J Young Pharm, 2022; 14(3) : 268-272
A multifaceted peer reviewed journal in the field of Pharmacy
www.jyoungpharm.org | www.phcog.net

Review Article

Clustered Regularly Interspaced Short Palindromic Repeats and its Associated Protein- 9: Drug Delivery and Therapeutic Applications

Chinthaginjala Haranath[1,*], Rachana Jonnala[1], Eranti Bhargav[1], Hindustan Abdul Ahad[2], Pavan Kumar Chintamaneni[3]
[1]Department of Pharmaceutics, Raghavendra Institute of Pharmaceutical Education and Research (RIPER)- Autonomous, Ananthapuramu, Andhra Pradesh, INDIA.
[2]Department of Industrial Pharmacy, Raghavendra Institute of Pharmaceutical Education and Research (RIPER)- Autonomous, Ananthapuramu, Andhra Pradesh, INDIA.
[3]GITAM School of Pharmacy, GITAM Deemed University, Hyderabad, Telangana, INDIA.

ABSTRACT
Genomic editing, which involves modifying genetic DNA at a specific target site, is now being sought by scientists all over the world in a variety of domains. To achieve the desired result, genome editing entails adding, removing, and exchanging DNA in a wide variety of cell types of organisms (inactivation of target genes, correction of malfunctioning genes, acquiring new genetic traits, etc.). CRISPR-Cas9 systems are gradually being recognized for their simple design, high efficiency, reproducibility, and cost-effectiveness in achieving such precise genome editing. CRISPR-Cas9 has shown to be a game-changing method for gene editing in a variety of animals and cell types. However, due to its low transfection effectiveness, this technique's clinical usefulness is limited the review article focuses on the development of CRISPR-Cas9, the procedure involved, and the medical applications of CRISPR-Cas9 in various disorders, with an acknowledgment of the various physical, non-viral, and viral methods for transferring CRISPR-Cas9 systems to mammalian cells, as well as a few of them in clinical trials.
Keywords: Drug delivery, Gene editing, Carriers, Clinical trials, DNA.

Correspondence

Dr. Chinthaginjala Haranath,

Associate Professor and Head, Department of Pharmaceutics, Raghavendra Institute of Pharmaceutical Education and Research (RIPER) Autonomous, Ananthapuramu-515721, Andhra Pradesh, INDIA.

Email id: haranathriper@gmail.com

DOI: 10.5530/jyp.2022.14.54

INTRODUCTION

In Prokaryotic organisms, CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) were first developed as a defense mechanism against invading plasmids and viruses. Ishino was the first to discover the CRISPR structure in 1987.[1] After discovering many similar structures in other bacteria and archaea, Jansen coined the moniker CRISPR in 2002.[2-3] Later, Mojica and colleagues postulated that the CRISPR pattern and its associated protein guard against genetic influences and may have immune-defense activities.[4] However, the three key contributors to this area are Charpentier, Doudna, and Zhang. The mechanism of CRISPR Cas-9 was first elucidated by Charpentier. Later Charpentier and Doudna reported Cas-9 mediated biochemical characterization and system optimization.[5] Zhang was the first to implement CRISPR Cas-9, genetic modification in multicellular organisms.[6]

Cas-9 Protein is the related nuclease in this technique and the most often used protein among several types of Cas proteins due to its ease of implementation, high performance, selectivity, and ease of use. It can repair genetic diseases at the DNA level by recognizing a specific genome sequence and acting as a pair of scissors to divide that sequence of DNA. It utilizes a specific guide RNA (sgRNA) to produce base pairs with target DNA and can repair gene mutations at the DNA level by recognizing a specific genome sequence and acting as a pair of scissors to divide that sequence of DNA.[7] This system is the most efficacious and powerful gene-editing tool as it can operate any DNA sequence. CRISPR Cas-9 framework has caused a worldwide work revolution in academia and industry. The major application is in rapid production of animal models, knockout cell lines and also in the fields of human medicine, biotechnology and agricultural development.[8]

Due to the high efficiency of CRISPR in gene editing, scientist all around the world have shown great interest towards it and several works towards applications of CRISPR for clinical uses in humans were carried out. Particularly CRISPR Cas-9 have shown favourable results in correcting generated genes in cancer. Oncolytic viruses have also been created to destroy tumor cells.[9] CRISPR Cas-9 method has also been employed to replace unique human T cells gene sequence with modified sequences.

