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Review Article

CRISPR/Cas9: a Revolution towards Treatment for Neurodegenerative Disorders -

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ABSTRACT

Age-dependent disorders in terms of neurodegenerative disorders are highly reported for progressive neural dysfunction. Animal models are exceptionally meaningful to assimilate the pathogenesis and treatment of neurodegenerative disorders. Small animals evoke pathological limitations as compared to large animals. CRISPR/Cas9 is a novel technique widely used to modify genome of many species using gene therapy approach. CRISPR/Cas9 is capable to correct DNA sequences by non-homologous end joining and homologous directed repair mechanisms. In this review, we illustrate the use of CRISPR/Cas9 to generate embryonic DNA in brain cells. We also describe the use of CRISPR/Cas9 technology by using knock out approach to generate transgenic animal models for rare neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS), Friedreich Ataxia (FRDA), and Neuronal Ceroid Lipofuscinoses (NCLs) to treat their causative agents.

Keywords: Neurodegenerative Disorders; Gene Therapy; CRISPR/Cas9; Animal Model; Gene Knockout; Zebrafish

INTRODUCTION

Neurodegenerative disorder is a colossal term to implement on age-dependent neurodegeneration conditions. Neurons are the main constituents of central nervous system with no regeneration capability proved to be life threatening. A wide range of diseases- Alzheimer's Disease (AD), Amyotrophic Lateral Sclerosis (ALS), Friedreich Ataxia (FRDA), Frontotemporal Dementia (FTD), Neuronal Ceroid Lipofuscinoses (NCLs), Huntington's Disease (HD) and Parkinson's Disease (PD)- are clustered under the umbrella of Neurodegenerative disorders. The high prevalence rate of neurodegenerative disorders is associated with reduced life expectancy of humans. Most of the disorders are sporadic while rate of genetic mutations are quite low (4-5% only) [1]. The pathogenesis of these disorders is not clear and effective treatment is not available yet.

The evaluation and characterization of effective therapeutic agents execute by animal models for neurodegenerative disorders. Transgenic models have great potential of genetic variability with the aid of vast variety of genetic tools. Wide ranges of transgenic animal models -mouse, pigs, sheep, zebrafish and non-human primates- are established [2]. In case of neurodegenerative disorders the most prevalent symptom is emotional abnormalities which are not observed in mouse model due to their rapid growth rate. Furthermore it takes 150 days for human brain development to the full extent and only 21 days in mouse leading to less accumulation of misfolded protein in neural cells [3].

Several other transgenic models of large animals -pigs, sheep, and non-human primates- have been used successfully. Non-human primates have an advantage over other transgenic models as they can mimic the neurological and behavioral symptoms associated with patients [4]. The development of first transgenic monkey *(rhesus)* against Huntington's disease in the year 2001 verified this idea. The modification within the genome of large animals by endogenous modifying genetic engineering techniques is difficult due to lack of embryonic stem cell lines production. The development of recent genetic engineering techniques makes it possible to investigate large animal models for neurodegenerative disorders [5].

CRISPR/Cas9 is a versatile tool in the library of genetic engineering techniques and proves to be a revolutionary step in biological research, covering a wide range of organisms and cell types including bacteria, pig, mice, rat, zebrafish, human pluripotent and stem cells human somatic cells [6]. CRISPR was first discovered as set of short repeats downstream to *iap* gene in *E.coli* genome. It modifies DNA in germ-line cells without the need of embryonic stem cell establishment. CRISPR/Cas9 matches 23 base pairs to target in a sequence-dependent manner and creates non-specific mutations within genome [7]. CRISPR/Cas9 can disturb single or both alleles of a gene thus rendering the transfer of X-linked inherited disorders to next generation. In this review we discuss about the application of CRISPR/Cas9 in rare neurodegenerative disorders.

CRISPR/Cas9 technology

The most efficient and novel technology in genome editing, that has revolutionized the diverse field of gene therapy, is CRISPR/Cas9 technique [8]. CRISPR/Cas9 gene editing disrupts or knockout the defective gene and regulates the normal gene function by introducing the normal gene construct [9]. In 1987, the story of CRISPR (clustered regularly interspaced short palindromic repeats) emerged while Nakata and colleagues working on *iap* gene that was involved in alkaline phosphatases isozyme conversion in E. coli and reported an exclusive set of about 29 nucleotide repeat downstream of gene *iap* [10,11].

