Application of the gene editing tool, CRISPR-Cas9, for treating neurodegenerative diseases

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\textbf{A B S T R A C T:}

Increased accumulation of transcribed protein from the damaged DNA and reduced DNA repair capability contributes to numerous neurological diseases for which effective treatments are lacking. Gene editing techniques provide new hope for replacing defective genes and DNA associated with neurological diseases. With advancements in using such editing tools as zinc finger nucleases (ZFNs), meganucleases, and transcription activator-like effector nucleases (TALENs), etc., scientists are able to design DNA-binding proteins, which can make precise double-strand breaks (DSBs) at the target DNA. Recent developments with the CRISPR-Cas9 gene-editing technology has proven to be more precise and efficient when compared to most other gene-editing techniques. Two methods, non-homologous end joining (NHEJ) and homology-direct repair (HDR), are used in CRISPR-Cas9 system to efficiently excise the defective genes and incorporate exogenous DNA at the target site. In this review article, we provide an overview of the CRISPR-Cas9 methodology, including its molecular mechanism, with a focus on how in this gene-editing tool can be used to counteract certain genetic defects associated with neurological diseases. Detailed understanding of this new tool could help researchers design specific gene editing strategies to repair genetic disorders in selective neurological diseases.

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1. Introduction

Genetic abnormalities are involved in several degenerative disorders, which affect millions of people worldwide. Some of genetic disorders can be diagnosed in early childhood, but many of them do not show any signs or symptoms until adulthood. For example, the choreic movements that characterize Huntington’s disease (HD) often emerge only after adulthood. For most genetic disorders, there are few, if any, treatment options available. Therefore, gene-editing tools such as zinc finger nucleases (ZFNs), meganucleases, and transcription activator-like effector nucleases (TALENs), and CRISPR (clustered regulatory interspaced short palindromic repeats)-Cas9 (CRISPR-associated), which can edit, replace, modify defective areas on the genome, have gained a great deal of interest as a means of treating certain neurodegenerative diseases. The application of these tools for introducing healthy genes to the defective area of the genome, may slow down the disease progression, but concerns of precisely excising only the defective areas of the gene persist. Among all the existing gene-editing tools, CRISPR-Cas9 seems most promising because of its ease in construction, efficacy, cost-effectiveness, and its capability of editing multiple genes at the same time. The principal objectives of this review article is to provide a basic understanding of the mechanism underlying how CRISPR-Cas9 could be used for gene editing in neurological diseases, such as the experimental paradigm we designed as a potential treatment for Huntington’s disease.

1.1. Basics of CRISPR-Cas9

CRISPR is a bacterial adaptive immune mechanism, which can target specific sequences of foreign nucleic acids (Jansen et al., 2002). CRISPR consists of unique spacer sequences delineated by short, repetitive, palindromic sequences, and sequences encoding Cas proteins (Koonin et al., 2006). The CRISPR-Cas9 system has proven to be applicable in eukaryotes for gene editing, which was initially adapted from S. pyogenes (Barrangou et al., 2007). After many years of research on the mechanism of the CRISPR-Cas 9 system, its application for gene editing, including knock-in and knock-down manipulations, as well as for correcting mutated regions in the genome is now within reach (Sander and Joung, 2014).

1.2. History of CRISPR-Cas9

Viruses infect the bacteria and propagate by integrating viral nucleic acid into the bacterial DNA. CRISPR-Cas9 is a type of adaptive immune mechanism present in most archaea and 40% of the bacteria to protect them from viral invasion (Van der Oost et al., 2009). CRISPR-DNA locus consists of CRISPR, and CRISPR and Cas gene (Westra et al., 2012). The CRISPR/Cas9 system is capable of incorporating sequences derived from phage DNA/RNA (Grissa et al., 2009). Cas represent a special kind of protein which helps in establishing a molecular memory in the bacterial cells, in the form of a genomic (viral) sequence integration into the spacer regions present in the CRISPR array (Jansen et al., 2002). Upon transcription and translation of this CRISPR locus, guide RNA (g-RNA), homologous to the viral nucleic acid, is formed, along with the Cas proteins (Haft et al., 2005). Cas9 binds g-RNA which guides it to the target viral DNA sequence (Makarova et al., 2011). Upon reinfection, bacteria utilize the stored viral sequence, in order to destroy the invading pathogenic nucleic acid (Garneau et al., 2010) (Fig. 1).