MECHANISM OF THE CRISPR CAS-9 GENE-EDITING SYSTEM

The CRISPR Cas-9 system is a protein family that is separated into two classes: class 1 (Classes I, III, and IV) and class 2 (Classes I, III, and IV) (Types II, V, VI). When compared to class 1 complex cas proteins, the class 2 CRISPR Cas system contains a single multi-domain protein such as Type II nuclease Cas9, which is preferred for gene delivery due to its ease of use.[10] CRISPRCas-9 works in three steps viz., Acquisition, Expression, and Interference as represented in Figure 1.

Acquisition

In this stage, fragments of foreign DNA are introduced as new spacers into CRISPR arrays from intruding plasmids or pathogens. Based on Protospacer adjacent motif (PAM), foreign target sequences (protospacers)are selected from foreign DNA and these spacers provide specific memory against the virus.[11]

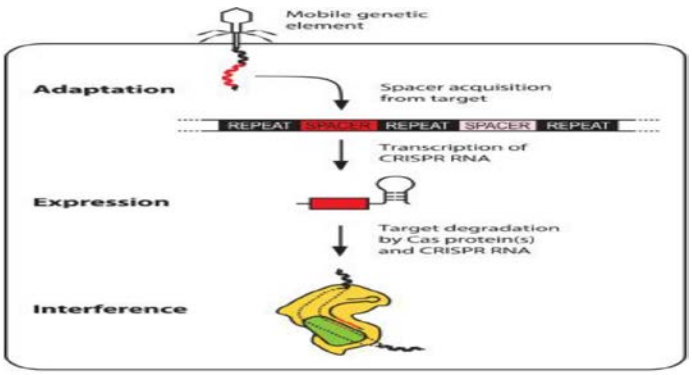

Figure 1: Mechanism of CRISPR Cas-9 gene editing system.

Expression

Here CRISPR is transcribed to pre-crRNA and then to developed crRNA. CrRNA recognizes all the genetic factors because each crRNA is complementary to foreign DNA.[12]

Interference

In this, crRNA guides the Cas nucleases to target and breakdown the protospacer sequences and blocks the dissemination of foreign DNA.[13]

The cas-9 nucleases have two domains, RuvC and HNH, with RuvC piercing non-complimentary bases and HNH piercing complementary DNA strands. Trans-activating crRNA and crRNA are combined to form sgRNA where tracrRNA hybridized to crRNA and binds with cas-9 forming a CRISPR Cas-9/sgRNA. TracrRNA and crRNA have two segments where at 3'end it binds to cas-9 and at 5'end binds to target DNA sequence. Cleavage by the targeted nuclease results in double-stranded break (DSB) formation. This can be remediated in eukaryotes by two pathways, i.e. Non homologous end joining (NHEJ) and pair directed by homology (HDR).For the knock-out animal models, NHEJ produces insertions/deletions (indel) and causes a frameshift mutation or the introduction of a premature stop codon.[14] The pathway HDR (knock-in) is less effective than NHEJ.

VARIOUS CRISPR CAS-9 GENE EDITING STRATEGIES

CRISPR Cas-9 utilizes three strategies to alter the genes. They are:

Using a plasmid-based CRISPR Cas-9 system encoding cas-9 and sgRNA from the similar vector.

The first strategy i.e., Plasmid based CRISPR Cas-9 is the most convenient and easy way, thus preventing several transfections of various components and have significant stability compared to other strategies.[15] However, Plasmid based system has challenges like difficulty in delivery of the plasmid to nucleus and more time required to edit the target for the plasmid to be conveyed to cas9mRNA inside the cells, and also it produces more off-targets. From the same plasmid, cas9 and sgRNA were communicated in PX260 0r PX334 vector systems and contains three cassettes i.e. CRISPR RNA array, tracr RNA, S. pyogenes cas9. In pX330 or pX335 vectors, contains two cassettes, tracr RNA and *S. pyogenes* cas 9. Both the vectors that are intended for specific targeting sites are designed with restriction enzymes and utlized for annealed oligonucleotides ligation.