CRISPR-Cas was discovered as a microbial defense system against phages and viruses by CRISPR RNA-based DNA targeting (crRNA) and DNA cleavage mediated by Cas nuclease [12]. Over a wide range of archaea and bacteria mainly three types of CRISPR systems I, II and III were identified on the basis of structures and sequences of Cas protein [9]. The observed ratio of CRISPR-Cas in sequenced archaea and bacteria is about 90: 40 percent of their genomes.

For identification and disruption of targeted nucleotide sequences type I and III retained multiple Cas proteins [13]. These multiple protein is known to form complexes with CRISPR RNA (crRNA). In type I Cascade complex is formed while in case of type III Cmr or Csm RAMP complexes are formed [11]. While in case of type II Cas proteins significantly decrease in number. However, increasingly comprehensive mapping and interpretation of CRISPR loci across wide range of microbial species, their biological importance remained evasive [14].

CRISPR locus comprises a series of conserved array of repetitive elements interspaced by distinct nonrepetitive array (sequences) known as protospacers. In CRISPR-Cas system, foreign DNA that is invaded, further processed by Cas 9 nuclease resulting into smaller DNA fragments. These smaller DNA fragments are then further integrated into host`s genomes as spacers at CRISPR locus [8]. Upon phage and viruses infections, these spacers are served as transcriptional templates for crRNA production, which directs Cas to disrupt target DNA sequences of introducing phages and viruses. Several families of Cas protein was identified, imparting greater role biogenesis of crRNA, incorporation of spacers and DNA cleavage that have been invaded (Figure 1) [15].

The Type II CRISPR system is the most efficient among the other

types because it requires only Cas 9 as cas protein, that retains RuvC and HNH like nuclease domains. The crRNA formed a complex with tracr- RNA (Trans activating crRNA), that direct the Cas9 toward the target sequences. At the target site cleavage mechanism requires DNA sequence protospacer complement to crRNA and a short PAM (protospacer adjacent motif) [16]. After the target site binding cleavage of single stranded DNA which is complementary to crRNA and opposite strand are promoted. This respective cleavage is directed by HNH and RuvC nuclease domain of Cas9, producing a double strand break at targeted site [17].

For efficient genome editing, scientists designed a gRNA (guide RNA), which was a chimeric RNA retaining all crucial crRNA and tracrRNA elements. In this way a diverse range of CRISPR cas 9 variants was synthesized, recognizing 20 - 24 nucleotide sequences complementary to the synthetic gRNA and 2 to 4 nucleotide in PAM sequences at targeted site. In such a manner CRISPR Cas 9 target discrete 22 to 29 nucleotide sequences that is specific in various genome [18]. However, recent research work revealed that CRISPR Cas9 had high mismatches tolerance among gRNA and its complementary sequence at target site. For instance, mismatches up to six bases were reported to be tolerated by CRISPR Cas 9 system in Streptococcus pyogenes [19].

The double strand break produced by CRISPR Cas 9 will stimulate the cellular DNA repair mechanisms. This DNA repair mechanisms involves NHEJ (Nonhomologous End-Joining) may having an error and HDR (homologous directed repair) which is an error free DNA repair pathway [20]. DNA repair in NHEJ can quickly ligate Double Strand Break (DSB) but inducing small deletion and insertion mutation at the targeted site. This type of mutation open a new way for researchers to abolish or disrupt the function of genes at targeted site in the genome [21]. DSB can also be eliminated by HDR (Homologous Directed Repair) which is complex than NHEJ repair mechanism. HDR error free mechanism requires donor DNA sequence having homology which serves as a repair template. (Figure 2) To date several researches conducted involving this strategy in genetic engineering without leaving an error behind [22].

CRISPR genes could be delivered ex-vivo into various forms, like DNA, mRNA and ribonucleoproteins (Table 1) [23].

Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS), first discovered in the beginning of 19th century, known as rapidly degenerating progressive disorder of motor neuron and muscle denervation coinciding the area of cerebral cortex, brain and spinal cord [24]. According to the statement of Jean-Martin Charcot delivered in 1887, la sclérose amyotrophique": The diagnosis as well as the anatomy and physiology of the condition ALS are one of the most completely understood conditions in the realm of clinical neurology. Discussing about its two types: Sporadic and familial (inherited) with prevalence ratio of about 90: 10 [25]. The incidence of ALS is uniform in the region of Europe, 2-16 persons per 100000 per year with no age restrictions. The incidence rate is greater in men as compared to women, about 1.5:1 in case of familial ALS [26]. Up till now more than 20 genes have been discovered to be a cause of ALS, most important of them are: SOD1, TARDBP, FUS and ANG (Figure 3) [27].

The sphincter muscles are affected with dementia and impairment of connective tissues in 50% patients. Symptoms include reduced exercise capacity fatigue, dysphagia, weight loss, malnutrition, breathing issues, exertional dyspnea, orthopnea, early morning headaches, respiratory failure and ultimately death [28].

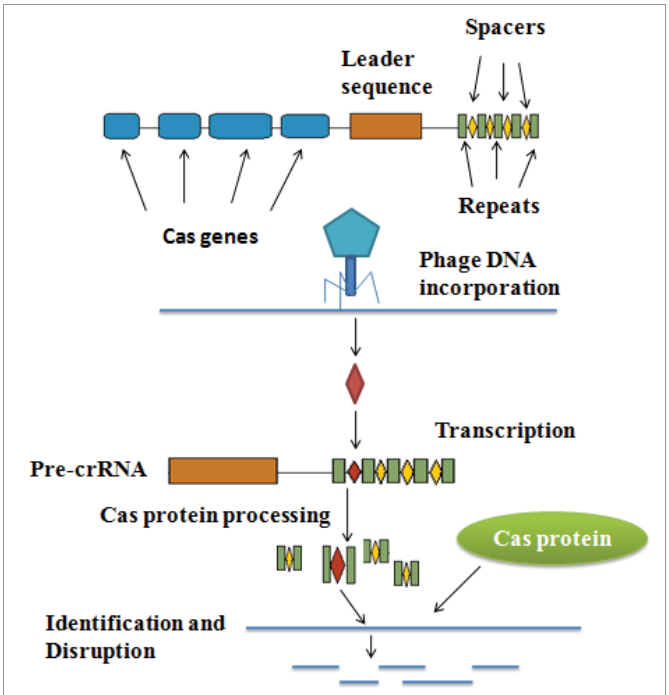

Figure 1: Cas protein mediated DNA disruption and identification.

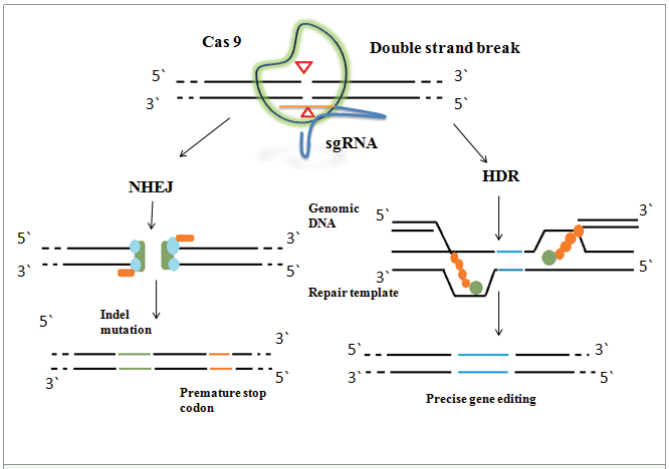

Figure 2: CRISPR/Cas-9 cascade following Non-Homologous End Joining (NHEJ) and Homologous Directed Repair Mechanisms (HDR).

No specific diagnostic test has been developed for ALS. Diagnostic criteria depend on assessing the upper motor neuron and lower motor neurons of voluntary muscles in patients with a time span of 14 months due to delayed onset of symptoms. About 50% patients die during the time span of 30 months and survival rate is 20% after onset of symptoms [29,30]. A number of risk factors are associated with onset of disease involving genetic as well as environmental factors. Exposure to electromagnetic waves, electric shocks, smoking, heavy metals, formaldehyde, chemicals, organic solvents, pesticides and trauma is strongly associated with ALS onset. An inverse relation is reported between BMI and vitamins. No effects of high physical activity observed in ALS [31].