This system can be easily implemented in eukaryotes by picking a gRNA and Cas9 protein (Wu et al., 2013). The Cas9 protein cuts the DNA at the target site, which is repaired by the endogenous DNA repair mechanism by two types of the gene-editing processes, the non-homologous end joining (NHEJ) and the homology-direct repair (HDR) mechanisms, which can be observed in eukaryotes (Fig. 2). NHEJ results in addition or deletion of the nucleotides, whereas, in HDR editing results in full DNA sequence restoration and can only be used when there is a homologue DNA sequence present. In HDR, genome-engineered constructs with “homology arms” are used for targeted introduction of “donor sequence” into eukaryotic genome following the CRISPR/Cas9-induced double strand DNA breaks (Auer and Del Bene, 2014). Most scientists have reported minimal off-target mutations caused by Cas9, and many strategies have been initiated to cut off-target effects even further (Wei et al., 2013). CRISPR-Cas9 system has already proven to have therapeutic applications in both in vitro and in vivo disease models (Jusila et al., 2016) as well as therapeutic gene-editing applications with enormous translational potential (Hsu et al., 2014; Wright et al., 2016).

2. Advantage of CRISPR-Cas9 over other gene editing tools

Other gene editing tools namely, TALENs and ZFNs, require specific engineering to target the DNA for precise DSBs (Mao et al., 2013), a procedure which requires a great deal of effort, time, and money. Additional problems involve size, off-target cleavage, and cytotoxicity (Carlson et al., 2012). Cytotoxicity and off-target effects are seen with ZFNs, and the efficiency of this method is relatively low (Kim and Kim, 2014). On the other hand, TALENs have a higher efficiency than ZFNs but require huge resources, and its delivery into cells is often a challenge (Carlson et al., 2012; Kim and Kim, 2014). CRISPR-Cas9 can facilitate DSBs with high efficiency, low cytotoxicity, and with relatively low off-target effects (Carlson et al., 2012). The advantages of CRISPR-Cas9 over other gene-editing tools, in terms of manipulating the genes, is detailed below.

2.1. Gene editing

Like other designer nucleases, the CRISPR-Cas9 system can be used to induce DSBs and single-stranded breaks that can be repaired through NHEJ and HDR (Sander and Joung, 2014; Strauf and Lahaye, 2013). Through NHEJ, insertions and deletions can cause mutations and alter the genome of organism (Sander and Joung, 2014). For example, CRISPR-Cas9 used in medaka embryos
produced 100% mutagenesis efficiencies for a gRNA targeting the Dj-1 gene (Ansai and Kinoshita, 2014). In another study, targeting the Tet1 gene, a gene encoding Tet proteins, which are essential in the DNA methylation process, Cas9 and Tet1-targeting-gRNA were co-transfection resulted in 55% mutagenesis (Horii et al., 2014). Although various studies have shown different percentages in mutagenesis, Ansai and Kinoshita (2014) have determined that using different gRNAs could produce frequencies of mutations ranging from 44.8% to 91.3%, indicating that different gRNAs are more efficient at producing mutations than others.

Gene knock-in is also possible with the CRISPR-Cas9 system through HDR (Beumer and Carroll, 2014). Researchers have utilized the ability of this system to induce targeted gene knock-in by injecting a donor oligonucleotide DNA strand with Cas9 and gRNA.
The highest rates of gene insertion in this experiment were observed with gRNAs targeting the UPTR gene, encoding Uracil phosphoribosyl transferase enzyme. UPTRT-targeting gRNAs reached insertion rates between 69.0% and 78.1% (Shen et al., 2014). The mutagenic and HDR rates with CRISPR-Cas9 match those of ZFNs and TALENs (Kim and Kim, 2014). A study comparing the efficiency of TALENs and CRISPR-Cas9 showed that CRISPR-Cas9 indel mutation rates ranged between 42% and 100% while TALENs was about 33% (Auer and Del Bene, 2014). A different study recorded mutagenesis rates of 11% for TALENs and 76% for CRISPR-Cas9, for the same gene (Veres et al., 2014). Knock-in approaches for both TALENs and CRISPR-Cas9 have been reported to be about 10.3% for TALENs and 8.3% for CRISPR-Cas9 (Auer and Del Bene, 2014). A cloning-free CRISPR-Cas9 system, which was devised by using synthesized dual-crRNA and tracrRNA, along with the Cas9 protein has also been used as a gene knock-in mechanism in mice (Aida et al., 2015).