Deliver the cas9 mRNA and sgRNA mixture

When there is direct conveyance of cas 9 mRNA and sgRNA into the target cells to edit the genes, the Cas 9/mRNA complex is formed within the cells. mRNAs delivery has lower off-targets than the CRISPR Cas-9 plasmid-based method. mRNA enters the cytoplasm to apply its effects and exhibits low cytotoxicity in the essential cells and cell lines.[16] The fundamental benefit is the transient articulation of mRNA and the drawback is poor stability.

Deliver the cas9 protein and sgRNA mixture

The purified cas9 protein is positively charged and forms a CAS9/SgRNA ribonucleoprotein complex (RNPS) complex with sgRNA. The most extensively employed technique recently is cas-9protein combination delivery with sgRNA. RNPS delivery has many advantages involving reduced off-targets, toxicity, and high gene editing efficiency.[17]

PHYSICAL AND NON-VIRAL DELIVERY OF CRISPR CAS-9

CRISPR Cas-9 has been delivered to target cells via physical and non-viral methods such as electroporation, nanoparticles, and hydrodynamic injection. Safety, lack of transgenic DNA size constraints, cost-effectiveness, availability is some of the advantages of non-viral delivery systems and can apply for human patients preferably than viral vectors.[18] The following are some of the most prevalent physical and non- viral delivery methods.

Electroporation

Electroporation is the most common and widely used method which deliver proteins nucleic acids and is suitable and for all CRISPR Cas-9 type systems. This method delivers plasmid-based CRISPR Cas-9 and RNPs to embryonic stem cells. CD_4^+ human Tcells. Through electroporation, gene editing has been done by plasmid-based CRISPR Cas-9 systems for zebrafish fin regeneration, mouse brain development, chicken development.[19]

Microinjection

Microinjection is one of the common laboratory techniques to deliver DNA or Protein into single cells. It involves injecting foreign molecules directly into living cells at the microscopic level using a glass micropipette at the microscopic level i.e., CRISPR Cas-9 framework into early-stage cells or cells with high reproducibility and specificity.[20] To get a knockout mouse model and microinjection of cas9 mRNA/sgRNA into pronucleus, this method is simple, convenient, effective, and reliable. It effectiveness is higher than the cytoplasm. The advantage is this can be focussed in a single cell. However, this technique suffers from setbacks of requiring high skills, induction of cell death.

Induced transduction by osmocytosis and propanebetaine (iTOP)

iTOP is an efficient and small molecule-based process for the transfer of native proteins and other compounds or molecules into the cells. NaCl hypertonicity and propanebetaine induce macro-pinocytosis leading to the transduction of proteins into cells. iTOP is used in the manipulation of primary cells. This method is effective in the direct delivery of RNPS or intracellular delivery of cas9&sgRNA. This method has a lower gene editing efficiency compared to electroporation, cationic lipids, and cell penetrating peptide (CPP).[21]

Mechanical Cell Deformation

The mechanical deformity of cells creates transient holes in the cell membrane, allowing the passive diffusion of biomaterials into the extracellular medium to the cytoplasm.[22] Delivery of plasmid-based CRISPR Cas-9 system achieved 90%&70% knockout efficiency by using

a microfluidic device in EGF breast cancer and lymphoma cells. The advantage of this method was it has high delivery efficiency and reduced cell death. The mechanical deformity theory provides an excellent solution for the delivery which has benefits over any of the current approaches.