CRISPR/Cas9 approach in ALS treatment

FUS protein is crucial to avoid for ALS disorder. Taking protein into account, FUS (fusion), along with EWSR1 and TAF-15 interact

Table 1: CRISPR gene ex-vivo delivery approaches.

CRISPR genes	Delivery method	Pros and Cons	Solution
DNA plasmids	1. Transfection 2. Electroporation like • Nucleofector 4D™ • Neon® Transfection System	Gene silencing, off-target effects and immune responses	-
DNA packaging within virus	Adeno-Associated Viruses (AAV)	Reduced efficacy	Smaller Cas9 orthologs form other species
mRNA transcript	Microinjection	mRNA immunogenicity and stability	• Addition of Tool-Like receptors for enhanced immunogenicity • Capping and tailing to increase stability
RNA packaging within virus	Lentiviruses	Limited to specific cell types could be infected by lentivirus	-
Ribonucleoproteins	Non-standard techniques • Lipid-based transfection • Non-lipid based transfection	Rapid degradation	-
Ribonucleoproteins	Covalent functionalization	Cell toxicity	-

Table 2: Classification and characteristics of neuronal ceroid lipofuscinoses (NCLs).

Gene	Locus	Gene Product	Disease	Size (amino acids)	Clinical Phenotype	Eponym
CLN1	1p34.2	PPT1	CLN1	306	Infantile classic, late infantile, juvenile, adult	Haltia–Santavuori
CLN2	11p15.4	TTP1	CLN2	563	Late infantile classic, juvenile	Janský–Bielschowsky
CLN3	16p11.2	CLN3	CLN3	438	Juvenile classic	Spielmeyer-Sjögren
CLN4	20q13.33	DNAJC5	CLN4	198	Adult autosomal dominant	Parry
CLN5	13q22.3	CLN5	CLN5	407	Late infantile variant, juvenile, adult	Finnish variant late infantile
CLN6	15q23	CLN6	CLN6	311	Late infantile variant, adult (Kufs type A)	Lake-Cavanagh early juvenile /Indian variant late infantile, adult Kufs type A
CLN7	4q28	MFSD8	CLN7	518	Late infantile variant, juvenile, adult	Turkish variant late infantile
CLN8	8p23.3	Unknown	CLN8	286	Late infantile variant, progressive epilepsy with mental retardation	Northern epilepsy/progressive epilepsy with mental retardation
CLN10/CTSD,	11p15.5	Cathepsin D	CLN10	412	Congenital classic, late infantile, adult	congenital
CLN11	17q21.31	Progranulin (GRN)	CLN11	593	Adult	Adult variant
CLN12/ATP13A2	1p36.13	ATPase Type 13A2	CLN12	1180	Juvenile, Kufor-Raheb syndrome	Juvenile variant
CLN13/CTSF	11q13.2	Cathepsin F	CLN13	484	Adult Kufs type	Adult Kufs type B
CLN14/KCTD7	7q11.21	KCTD7	CLN14	289	Infantile, progressive myoclonus epilepsy 3	Infantile

with transcription factor TFIID and C-terminal of RNA polymerase II, is member of conserved RNA-binding protein family (FET family) which resides between cytoplasm and nucleus. FUS protein, first identified as an oncogene, is encoded by 526 amino acids. It has long N-terminal domain of prion like (LC) and glycine rich regions, and C-terminal with two Arg-Gly-Gly (RGG)-repeat motifs, RNA Recognition Motif (RRM), RanBP2-type Zinc finger domain and non-conventional nuclear localization signal NLS [32]. FUS gene is involved in transcription, post-transcriptional splicing of pre mRNAs, translation and DNA repair. It also acts as synaptic modulator in Central Nervous System [33]. FUS binding with RNA occur due to the activity of zinc finger motif with GGUG motif of RNA. Due to prion like activity of FUS gene it is used in *Saccharomyces cerevisiae* to form protein aggregates just like in human diseases [34]. FUS is involved in DNA damage repair by recruiting it to damage site to accumulate γH2AX at target site. Double stranded breaks translocate FUS to cytoplasm from nucleus and phosphorylate the C-terminus. FUS DNA repair mechanism entirely depends on its interaction with HDAC1. By controlling DNA repair, FUS also regulates RNA processing phenomenon [35].