2.2. Multiplexing strategy of CRISPR-Cas9 system in gene editing

The CRISPR-Cas9 system is capable of multiplexing (Liu et al., 2014). With multiple gRNA targeting different sites and one Cas9 protein, multiple sites can be altered at the same time (Liu et al., 2014). This is useful when one gRNA is not enough to disrupt a targeted gene (Auer and Del Bene, 2014) or when modifying two or more genes at the same time (Zhou et al., 2014). Using two different gRNAs to target the same gene has been shown to increase mutagenic frequencies (Zhou et al., 2014). On the other hand, targeting more than one gene at the same time is also useful because it can decrease the time and money required to produce animal models with different mutations (Zhou et al., 2014). Multiplexing offers a unique ability of CRISPR-Cas9 that has not been successfully utilized by ZFNs or TALENs, due to the complexity in those systems, which requires the designing of protein for each targeted gene, resulting in an increase in the variable efficacy at each gene (Liu et al., 2014).

Multiplexing with CRISPR-Cas9 was performed shortly after the system was first established for use in eukaryotes (Liu et al., 2014). One of the first multiplexing experiments was completed in mice with two gRNAs targeting the Tet1 and Tet2 genes (Wang et al., 2013). The two gRNAs were co-injected with Cas9 to yield bi-allelic mutations of both genes at 80% efficiency (Wang et al., 2013). A similar experiment in rats resulted in bi-allelic mutagenesis of three different genes at a rate of 60% (Li et al., 2013). As many as six genes have been targeted at one time, with ten gRNAs, resulting in multiplexed gene alteration (Liu et al., 2014).

Multiplexing with CRISPR-Cas9 was also used to generate mouse models with mutations in multiple immune response genes (Zhou et al., 2014). For example, when five different gRNA were injected with Cas9 into nine mice, the results showed that 22.2% of the mice had four mutations, 33.4% had three mutations, and 44.4% had two mutations (Zhou et al., 2014). None of the mice had five different genes altered. The multiplexing efficiency increased to 85% when multiple gRNA targeting the same genes were used at the same time (Zhou et al., 2014).

Multiplexed-editing has also shown heritability. In one experiment, researchers were able to alter four genes simultaneously with four different gRNA (Ma et al., 2014). Offspring genomes from this study were analyzed and it was found that the same mutations observed in the offspring were also found in the parents.

Multiplexing strategies have been applied in many other settings. For example, it has been implemented in Saccharomyces cerevisiae as an exploratory analysis of all possible single-, double-, triple-, quadruple-, and quintuple-gene disruptive combinations for the search of strains with high mevalonate production (Jakociunas et al., 2015), which is an important protein for the isoprenoid biosynthesis pathway. Another group of scientists (Chen et al., 2015) used enriched gRNAs to explore the specific mutations which are responsible for loss-of-function that drive metastasis and tumor growth in lung carcinoma. In yet another application, dystrophin reading frame has been restored in Duchenne muscular dystrophy (DMD) using multiplexing strategy by targeting the mutation at exons 45–55, where a single large deletion of up to 62% of DMD mutations was demonstrated (Ousterout et al., 2015).

2.3. Gene regulation through CRISPR/Cas9 system

In addition to multiplexing, gene regulation is another characteristic of CRISPR-Cas9 systems that has not been demonstrated with either ZFNs or TALENs. The ability to regulate genes is an important ability of the system because there are many diseases that result from too much, or too little, production of a protein. Using the dCas9 protein (enzymatically inactive Cas9), scientists can activate or repress transcription of a gene (Gilbert et al., 2013). CRISPR-Cas possesses the gene regulating ability in the bacteria through the destabilization of bacterial lipoprotein mRNA (Louwen et al., 2014), the same principle can also be applied to gene regulation in eukaryotes. dCas9, with alteration in both HNH and RuvC nuclease domains, has applications through transcriptional control in gene regulation (Fineran and Dy, 2014). dCas9-guideRNA complex can bind to the targeted DNA sequence but it does not act as an endonuclease that has the ability to cleave DNA (Xu et al., 2014). Instead, dCas9 can be combined with repressor proteins or activators to silence a gene or increase gene expression, respectively (Mali et al., 2013). The altered dCas9 protein is also capable of binding to the DNA at the transcriptional promoter region and competitively block the initiation or elongation of transcription (Xu et al., 2014).

Hu et al. (2014) have achieved this gene activation by fusing a dCas9 protein to a transcription activator domain, VP64, and used gRNA that targeted a gene promoter. Other transcription activator domains, VP48 and VP160, have been used in human HeLa cells, HEK293T cells, and mouse embryo cells to activate both reporter and endogenous genes (Cheng et al., 2013).

