs, CRISPR/Cas9 (RGENs) |
| Molecular target | RNA | DNA |
| Modulation of targeting | Knock out | Knock out or knock in |
| Method of delivery | Nanoparticles, viral vectors, bioconjugates | ZFNs, TALENs: viral vectors, CRISPR/Cas9: viral vectors, electroporation, PEI-mediated transfection, nanoparticles |
| Off-target risk | High | Low or moderate |

RNAi: RNA interference, ASO: antisense oligonucleotides, miRNA: microRNA, ZFNs: zinc-finger nucleases, TALENs: transcription activator-like effector nucleases, CRISPR/Cas9: clustered regularly interspaced short palindromic repeat/CRISPR associated protein 9, RGENs: RNA-guided engineered nucleases, PEI: polyethylenimine.
scription activator-like effector nucleases (TALENs), and Cas9. Gene editing has low off-target effects, shows an ease of multiplexing and has greater target specificity compared to gene silencing.

There are three common requirements for any effective gene therapy modality: 1) the identification of the target gene that is mutated in the disease; 2) a delivery system for the genes or materials; and 3), an approach for regulating the expression of the target gene. Delivery tools for the genetic material in gene therapy are divided into viral and non-viral categories. Adeno-associated viruses (AAVs) and lentiviruses are commonly used for movement disorders. AAVs and lentiviruses have the capability to infect both dividing and non-dividing cells, and the latter can integrate into the genome of host; however, the former does not. However, they are still not safe to apply to clinical trials even though their viral genomes have been modified to remove virulence genes, including those that are self-replicating. Naked plasmid DNA and cationic lipid methods belong to the non-viral set of delivery tools. Unfortunately, non-viral delivery tools are not sufficient for the treatment of chronic neurodegenerative conditions because they create only transient modification in gene expression. New delivery systems that induce permanent effects safely are thus required for clinical application.

## GENE EDITING

Gene silencing has helped researchers achieve the knockdown of specific gene targets cheaply, simply, and quickly. However, it has critical limitations, including incomplete gene silencing, temporary effects, and off-target errors, which limit its broader clinical application. In the past decade, a new strategy has emerged that enables researchers to manipulate practically any gene in cells and tissues. This core methodology is referred to as gene editing, which is a type of genetic engineering in which DNA is inserted in, deleted from or replaced in a genome using site-specific nucleases, which enable the precise modification of genes by introducing double strand breaks (DSBs) at the target location in the genome. These programmable nucleases include ZFNs and TALENs, which create site-specific DSBs at target locations. Distinct from these site-specific nucleases, CRISPR/Cas9 is an RNA-guided engineered nuclease (RGEN) system, in which a synthetic guide RNA (gRNA) introduces a DSB at a specific location in the target genome. Below is a brief review regarding the key features of these three types of programmable nucleases—ZFNs, TALENs and the CRISPR/Cas system (Table 2).

### Table 2. Comparison of different programmed nucleases

|                  | ZFNs          | TALENs        | CRISPR/Cas9 (RGENs) |
|------------------|---------------|---------------|---------------------|
| DNA targeting specificity determinant | Zinc-finger proteins | Transcription activator-like effectors | CRISPR RNA of sgRNA |
| Nucleases        | FokI          | FokI          | Cas9                |
| Restriction in target site | G-rich        | Start with T and end with A | End with NGG or NAG (lower activity) sequence (PAM) |
| Ease of engineering | Difficult   | Moderate     | Easy               |
| Ease of multiplexing | Low        | Moderate     | High               |
| Off-target effects | Moderate    | Low          | Variable           |
| Cytotoxicity     | Variable to high | Low          | Low                |
| Ease of in vivo delivery | Moderate: viral vectors | Moderate: viral vectors | Moderate: viral vectors, nanoparticles, PEI-mediated transfection |
| Cost             | High         | Moderate     | Low                |

RGENs: RNA-guided engineered nucleases, ZFNs: zinc-finger nucleases, TALENs: transcription activator-like effector nucleases, CRISPR: clustered regularly interspaced short palindromic repeat, Cas9: CRISPR associated protein 9, sgRNAs: single-guide RNAs, PAM: protospacer adjacent motif, PEI: polyethylenimine.
TALENs recognize specific target DNA through protein-DNA interactions. A TALEN target site consists of two TALE binding sites separated by a spacer. The DNA-binding domain of a TALEN is composed of multiple repeats and can recognize 33–35 nucleotides. Although there was a problem with low efficiency during the early stages of development, platinum TALENs have high efficiency in mammalian cells. Additionally, the most advantageous feature of TALENs is that they can be designed to target almost any given DNA sequence because the cutting of target DNA sequences with TALENs is achieved by Fok1, which is linked to complementary DNA sequences.

CRISPR/CAS9 SYSTEM

The CRISPR/Cas9 system is categorized as an RGEN that recognizes a target specific sequence with a 23-bp length, and the mechanism of action is different from that of ZFNs and TALENs. Unlike ZFNs and TALENs, CRISPR/Cas9 uses gRNA instead of a protein-DNA interaction to recognize genomic DNA and utilizes Cas9 as a nuclease. The gRNA can recognize approximately 20-bp nucleotides and requires a protospacer adjacent motif (PAM), which can recruit Cas9. Cas9 is guided by specific sequences of gRNA that are related to a trans-activating crRNA (tracrRNA) and form the complementary DNA target sequence, resulting in a site-specific DSB.

APPLICATIONS OF CRISPR/CAS9 SYSTEMS IN HEREDITARY MOVEMENT DISORDERS

Why is CRISPR/Cas9 applicable for hereditary movement disorders or neurodegenerative disorders?