More than 50 mutations have been reported in FUS gene majority of them are missense dominant in nature. Most of the FUS mutations cluster in NLS and RGG region of C-terminus and LC region of N-terminus. FUS mutations hold upto 35% prevalence rate in patients under the age of 40 years and could either result in loss or gain in function. Overexpression of FUS mutant gene or knockdown of FUS alters RN polymerase II distribution within nuclei and become a significant cause of neurodegenerative phenotype [36]. The over activity of FUS results in the formation of aggregates in nucleus and cytoplasm of motor neurons, consequently leading towards motor neurons death [37]. Loss of FUS gene results in alteration of non-homologous end joining ND homologous recombination in neuron. The consequences of mutation in FUS gene include the progression of ALS and eventually leading to neural death. The mutant gene form more stable complex with RNA and alter the DNA damage repair and RNA splicing process [38].

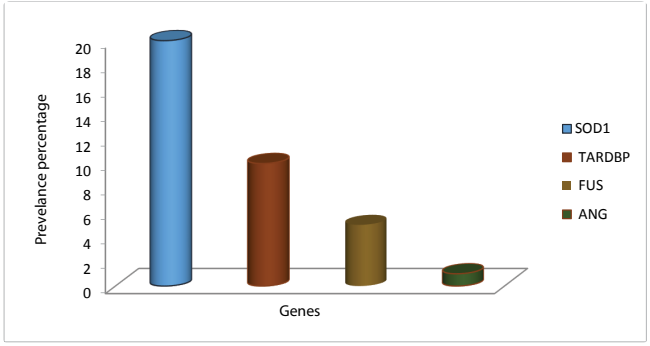

Figure 3: Prevalence of major genes associated with amyotrophic lateral sclerosis.

In a recent experiment conducted by scientists on zebrafish strains AB and TU, mRNA library was synthesized and mapped on zebrafish genome. Mass spectrometric analysis was performed using C18 column and a 240 minute LC gradient. Confocal microscope was used for Zebrafish trunks imaging. Fus morpholino of about1mM concentration was injected into 1-cell staged embryos. Embryos at 2-dys post fertilization stage was selected and analyzed for their movements by touching their tail with the aid of forceps.

To treat FUS mutation, CRISPR-Cas9 technology was used and experimented on zebrafish. The use of CRISPR-Cas9 is to disrupt gene function to explore the altered cellular biology of gene. The CRISPR-Cas9 using the knockout approach disrupt the gene with a single guide RNA targeting exon 3 deleting 8 base pairs and resulting in a frameshift mutation and a premature stop codon. The most suitable tissue to determine gene deregulation is brain. The mutant and transgenic lines were detected on Mass Spectrometry (MS) concluding that FUS protein was not produced in transgenic lines and function of protein is lost. High radiation sensitivity of zebrafish was observed with no apparent changes in transcriptome and proteome [33,39].

Neuronal ceroid lipofuscinoses (NCLs)

The Neuronal Ceroid Lipofuscinoses (NCLs), first discovered in the beginning of 19th century, is a rapidly progressive juvenile lysosomal storage neurodegenerative condition with altered lysosomal and an excess expression of ceroid lipofuscin within neurons [40]. It's the most prevalent disorder of childhood (1/7,500) with different incidence rate in different regions worldwide [41]. NCL could be classified based on the criteria for age for on-set of disease: Infantile Neuronal Ceroid Lipofuscinoses (INCL), Late-Infantile Neuronal Ceroid Lipofuscinoses (LINCL), Juvenile Neuronal Ceroid Lipofuscinoses (JNCL) And Adult Neuronal Ceroid Lipofuscinoses (ANCL). A total number of 13 genes with 360 mutations reported to cause NCLs being NCL1, NCL2 and NCL3 major reported types (Table 2) [42].