Improvements to this technique have even been made where gene activation showed six-fold improvements, when three different proximal gRNAs were used for targeting the same gene promoter (Cheng et al., 2013). Gene repression is also possible when dCas9 is fused to KRAB, a transcription repressor domain, or when dCas9 is used alone. Gilbert et al. (2013) used both dCas9 and dCas9-KRAB with the gRNAs targeting the CD71 and CXCR4 genes, and found a 60%–80% repression in expression of the genes (Gilbert et al., 2013). In a different study, Zhao et al. (2014a) used dCas9 alone to sterically block binding or elongation of RNA polymerase to disrupt transcription of miRNA (microRNA). The miRNA genes, miR-21 and miR-30a, were targeted with specific gRNA, which directed dCas9 to the sites of transcription and repressed miRNA expression (Zhao et al., 2014b). CRISPR-Cas9 system has also been used to demonstrate the functional relevance of topologically associated domains (TADs) in regulating gene expression and predicted the pathogenicity of structural variants, in particular, the non-coding domains (TADs) in regulating gene expression and predicted the pathogenicity of structural variants, in particular, the non-coding regions of the human genome (Ren and Dixon, 2015).

3. Prospects of CRISPR-Cas9 in neurodegenerative diseases

Several neuronal disorders are genetic in origin, and for most of those, variable treatments are still unavailable. Management of the neurological disorders is still at an elementary level, as the complex machinery of brain is poorly understood, including the pathophysiology and genetic components of many neurodegenerative
diseases, which remain enigmatic. All these factors contribute to a limitation in the discovery of newer pharmacological targets. Determination of the disease-causing factors could hasten the drug-discovery process. Due to the complexity of the behaviors, the current animal models of neurodegenerative disorders need refinement to meet the clinical translation. Many researchers are also modeling human iPSCs to utilize them for modeling the disease. At present, CRISPR is being used widely, not only for gene therapy, but also to generate animal models which can be utilized in the drug discovery process. Some of the key findings, inferences and application of CRISPR-Cas9 in human diseases have been summarized in Table 1. The CRISPR-Cas9 research is not limited to the animal research, but it being tested on humans in China to treat lung cancer (Cyranoński, 2016). The proposed idea was that the immune cells will be isolated from the blood of patients, and the PDCCD1 gene, which codes for the protein PD-1, will be disabled ex vivo. PD-1 proteins down-regulate immune system and foster self-tolerance to the programmed cell death. PDCCD1-edited-immune cells, when injected into the lungs of patient are expected to attack the cancer cells. Similarly, another research group used CRISPR-Cas9 to correct point mutations in G6PD and HBB through HDR technique in human zygotes, by injecting Cas9 protein complex with appropriate gRNA (Tang et al., 2017).

3.1. Alzheimer’s disease

Alzheimer’s disease (AD) is one among the leading causes of death in the elderly population. Genetic studies on AD patients have uncovered the relationship between the mutation in genes and the onset of symptoms. Early-onset familial AD is caused by the mutation present in the gene encoding presenilins (PSEN1 and PSEN2) and amyloid precursor protein (APP). Mutation in the APOE gene, which codes for the protein PD-1, will be disabled ex vivo. PD-1 proteins down-regulate immune system and foster self-tolerance to the programmed cell death. PDCCD1-edited-immune cells, when injected into the lungs of patient are expected to attack the cancer cells. Similarly, another research group used CRISPR-Cas9 to correct point mutations in G6PD and HBB through HDR technique in human zygotes, by injecting Cas9 protein complex with appropriate gRNA (Tang et al., 2017).

3.2. Parkinson’s disease

Parkinson’s disease (PD) is a long-term, progressive neurodegenerative disorder that mainly affects the motor system. Death of neurons in the substantia nigra results in the deficiency of dopamine in the brain and is the major pathology involved in PD. Symptoms include postural instability, resting tremors, rigidity, and Bradykininesia. Currently, dopamine agonists, anticholinergic agents and l-Dopa, a dopamine precursor, are available for the treatment. However, the severe side effects of those drugs and the resulting effects make drug-tolerance and management of PD very difficult. As such, alternative therapies are desperately needed.