Many genes have been identified to be critically involved in the pathogenesis of hereditary movement disorders or neurodegenerative disorders; hence, these are potential targets for the CRISPR/Cas9 system to develop disease modifying treatment strategies. Huntington's disease (HD) is a prototype disease among several trinucleotide repeat disorders, in which the expansion of a polyglutamine region stretches beyond a certain threshold and causes disease. Among autosomal dominant cerebellar ataxia, spinocerebellar ataxia types 1, 2, 3, 6, 7, and 17 are trinucleotide repeat disorders in which the accumulation of abnormal proteins with an expanded polyglutamine track is a common pathogenic mechanism in neurodegeneration. Although most cases of Parkinson's disease (PD), Alzheimer's disease and amyotrophic lateral sclerosis are sporadic onset and associated with multifactorial etiological factors, the accumulation of abnormal misfolded proteins is a common pathological feature. Genome engineering to modify abnormal protein production and prevent their accumulation appears to be effective in these diseases.

Some hereditary movement disorders occur in an autosomal recessive pattern, which is caused by loss-of-function mutation of certain genes. Given that CRISPR/Cas9 can knock in a specific transgene, these autosomal recessive movement disorders can also be good targets for the application of CRISPR/Cas9.
The application of CRISPR/Cas9 for the generation of model system for hereditary movement disorders

The CRISPR/Cas9 system is accelerating the development of biological research and enabling targeted genetic interruption in almost any cell type. Although CRISPR/Cas9 has an off-target problem, it has opened the door to the development of new in vitro and in vivo model systems for studying the complexities of the nervous system in regards to hereditary movement disorders, including applications for the study of synaptic and neural circuit function, neuronal development, and genetic neurological diseases.

Genome editing using CRISPR/Cas9 is possible in various cell lines, including human induced pluripotent stem cells, which can be utilized as a valuable in vitro tool for the investigation of specific mutations in the pathogenesis of various disorders. For example, Vannocci et al. developed a novel cellular model of Friedreich’s ataxia, which is an autosomal recessive ataxia caused by reduced levels of frataxin, using CRISPR/Cas9 to stably introduce the disease frataxin gene into cells.

Traditionally, transgenic experimental model systems using species such as mice, flies, fish and cells have provided neuroscientists with important and valuable information about the molecular pathology of many hereditary disorders. Transgenic mouse models are widely used because, in addition to knockouts, the genomes of mice can be modified to create pathologies based on gain-of-function mutations using a versatile set of genetic tools. However, rodent models are not sufficient to recapitulate the full range of pathological phenotypes when compared to patients with hereditary movement disorders. The ability to investigate genetically modified large animals, such as pigs, dogs, and non-human primates, has the potential to significantly enhance our understanding of the complex pathological process of the human disease. Large animal models are more capable of confirming therapeutic effects that cannot be adequately modelled in rodents. However, the transgenic modification of genes in large animals using traditional gene targeting technology is generally less successful due to the lack of available embryonic stem cell lines.

Recently, CRISPR/Cas9 was successfully used to generate the precise disruption of single and multiple genes in pigs and non-human primates, which can be used as large animal models of hereditary movement disorders or neurodegenerative disorders. Recently, Holm et al. suggested the use of CRISPR-mediated pig models for neurodegenerative disorders, including HD and PD. However, off-target effects and mosaic mutations are problems that need to be solved during the CRISPR/Cas9-mediated generation of large animal models. Although off-target mutations will be diluted quickly over generations in small animal models with short breeding times, this can be a serious problem in large animal models, such as monkeys, which have longer periods between generations. Moreover, somatic mosaicism and allele complexity can occur during CRISPR/Cas9-mediated mutagenesis through zygote injection. The generation of large animal models using CRISPR/Cas9 will be improved by reducing the off-target effects and mosaic mutations.

CRISPR/Cas9-mediated preclinical therapeutic applications for hereditary movement disorders or neurodegenerative disorders

Several approaches for gene therapy, including gene silencing and virus-mediated gene delivery, in hereditary movement disorders have been pursued both in preclinical studies and in early phase clinical trials. Meanwhile, CRISPR/Cas9-mediated gene editing is still in the early preclinical phase. Dr. Nicolas Merienne and his colleagues performed research to reduce mutant huntingtin aggregation by using CRISPR to delete the open reading frame of the HTT gene, leading to the loss of mHtt expression. In these studies, CRISPR/Cas9 reduced the aggregation of mutant huntingtin in the mouse striatum, demonstrating the potential of the CRISPR/Cas9 system as a gene therapy modality for hereditary movement disorders. Recently, Chen et al. showed that the CRISPR-mediated knock in of designer receptors exclusively activated by designer drugs (DREADDs) enables the precise regulation of human pluripotent stem cell (hPSC)-derived neurons by chemical compounds. When the hPSC-derived human midbrain dopaminergic neurons were transplanted into a PD mouse model, their motor function was able to be reversed or enhanced by DREADD ligands. Further, in June 2016, the US National Institutes of Health approved a pro-
posal to use CRISPR/Cas9 in the first human clinical trial to edit the genome of T cells to augment cancer therapies, which will be the starting point for subsequent CRISPR clinical trials in various human diseases. CRISPR-mediated gene therapies in HD, PD, dystonia, and hereditary ataxias can be challenging, but will be a feasible therapeutic option in the near future.

CONCLUSION

Although there are still many problems to be solved, such as off-target effects, delivery system, efficacy, safety concerns, and ethical issues, CRISPR/Cas9 is quickly being applied as an essential tool in biotechnology and will be applied to clinical practice sooner or later. CRISPR/Cas9-mediated preclinical research and clinical trials should be encouraged and performed in hereditary movement disorders or neurodegenerative disorders.

Conflicts of Interest

The authors have no financial conflicts of interest.

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