The accumulated material coincides the region of lysosomes in numerous forms: Curvilinear Profiles (CLP), Fingerprint Profiles (FPP), Granular Osmiophilic Deposits (GRODs) and Granular Osmiophilic Deposits (GRODs) [43]. Symptoms include cognitive and motor decline, vision loss, Seizures, ataxia, myoclonic epilepsy, learning difficulties, dementia and ultimately death. Diagnostic tests for NCL include enzyme testing, electron microscopy, ultrastructure assessment genome sequencing, skin biopsy, brain MRI and EEG [44].

CRISPR/Cas9 approach in NCL treatment

CSPα is a protein responsible for NCL disorder. Discussing the protein, CSPα with other 39 members belongs to a family of highly conserved J-proteins present in bacteria, virus, fungi, plants and animals. A significant J domain is present in every member who is of 70 amino acids distributed in 4 helices with n tripeptide motif of histidine, proline and Aspartic Acid (HPD) within helices II and III. Outside of J domain a 25 amino acids domain known as Cysteine String Protein (CSPs) (weighing 34kDa) having 13-15 cysteine residues is present. A hydrophobic region, N-terminal and C-terminal for alternate splicing also exists within J protein [45]. It activates the ATPase activity of Hsc70 to do conformational changes in proteins for their transport across mitochondrial membrane. N terminal is responsible for binding of protein to different substrates [46].

Mutation in CSPα in HPD [substitution of leucine-115 with an arginine (L115R) or deletion of the neighbouring leucine-116 (ΔL116)] domain results in misfolding of proteins with the consequences in a number of neurodegenerative disorders, type II diabetes and cystic fibrosis. The mutation result in adult NCL with onset at the age of 20-30 years. Its activation depends on its assembly with small glutamine-rich tetratricopeptide repeat-containing protein (SGT) and 70-kDa heat-shock cognate protein (Hsc-70) [47]. Misfolded protein accumulates in neurons, increases ROS stress and leads to their degeneration [48].

In a recent experiment conducted by scientists on zebrafish strain AB. At first they amplified the gene fragment by running PCR. The plasmid construction was performed by using different vectors- p5E-HuC, pME-EGFP, pDestTol2pA, and pME-mCherry and transformed into *E.coli.* Transgenic fish lines were created by microinjecting plasmids with Tol2 transposase mRNA at embryonic stge. Larvas at 3-dys post fertilization stage was selected, raised and out breed with wild type. After breeding F1 progenies were elected and experimented further. Taking help from online available tool, ZIFIT Targeter, CRISPR/Cas-9 target sites were constructed. Cas-9 mRNA and sgRNA (single guided RNA) were designed and template generation was performed with PCR. The transcription of mRNA is performed with mMESSAGE mMACHINE T7 ULTRA kit (Ambion) and that of sgRNA with *T7* polymerase. The transgenic male fish and wild time female fish were allowed to cross and resulting embryos were injected with mRNA (300 ng/µl) and sgRNA (60 ng/µl). Genomic DNA of 5 embryos at 48-72h was collected, incubated and neutralized. Real-time PCR was performed to amplify target sites of CRISPR. DNAJC5 gene mutation was treated using CRISPR-Cas9 technology and experimented on zebrafish. CRISPR-Cas9 is used to disrupt DNAJC5 gene function to explore the altered cellular biology of specific gene by using the knockout approach with the aid of sgRNA targeting. Random Indels were induced resulting in frameshift mutation and a premature stop codon with 40% reduced expression of gene product [49].

Friedreich ataxia (FRDA)

Friedreich Ataxia (FRDA) characterized as an autosomal recessive neurodegenerative abnormality caused by mutation in FXN gene which encodes mitochondrial frataxin protein. In 1988 the mutated gene was mapped to chromosome 9 in FRDA [50]. Over the period of 1863-1877 Friedreich Ataxia (FRDA) disease was described in five papers by Nicholaus Friedreich [51].

Most of Friedreich ataxia patients develop several neurological symptoms such as flexia, ataxia, sensory loss, dysarthria, and motor

dysfunction at 5-15 years of age but late onset are rarely reported with prevalence rate of 1 person per 50,000 people in USA populations [52]. Muscle weakness, foot deformity, scoliosis, diabetes and cardiac symptoms were also reported in previous studies [53]. Currently no possible therapeutic approaches available yet for Friedreich ataxia. But still several drugs are in development phases and testing to increase frataxin expression or mitochondrial function [54].