Although PD is idiopathic, some studies conducted on PD patients with family histories of PD have revealed the presence of a mutation in the gene encoding DJ-1, PINK1, or SNCA. Inclusion of Lewy bodies, filled with mutant α-synuclein protein, in the dying or dead neurons have been observed in postmortem analysis of the brains of PD patients. To date, SNCA and LRRK2 were identified to be responsible for the autosomal dominant phenomenon in PD (Sundal et al., 2012). Many transgenic models with combination of PARK: DJ1; and LRRK2 knock-outs were previously developed that have shown nuclear inclusions and other morphological changes. However, these models failed to exhibit behavioral defects that are seen in PD (Dawson et al., 2010; Le et al., 2014). To date, the 6-OHDA rodent model and the MPTP non-human primate model are used extensively for developing palliative therapies as they have been predictive of clinical outcomes. However, their utility in preclinical studies for exploring regeneration and neuroprotection has been consistently inimical. Chesselet (2008) has created a mouse model that overexpresses α-synuclein to mimic the PD pathology, but this model lacks overt degenerative pathology in dopaminergic neurons. Along with nigrostriatal DA systems, non-DA systems such as cardiac sympathetic denervation, sleep disturbances, and olfactory dysfunction, are affected in PD. Generation of robust genetic models with degeneration of the both the nigrostriatal DA system and non-DA systems are essential to accurately mimic the human pathology involved in PD (Langston, 2006).

CRISPR-Cas9 have been used generate pig models of PD by targeting the genes for Pink1, DJ1, and Parkin (Zhou et al., 2015; Wang et al., 2016), Zhou et al. (2015), including knock-outs of Parkin and PINK1. However, the classic symptoms associated with PD, such as rigidity, Bradykininesia and resting tremors, were not observed in 7-month-old live mutant pigs. Wang et al. (2016) also used a multiplexing strategy to knock-down Parkin, DJ-1, and PINK1. However, again, these mutant piglets exhibited normal behavior, and remained healthy at the age of 10 months.

To fully understand the function of all these genes and their causal relationship with each other in PD, animal models could be created with various gene knock-down combinations of LRRK2, PARK2, DJ-1, PINK1, or SNCA. Using CRISPR-Cas9 technology, Platt et al. (2014) created a transgenic mouse model that expresses Cas9, and demonstrated the application of multiple gRNAs edited target genes. When utilizing such animal models that express Cas9, either single or multiple genes implicated in PD can be deleted at the same time, and the resultant synergistic effect of multiple gene mutations in PD can be easily studied.
Examples of recent applications of CRISPR-Cas9 for gene correction and gene editing.