Hydrodynamic Injection

This is an effective method of delivering genetic material to mice hepatocytes via the venous blood. The Fah mutation is fixed in the mouse model by cloning Fah targeting sequence in the pX330 backbone, which also contains cas9 and expression vector. Fah protein articulation was observed in 1/250 hepatocytes after the corrected Fah plasmid was hydrostatically administered into animals. Only small animals are chosen and this infusion increments blood pressure, liver growth, and even death in animals can occur.[23]

Polymer Nanoparticles

Polymeric nanoparticles deliver different kinds of nucleic acids like plasmid DNA, RNA, and oligonucleotides, i.e., the CRISPR system efficiently distributes and modifies the genome of methicillin-resistant Staphylococcus aureus (MRSA). CRISPR nanoparticle is formed by conjugation of Bpei (a cationic polymer) to cas9 and complexed with sgRNA. Compared to cas9/sgRNA compounded with traditional lipids, polymer nanoparticles have greater editing efficacy.

Cell Penetrating Peptide (CPP)

Cell penetrating peptide has been developed for treating cells to be transfected and translocated across the membrane. In human cell lines, gene disruption occurs by conjugation of CPP with cas9 protein and CPP with gRNA where both are delivered to the same cells to edit the genes in different cell lines like embryonic stem cells, dermal fibroblasts, HeLa, etc. Cas9 and gRNA have been delivered as CPP-conjugated Cas9 and CPP-complexed gRNA, respectively. As a result, off-target modifications, host immune reaction, and cytotoxic were all minimised.[24]

DNA nanostructures

DNA nanostructures are made from DNA and have a lower size, good biocompatibility, and spatially communication application for diverse biomedical applications. Rolling circle replication (RCR) and DNA nanococoons (RNP delivery) methods have been developed to construct DNA nanostructures. Sun *et al*. developed charged polymer-coated DNA nanococoons to transport Cas9/sgRNA nuclei to human cells, and they discovered that a partial complement between the sgRNA guide sequence and the nanococoon sequence may improve genome editing. CRISPR Cas9 release by DNA nanostructures could be used in anticancer therapy.[25]

Gold Nanoparticles

The human body tolerates gold nanoparticles well and quickly connects to DNA. Gold nanoparticles are widely used for tumor therapy, thermo-triggered release and edit the genes against cancer. These nanoparticles are assembled with cas9 with tag peptide and sgRNA. The CRISPR Cas-9 plasmid - gold nanoparticles were tested on mice with tumours, and genes were edited. This achieves 90% efficient delivery and 30% gene editing efficiency.[26]

Lipid nanoparticles

When a negatively charged nucleic acids binds with cationic lipids through electrostatic interactions lipid nanoparticles are formed and to enter cells, use cellular uptake or macro-pinocytosis. To edit the genes, transfection lipids such as Lipofectamine 2000, Lipofectamine 3000, and RNAiMAX are used to deliver viral vector CRISPR Cas9, cas9 mRNA/sgRNA combination, and RNPs to distinct cell lines. To deliver RNPs, RNAiMAX outperformed lipofectamine in terms of capability and toxicity. Because cas9 is polarised, cationic lipid functionalization and RNPs are found in cas9-sgRNA RNPs, lipid nanoparticles should be updated. High editing efficiency and lower off-targets are observed in RNPs than plasmid-based CRISPR Cas9. Lipid nanoparticles will deliver RNPs to the brain of the mouse for Invivo gene editing. Lipid nanoparticles based CRISPR Cas9 delivery system delivers >97% knockdown of serum transthyretin in a single administration.

VIRAL DELIVERY SYSTEMS FOR CRISPR CAS-9

The viral delivery system is the most powerful and widely utilized method for delivering plasmid-based nucleic acids to human cells *in vivo* and *in vitro*.[27] Virus vectors have been engineered to ensure several nucleic acid-based medicines.

Adeno- associated virus (AAV)

AAV virus is a non-pathogenic virus that infects both proliferating and non-dividing cells. Adeno-associated virus has become the basis for the treatment of Leber's congenital amaurosis2 (LCA2), a retinal disorder which is a gene therapy approved by the FDA. Streptococcus pyogenes, Spcas9 exhibits strong cas9 genome editing activity. To get around a single Adeno-associated virus packaging constraint, dual AAVs are employed to distribute cas9 expressing DNA and sgRNA independently.[28] In an animal model of metabolic liver infection, a dual Adeno-associated virus system repaired 10% abnormalities in hepatocellular and delivered CRISPR Cas9 for the treatment of metabolic liver infection.