CRISPR/Cas9 approach in FRDA treatment

Frataxin protein encoded in nucleus and translated by cytoplasm then transported to mitochondria for its maturation and processing by Mitochondrial Peptidases (MPP) [55]. FXN is a vital protein of about 210 amino acid residues mostly conserved from bacteria to human. Frataxin is a globular protein incorporated into the internal mitochondrial membrane comprising six antiparallel β-sheets one side flanked by two α-helices. Its α1-helix has twelve acidic residues and adjacent anionic patch formed by β1-sheet rendering charged dipole on protein molecule [56]. The nature and size of this configuration determine potential interaction with other ligands or proteins [57]. This frataxin protein impart greater role in iron sulfur cluster biogenesis which are the part of enzymes involved in electron transport chain [58]. Frataxin deficiency leads to a variety of cellular abnormalities, reduced level of ATP production and level of iron increases in mitochondria ensuing to oxidative stress and cell death particularly neurons which are sensitive to oxidative stress [59].

Friedreich ataxia mainly caused due the mutation in both alleles of FXN gene which encode frataxin protein. An expansion of GAA (Guanine-Adenine-Adenine) triplet is repeated frequently in first intron of FXN gene. In Friedreich ataxia 98% chances of mutation is due to expansion of GAA repeat in first intron of both of two alleles while remaining 2% is due to GAA triplet repeat in one allele and deletion or point mutation in other allele. This expansion promote epigenetic modification that occur upstream of gene by silencing it [60]. The repeats in normal allele is about 35 or lesser in number while mutated allele may have more than 1000 repeats. Transcription of frataxin messenger RNA (mRNA) disrupted due to expansions of GAA repeat decreasing the mitochondrial frataxin protein level, found throughout the body [55].

Recently, for the treatment of Friedreich ataxia CRISPR/CAS 9 knockout technologies were used to transfect the human cells. In this experiment, two different CRISPR-13 and CRISPR-14 (RNA editing tools) were designed using an Addgene kit. Using the sequence library of about 190000 sequences, sngRNA was designed to target FXN exons. For the detection of FXN specific modifications induced by CRISPR, T-REx293-cFXN cells were transfected with CRISPR expression vector and amplified through PCR. Surveyor assay analysis of PCR product predicted the relative, efficient cutting specificity of both CRISPR-I3 and -I4 at their target site. The cutting mechanism of CRISPR-13 reported to be more efficient in this experiment. Indels occur at Random site in exon 4 as a result disruption of FXN gene occurs. Then the normal expression of frataxin protein can be supplemented by engineering these cell lines using CRISPR/CAS-9 and an exogenous, inducible FXN gene incorporated in to the genome that compensate the endogenous FXN gene bi-allelic knockout [55].

Effective targeting of FXN gene in Friedreich Ataxia (FRDA), CRISPR/Cas-9 technology was used to design animal model allowing proper consideration about the disease progression by removing the GAA repeat through CRISPR/Cas9 approach, evaluating the effect of decreased level of frataxin protein and molecular basis of Friedreich ataxia [55]. Reduced sensitivity to oxidative stress of FRDA fibroblasts was suspected when herpes simplex virus given to these fibroblasts. No effect of iron level was reported in nervous system of Friedreich ataxia patient and in conditional knockout (mouse) model [61]. Using a pair of sgRNA CRISPR/Cas-9 and a plasmids expressing the *sp*Cas9, it was reported that the GAA excision enhances the expression of FXN protein enough to abrogate the Friedreich ataxia symptoms in the YG8sR model [62]. Not only the GAA triplet was removed but a normal copy of FXN gene also supplanted by NHEJ. It was suggested in experiment that CRISPR using a pair of sgRNAs and a plasmids expressing the *sp*Cas9 was done by electroporation in vivo data, allowed to verify whether the GAA excision enhances the expression of FXN protein enough to abrogate the Friedreich ataxia symptoms in the YG8sR model (Figure 4) [62].