<table>
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<tr>
<th>Organism</th>
<th>Cell type</th>
<th>In vivo</th>
<th>In vitro</th>
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<th>Delivery methods</th>
<th>Strategy used</th>
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<td>Human</td>
<td>iPSC cells</td>
<td>In vitro</td>
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<td>2A-GFP reporter was inserted before the stop codon of the MYF5 gene</td>
<td>Achieved 55% efficiency in the insertion of the GFP gene. Used Cas9-nickase, and</td>
<td>Southen</td>
<td>HDR</td>
<td>Wu et al., 2016</td>
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<td>suggested less off-target effects. Cas9D10A mutant, functions same as a nickase in vitro, and yielded similar HR but lower nonhomologous end joining (NHEJ) rates. Deactivating one of the Cas9 nuclease domains increased the ratio of HR to NHEJ and reduced toxicity. Every single cell expressed CRISPR-Cas9, gRNA1 and gRNA2 reduced mutant huntingtin protein up to 79% and 57%, respectively. gRNA location (Kozak sequence) play an important role in efficient gene-silencing. NHEJ resulted in large number of nucleotide deletions ranging from 72 to 1, in randomly sequenced cells.</td>
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<tr>
<td>Human</td>
<td>HEK293</td>
<td>In vitro</td>
<td></td>
<td>Tested Knock-in rates between 3% and 8%</td>
<td>More than 90% of the mutations led to out-of-frame insertions or deletions resulting in elimination of NMDA receptor and GluA2 function. Two different gRNAs, one located on the plus strand and the other gRNA located on the minus strand were tested, separately. gRNA on the plus strand have shown greater efficiency than the gRNA located on the minus strand</td>
<td>Southen</td>
<td>HDR</td>
<td>Mali et al., 2013</td>
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<td>In vivo delivery of the same vectors resulted in indel mutations in 68% after two weeks of viral delivery. In vivo multiplied-CRISPR and Cas9 vector resulted in indel mutations for two genes in 62% of the cells, whereas for all the three genes, indel mutation was found in only 35% cells.</td>
<td>Southen</td>
<td>NHEJ</td>
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<td>Mouse</td>
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<td>Edited human Hunting transgene using 2 different gRNA, separately in the cells.</td>
<td></td>
<td>Southen</td>
<td>HDR</td>
<td>Mouse: in-utero electricroporation of the plasmid construction Rat: Biolistic transfection</td>
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<tr>
<td>Rat</td>
<td>hippocampal slice cultures</td>
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<td>Edited GluN1 subunit of NMDA receptor and GluA2 subunit of the AMPA receptor.</td>
<td></td>
<td>Southen</td>
<td>NHEJ</td>
<td>Incontro et al., 2014</td>
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<td>Southen</td>
<td>AAV 1 &amp; 2</td>
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<tr>
<td>Mouse and rat</td>
<td>pyramidal neurons (mitotic precursor cells)</td>
<td>In vitro</td>
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<td>knock-out Grin1, the gene encoding NMDA receptor subunit receptor GluN1</td>
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<td>Southen</td>
<td>NHEJ</td>
<td>Staaahl et al., 2017</td>
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<td>Mouse</td>
<td>Neurons</td>
<td>In vitro and In vivo</td>
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<td>In vitro and in vivo comparison of MeCP2 editing Tested multiplexing strategy in vivo for editing Dnmt1, Dnmt3b, Dnmt3a</td>
<td>Explored the efficiency of short-lived Cas9 ribonucleoprotein (RNP) complex RNP complex can overcome the problem with the cas9 permanent integration in the target cells. Variants of Cas9 with multiple SV40 nuclear localization sequences has shown a tenfold increase in the efficiency of editing in vivo.</td>
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<td>NHEJ</td>
<td>Ousterout et al., 2015</td>
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<tr>
<td>Mouse</td>
<td>Neural progenitor cell, Neurons</td>
<td>In vitro and In vivo</td>
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<td>Southen</td>
<td>NHEJ</td>
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<tr>
<td>Human cells &amp;</td>
<td>Human myoblasts</td>
<td>In vitro</td>
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<td>Multiplexing strategy to perform exon-skipping. Restoration in the expression of dystrophin gene in cells (DMD)</td>
<td>Tagging T2A skipping peptide to the Cas9 vector resulted in generation of a single large deletion (exon 45—55) that can correct up to 62% of DMD mutations.</td>
<td>Southen</td>
<td>HDR</td>
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<td>transplanted</td>
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<td>Southen</td>
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<td>Ousterout et al., 2015</td>
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<tr>
<td>Human</td>
<td>iPSCs</td>
<td>In vitro</td>
<td></td>
<td>Generated two isogenic disease-free iPSCs cell lines by knocking-out FUS(A275G) and SOD1(A425V) genes that cause ALS.</td>
<td>The gene targeting efficiency at FUS gene and SOD1 gene was about 1% and 20%, respectively. However, the reason for differential efficiency in HDR with these two genes was not explained.</td>
<td>Southen</td>
<td>HDR</td>
<td>Wang et al., 2017</td>
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3.3. Huntington’s disease and Kennedy’s disease

Huntington’s disease and Kennedy’s disease are inherent neurodegenerative diseases caused by the characteristic polyglutamine repeat expansion in the genes encoding Huntingtin protein and androgen receptor (AR), respectively. In both genes, mutation of this trinucleotide repeat exists in the exon1 region. However, the exact pathology involved with the extended polyQ region is yet to be uncovered. HD is autosomal dominant in nature, and is a gain-of-function type of disorder. The pathological hallmark of HD is a loss of striatal neurons, and aggregation of mutant huntingtin protein in the neurons. Huntingtin protein is essential for the development process, but its function in adults is not fully understood (Zeitlin et al., 1995).

Manifestations of Kennedy’s disease include slow progressive muscular weakness, fasciculation and atrophy (Sobue et al., 1989). AR is not only expressed in reproductive organs, but also in non-reproductive organs, such as the kidney, adrenal gland, skin, skeletal muscle, and nervous system, which indicates the importance of AR in performing various activities in a variety of tissues (Adachi et al., 2007). AR was found to be expressed at relatively high levels in spinal and brainstem motor neurons, the same cells that are prone to be vulnerable in KD, which include nuclear inclusions that contain mutant AR (Li et al., 1998). HD is a fatal disorder, but KD is not. At present, there is no cure for either disease and only palliative treatment options are available. The life expectancy of an HD patient is very short, usually about 10–15 years after the onset of the hallmark choreic symptoms, which includes non-directed involuntary motor movements.

A study conducted by Drouet et al. (2009) has demonstrated that in the mouse models of HD, no worsening of pathology was observed when both mutant and normal huntingtin alleles were partially (up to 50%). Similar studies, conducted in mice (Duyao et al., 1995) and humans (Ambrose et al., 1994; Persichetti et al., 1996), have demonstrated that loss-of-function of the wild-type allele will not worsen the HD pathology. However, there are other studies which suggest that the survival of the neurons will be affected by a long-term inactivation of the huntingtin protein in the adult brain (Cattaneo, 2003; Chen et al., 2005).