Lentivirus

Lentiviral mediated delivery of CRISPR Cas-9 disrupts not only gene expression but also MicroRNAs (miRNA) expression very effectively. One of the studies indicate that CRISPR Cas-9 lentiviral system reduces cell proliferation, migration and invasion of premiR-2 (MicroRNA) sequences by four gRNA lentiviral vectors in ovarian cancer cell lines.

THERAPEUTIC APPLICATIONS OF CRISPR CAS-9

Sickle Cell Anemia

Sickle cell disease is a category of blood recessive genetic inherited disorder that affects hemoglobin, the molecule that provides oxygen to cells in the body. It is caused by a single nucleotide substitution called sickle hemoglobin (HbS) in the ß-globin gene (HBB), which causes red blood cells to be misshapen or "sickled shaped".[29] Using genome editing technology, there are two methods to make stem cells of engineered SCD ideal for autologous transplantation. Firstly, the direct solution is to rectify the point mutation in the HBB gene. In 2016, Kohn *et al*. showed the use of CRISPR/Cas9 to rectify the mutation of SCD-derived HSPCs from patient bone marrow.[30] The second strategy involves lowering or interrupting BCL11A development, which can help hbf production resume. The rationale for this is that the protein BCL11A mediates fetal to adult hemoglobin transfer, and the participation of HbF in SCD can protect against sickling red blood cells.[31]

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a severe neuromuscular disorder that occurs due to recessive DMD gene mutations on the X chromosome.[32] As a result, dystrophin, a protein required for binding the actin cytoskeleton to the extracellular matrix in muscle cells and maintaining membrane integrity, is missing. In DMD patients, CRISPR/Cas9 appears to be beneficial as it can permanently fix the genetic mutation, which minimizes the expense of long-term care. Researchers

have created genetically modulated mice bearing exon 44 deletions similar to the one present in patients, to show that this type of treatment could be delivered to aliving organism.

Huntington's Disease

Huntington's disease (HD) is an uncommon neurological autosomal genetic disease, that causes aberrant limb motions as well as psychiatric symptoms and cognitive deficits.[33] A genetic mutation in the huntingtin gene (mHTT), which codes for the huntingtin protein (HTT), causes the disease by increasing the polyglutamine (CAG) replicate core to more than 35 CAG repeats.[34] The CRISPR-Cas9 method has the capability of being a more expense technique of mHTT genetic manipulation and having a relatively high level of efficiency.[35] HTT gene is targeted by both mutant and wild type alleles. Then sgRNA targets either beginning of the coding sequence or core region within the promoter to disrupt HTT transcription or translation. So, this would drastically reduce or inactivate HTT expression.[36-37]

Cancer disease

Cancer is the world's second greatest cause of mortality after cardiovascular diseases.[38] CRISPR Cas-9 system has been used widely in cancer treatment which enables the modification of genes and allowing target corrections of mutations.[39] ORF57 was knocked out completely in iSLK/Bac16 and HEK293/Bac36 cells in a sarcoma study by using many rounds of solitary replication and selection from a vector representation to create one Cas9 protein and two guiding Transcripts simultaneously.[40] A genome-wide loss of function (LOF) genetic screen was conducted by Edwin *et al*. in colorectal cancer HCT116 cells containing mutant or wild type KRAS.[41] They discovered that NAD kinase or ketohexokinase were metabolic genes and possible therapeutic targets, with pharmacological suppression of these enzymes inhibiting colonic tumor cell proliferation.

Crohn's disease

Crohn's disease is an inflammatory bowel disease (IBD) that can affect any region of the large intestine.[42] CRISPR/Cas9 system may be quite strong enough to start cutting the genes involved in IBD and therefore reduce the burden of financial health care in the treatment of IBD. In a study, CRISPR/Cas9 system was used to modify HSCs, also known as Lin-Sca1 + Kit+ cells (LSKs), in rats, and investigated the hypothesis by coupling CRISPR technology and LSK transplants, genetic modifications in the entire immune system of the reconstituted animals were introduced. Prior to this, LSKs were employed to deliver CRISRP/Cas9 to recipients.[43-44]