FXN gene Exon 4 of FXN is shown and CRISPR-I4 target site was shown with vertical red arrow. The targeting construct is shown below the *FXN* gene with dotted lines indicating regions of homology with the target locus. Black and red horizontal arrows indicate target-specific and construct-specific PCR primers, respectively. Underneath are the expected PCR product and its expected size (1.8 kb).

The cutting mechanism of CRISPR-13 induced indels at Random site in exon 4 as a result disruption of FXN gene occurs. Then the normal expression of frataxin protein can be supplemented by engineering these cell lines using CRISPR Cas 9 and an exogenous, inducible FXN gene incorporated in to the genome that compensate the endogenous FXN gene bi-allelic knockout. In this way CRISPR CAS 9 impart vital role in gene engineering at the FXN rescuing the phenotype in Friedreich ataxia [55].

DISCUSSION

Neurodegenerative disorders occur from accumulation of misfolded protein causing the dysfunction or death of neurons with mutations in genome. Recent research using CRISPR/Cas9 on neurodegenerative disorders have provided us with great opportunities to deal with them. Great research has been performed on Alzheimer's Disease (AD), Huntington's Disease (HD) and Parkinson's Disease (PD). Whereas Amyotrophic Lateral Sclerosis (ALS), Frontotemporal Dementia (FTD) and Neuronal Ceroid Lipofuscinoses (NCLs) are in preclinical phase with some specific sort of small transgenic animals like zebrafish. It is the need of the hour to develop large animal models to find a cure for these disorders as well as consider other disorders.

Despite the fact that the use of CRISPR/Cas9 has brought a great revolution, it still has ethical and social concerns. Several limitation of CRISPR/Cas9 reported since its development. CRISPR/Cas9 induced mutations diluted in small animals over short time span but it requires very long time span for large animals. Other limitation with CRISPR/

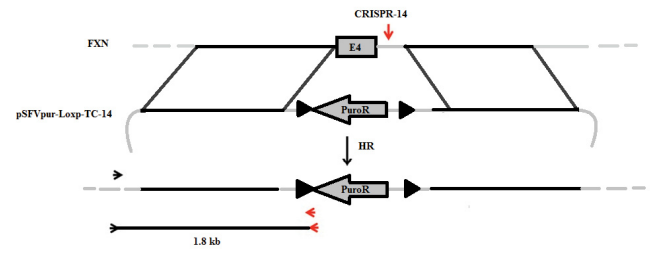

Figure 4: FXN gene disruption by CRISPR-I4.

Cas9 accounts for mosaic mutations which should be considered and monitored carefully. To make the knock-out of a gene confirm, multiple target sites of CRISPR/Cas9 are designed. Another issue reported with the use of CRISPR/Cas9 is off-target effects [63]. This issue is resolved by decreasing the concentration of Cas9 and using specific gRNAs [64]. The low rate of homologous recombination also hinders the activity of CRISPR/Cas9 [65]. Double Stranded Breaks (DSB) also contribute to about 1500 time off target effects which overcome by synthesizing a pair of Cas9 nickases that create Single Strand Break (SSB) rather than Double Stranded Breaks (DSB) [66]. Creating truncated sgRNA, CRISPR Cas9 in combination with other nucleases and peptide mediated delivery, reported to reduce the chances of off-target effects also enhanced genetic engineering efficacy of CRISPR Cas9 system [67]. To address all the issues further development is required in CRISPR/Cas9 system.

CONCLUSION AND FUTURE PERSPECTIVES

Transgenic approach and CRISPR/Cas9 proves as an umbrella to combat variety of disorders. CRISPR/Cas9 is a tremendous genome editing tool with several advantages over primitive techniques. It has shown therapeutic potentials over a range of animal models and cell lines being diagnosed for number of disorders (genetic, neurodegenerative). Before CRISPR/Cas9 covers journey from pre-clinical to clinical trials, many hurdles should be overcome- ethical concerns, mosaic mutations, off-target effects. Compared with other disorders CRISPR/Cas9 studies on neurodegenerative disorders lag behind. To prospect future applications of CRISPR/Cas9, it may revolutionize gene therapy research department and become most versatile tool in gene therapy practices.

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