Use of a knock-out animal model using CRISPR-mediated HDR strategy targeting the CAG repeats, will help determine the precise impact of the polyQ region. CRISPR-mediated HDR can also be used in therapeutic implications to treat HD and KD by using an ‘on-skipping’ strategy, which has been shown to completely reverse the disease pathology in a mouse model of muscular dystrophy (Long et al., 2014; Ousterout et al., 2015). Kolli et al. (2017) have recently demonstrated that CRISPR-Cas9 NHEJ mechanism can produce large nucleotide deletions of up to 72 nucleotides. This study suggests a possibility that CRISPR-Cas9 may be effectively used to reduce the number of CAG repeats, and create truncated, but fully-functional, HTT.

4. Challenges, limitation and rectifications in CRISPR/Cas9 technique

Despite the huge potential of CRISPR/Cas9 for gene therapy and genome editing, there are challenges that need to be overcome when generating and treating animal models of human diseases.

4.1. Off-target effects

One of the most serious issues with CRISPR is the off-target effects, for which the key factors are the gRNA and the Cas9. Because the gRNA of the CRISPR-Cas9 targeting system is only 17–24 bp in size, there is high chance that the selected-gRNA exists elsewhere other than the target regions. Also, there is a good chance that a sequence with only minute changes to the selected-gRNA might exist on the genome and cause mis-targeting. However, recent studies suggest that Cas9 can withstand mismatches, and is dependent upon the distribution and number of mismatched nucleotides (Hsu et al., 2014; Mali et al., 2013; Hu et al., 2014), particularly mismatches at the 5’-terminal have proven to be tolerated well. Another aspect to lower off-target effects is to pick a gRNA sequence with low GC content (<35%), because high GC content makes nucleic acid hybrids more stable and thus increases the tolerance of the mismatches. At present, many on-chip data bases, such as IDLV capture, GUIDE-seq (Tsai et al., 2015), Digencode-seq (Kim et al., 2015), direct in situ break labeling, and enrichment of streptavidin (Ran et al., 2015) strategies, are available to detect potential off-target editing.

Off-target effects can be reduced in CRISPR-Cas9 system by altering the Cas9 enzyme to act as a nickase, which is capable of cleaving only one target DNA strand (Kim and Kim, 2014). The Cas9 nickase is applicable in the genome editing by creating a nick which can be repaired by HDR. This system can also be used with two gRNAs which are complementary to opposite sides of the target DNA sequence to produce paired nicks for gene editing via both NHEJ and HDR pathways (Xu et al., 2014). Reducing Cas9 protein availability in the cells could be another strategy which can be made possible by decreasing prolonged Cas9 expression in the cells, or by direct injection of Cas9 protein (Hsu et al., 2013).

4.2. Homology-direct repair

Although CRISPR/Cas9-mediated gene editing through NHEJ has a high efficiency, the rate of HDR is relatively low. Many studies performing HDR have reported a rate of knock-in frequencies between 3.5% and 15.6% (Hruscha et al., 2013; Mali et al., 2013). Other experiments have demonstrated efficiencies as high as 45% HDR rate in Toxoplasma gondii (Shen et al., 2014). When there is a disruption or damage in the DNA strands, the endogenous DNA repair mechanisms react spontaneously, leading to an NEJM type of editing. This is because NEJM takes place at growth 1 (G1) and mitotic (M) phases (Daley and Sung, 2014), whereas HDR occurs at the synthesis (S) and premitotic (G2) phases (Heyer et al., 2010). Thus, if NEJM is suppressed by using molecules, such as KU70 and DNA ligase IV inhibitor (Chu et al., 2015; Maruyama et al., 2015), high efficiency of HDR can be achieved. Studies also suggest that the rate of HDR is dependent on gRNA concentration and Cas9 protein availability in the embryos (Hwang et al., 2013; Guo et al., 2014). As high concentrations of gRNA and Cas9 proteins potentiate off-target effects, it is very important to have an optimal balance between the concentration of the gRNA and Cas9 proteins to optimize the efficiency of HDR obtained.