Neurofibromatosis

Neurofibromatosis type 1 (NF1) is a autosomal genetic disorder occurring in 1:2000 or 3000 births.[45-46] NF1 is a 300kb human gene located on chromosome 17q11.2.[47] Scherer *et al*. highlights newer malignant peripheral nerve sheath tumors (MPNST) mouse models that use somatic CRISPR/Cas9 carcinogenesis to develop genomically identical tumor cells in multiple wild-type mice historical strains.[48] This could be the first study to examine the influence of the host strain on CRISPR Cas-9 mice models in depth, and it uncovers a number of important strain-dependent characteristics, such as effects on tumour initiation and the tumour immune system.

CONCLUSION

Even in these days, with advances in fields of medicine, biotechnology, and genetics there are several disorders like cancers, neurofibromatosis, Crohn's disease, due to absence or malfunction of gene are still existing. CRISPR CAS 9 is an excellent solution to address all these genetic disorders. However, clinical translation of CRISPR/Cas9 genome editing still need optimization, as the CRISPR CAS9 has to protected in the blood stream without being degraded and it should also reach the target site for action. The advances in the drug delivery technologies readily answers the limitations associated with CRISPR CAS9 delivery and helps in achieving maximum outcome and will help in creating a new path to combat several genetic disorders.

ACKNOWLEDGEMENT

Authors are thankful for the management of RIPER for provided support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. J Bacteriol. 1987;169(12):5429-33. doi: 10.1128/jb.169.12.5429-5433.1987, PMID 3316184.
2. Jansen R, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol. 2002;43(6):1565-75. doi: 10.1046/j.1365-2958.2002.02839.x, PMID 11952905.
3. Jansen R, Van Embden JD, Gaastra W, Schouls LM. Identification of a novel family of sequence repeats among prokaryotes. Omics. 2002;6(1):23-33. doi: 10.1089/15362310252780816, PMID 11883425.
4. Mojica FJ, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol. 2005;60(2):174-82. doi: 10.1007/s00239-004-0046-3, PMID 15791728.
5. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, *et al*. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature. 2011;471(7340):602-7. doi: 10.1038/nature09886, PMID 21455174.
6. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337(6096):816-21. doi: 10.1126/science.1225829, PMID 22745249.
7. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, *et al*. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339(6121):819-23. doi: 10.1126/science.1231143, PMID 23287718.
8. Veres A, Gosis BS, Ding Q, Collins R, Ragavendran A, Brand H, *et al*. Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. Cell Stem Cell. 2014;15(1):27-30. doi: 10.1016/j.stem.2014.04.020, PMID 24996167.
9. Yuan M, Webb E, Lemoine NR, Wang Y. CRISPR-Cas9 as a powerful tool for efficient creation of oncolytic viruses. Viruses. 2016;8(3):72. doi: 10.3390/v8030072, PMID 26959050.
10. Van Der Oost J, Westra ER, Jackson RN, Wiedenheft B. Unravelling the structural and mechanistic basis of CRISPR–Cas systems. Nat Rev Microbiol. 2014;12(7):479-92. doi: 10.1038/nrmicro3279, PMID 24909109.
11. Yosef I, Goren MG, Qimron U. Proteins and DNA elements essential for the CRISPR adaptation process in *Escherichia coli*. Nucleic Acids Res. 2012;40(12):5569-76. doi: 10.1093/nar/gks216, PMID 22402487.
12. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, *et al*. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science. 2008;321(5891):960-4. doi: 10.1126/science.1159689, PMID 18703739.
13. Westra ER, Buckling A, Fineran PC. CRISPR–Cas systems: Beyond adaptive immunity. Nat Rev Microbiol. 2014;12(5):317-26. doi: 10.1038/nrmicro3241, PMID 24704746.
14. Shen B, Zhang J, Wu H, Wang J, Ma K, Li Z, *et al*. Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. Cell Res. 2013;23(5):720-3. doi: 10.1038/cr.2013.46, PMID 23545779.
15. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 2013;8(11):2281-308. doi: 10.1038/nprot.2013.143, PMID 24157548.
16. Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, *et al*. Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nat Methods. 2014;11(4):399-402. doi: 10.1038/nmeth.2857, PMID 24584192.
17. Kim S, Kim D, Cho SW, Kim J, Kim JS. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res. 2014;24(6):1012-9. doi: 10.1101/gr.171322.113, PMID 24696461.
18. Qin W, Dion SL, Kutny PM, Zhang Y, Cheng AW, Jillette NL, *et al*. Efficient CRISPR/Cas9-mediated genome editing in mice by zygote electroporation of nuclease. Genetics. 2015;200(2):423-30. doi: 10.1534/genetics.115.176594,