4.3. Allele-specific editing

Many genetic disorders are often dominant in nature. Some dominant genetic mutations might result in the toxic-gain-of-function. In such cases, along with editing the mutation on one of the alleles, it is also important to spare the functioning of the other normal allele in order to minimize a possible loss-of-function of the proteins transcribed from that gene. In CRISPR-Cas9 system, gRNA is about 17–24bp in length and is homologous to the target site where the DBS on the gene is intended. Cas9-gRNA complex binds to a particular sequence, PAM, located next to the gRNA. PAM is usually -NGG (for S Pyogenes-derived Cas9), where N can be either an adenine, guanine, cytosine, or thiamine base. Specific allele targeting can be brought about in two ways. One uses the introduction of the single nucleotide polymorphisms (SNPs), and the second
utilizes the identification of naturally existing SNPs in the mutant allele. The first has already been tried using an in vivo gene editing process, which employs the CRISPR-Cas9 system. Courtney et al. (2016) have shown that NEJM-mediated-addition of nucleotides has introduced a SNP in heterozygous dominant disease causing alleles, which resulted in a novel PAM sequence. By making use of this new PAM in the dominant allele, these scientists have shown the potential of the CRISPR-Cas9-based system to edit the mutation alone, while sparing the function of the other normal allele. The latter, which is the identification of naturally existing SNPs, could be more challenging because the PAM sequence is often not located where SNP exists in the gene. In addition, a compelling factor associated with efficient silencing would be the location of the selected gRNA.

4.4. Delivery methods

Delivery of the nucleic acids is challenging and include such methods as (1) direct plasmid administration (electroporation, microinjection, etc.), (2) non-viral (e.g., chemical, lipofectamine, nano-particles, dendrimer, cationic polymers, and viral-mediated vehicles, such as adenovirus vector, adeno-associated virus, retrovirus, lentivirus, etc.). Direct introduction of the plasmid with gRNA and Cas9 protein through nucleofection, electroporation, or transfection techniques lack efficiency (Kim and Kim, 2014). However, the delivery of nucleic acids using viral and non-viral delivery mechanisms has proven to be more efficient. Unlike the siRNA, TALENs, ZFNs, and microRNA, the CRISPR-Cas9 plasmid is huge (>10 kb), which leaves minimal options for its delivery, in vivo or in vitro. In addition, chemical methods of nucleic acid delivery are inefficient and are cytotoxic in primary cells. To circumvent this problem, the microinjection or electroporation method is favored, but is not optimal, due to its invasiveness and higher cell mortality rate.

At present, viral vectors, such as adenoviral vectors (AdVs), integrase-defective lentiviral vectors (IDLVs), and recombinant adenovirus-associated viral vectors (rAAVs), are being used extensively to deliver CRISPR-Cas9 for both in vitro and in vivo applications. The advantage of using IDLVs-Cas9 is its transient expression as it does not integrate into the host DNA and thus, the efficacy of CRISPR-Cas9 system is greatly enhanced (Wang et al., 2015). However, the downfall of using this vector is the persistence of the Cas9 expression, leading to off-target effects. AdVs and rAAV vectors are also commonly used viral vectors because of their non-integrating nature, low immunogenicity, and non-pathogenicity (Swiech et al., 2015). The only disadvantage of using rAAV over IDLVs (accommodate – 10 kb) is its smaller accommodation size (~4.5 kb) (Mefford et al., 2015). CRISPR-Cas9 components are about 8–10 kb. Thus, packaging all of the CRISPR-Cas9 components in a single rAAV is impossible. Researchers have achieved gene-editing using two separate rAAV, but this adaptation compromises the efficiency of the CRISPR-Cas9 system (Ran et al., 2015).

5. Conclusion

Compared with the other gene editing tools, such as ZFNs, TALENs, and the CRISPR-Cas9 system is being rapidly employed for both gene editing and gene correction as a treatment strategy for many genetic disorders, primarily due to its ease of construction, efficacy, cost-effectiveness, and capability of editing multiple genes at the same time. Its application has not been limited to the gene editing, but is widely applied in regulating the genes. Despite some potential off-target effects that were experienced initially, recent research in this area continues to lead to significant improvements of this system. Owing to the plethora of applications, CRISPR-Cas9 system is thought to have enormous potential for treating many genetic disorders. At present, CRISPR-Cas9 has been successfully applied in animal model of various diseases, but further research is needed before translating these techniques to humans. Certainly, CRISPR is a revolutionary technology with a huge potential for facilitating drug discovery, gene therapy, and uncovering pathological aspects of many diseases.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

Support for this study was provided by the Field Neurosciences Institute and from the Office of Research and Sponsored Programs, the College of Medicine, the Program in Neuroscience, the College of Humanities and Social & Behavioral Sciences at CMU, and the John G. Kulhavi Professorship in Neuroscience at CMU.

References