PMID 25819794.

19. Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G, *et al*. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N Engl J Med. 2014;370(10):901-10. doi: 10.1056/NEJMoa1300662, PMID 24597865.
20. Crispo M, Mulet AP, Tesson L, Barrera N, Cuadro F, Dos Santos-Neto PC, *et al*. Efficient generation of myostatin knock-out sheep using CRISPR/Cas9 technology and microinjection into zygotes. PLOS ONE. 2015;10(8):e0136690. doi: 10.1371/journal.pone.0136690, PMID 26305800.
21. Suresh B, Ramakrishna S, Kim H. Cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA for genome editing. Methods Mol Biol. 2017;1507:81-94. doi: 10.1007/978-1-4939-6518-2_7, PMID 27832534.
22. Worthen CA, Rittié L, Fisher GJ. Mechanical deformation of cultured cells with hydrogels. Methods Mol Biol. 2017;1627:245-51. doi: 10.1007/978-1-4939-7113-8_17, PMID 28836207.
23. Suda T, Liu D. Hydrodynamic gene delivery: Its principles and applications. Mol Ther. 2007;15(12):2063-9. doi: 10.1038/sj.mt.6300314, PMID 17912237.
24. Kang YK, Kwon K, Ryu JS, Lee HN, Park C, Chung HJ. Nonviral genome editing based on a polymer-derivatized CRISPR nanocomplex for targeting bacterial pathogens and antibiotic resistance. Bioconjug Chem. 2017;28(4):957-67. doi: 10.1021/acs.bioconjchem.6b00676, PMID 28215090.
25. Sun W, Ji W, Hall JM, Hu Q, Wang C, Beisel CL, *et al*. Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing. Angew Chem Int Ed Engl. 2015;54(41):12029-33. doi: 10.1002/anie.201506030, PMID 26310292.
26. Mout R, Ray M, Yesilbag Tonga G, Lee YW, Tay T, Sasaki K, *et al*. Direct cytosolic delivery of CRISPR/Cas9-ribonucleoprotein for efficient gene editing. ACS Nano. 2017;11(3):2452-8. doi: 10.1021/acsnano.6b07600, PMID 28129503.
27. Liu C, Zhang L, Liu H, Cheng K. Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. J Control Release. 2017;266:17-26. doi: 10.1016/j.jconrel.2017.09.012, PMID 28911805.
28. Gaj T, Epstein BE, Schaffer DV. Genome engineering using adeno-associated virus: Basic and clinical research applications. Mol Ther. 2016;24(3):458-64. doi: 10.1038/mt.2015.151, PMID 26373345.
29. Park SH, Lee CM, Deshmukh H, Bao G. Therapeutic Crispr/Cas9 genome editing for treating sickle cell disease. Blood. 2016;128(22):4703. doi: 10.1182/blood.V128.22.4703.4703.
30. Hoban MD, Lumaquin D, Kuo CY, Romero Z, Long J, Ho M, *et al*. CRISPR/Cas9-mediated correction of the sickle mutation in human CD34+ cells. Mol Ther. 2016;24(9):1561-9. doi: 10.1038/mt.2016.148, PMID 27406980.
31. Xu J, Peng C, Sankaran VG, Shao Z, Esrick EB, Chong BG, *et al*. Correction of sickle cell disease in adult mice by interference with fetal hemoglobin silencing. Science. 2011;334(6058):993-6. doi: 10.1126/science.1211053, PMID 21998251.
32. Mendell JR, Shilling C, Leslie ND, Flanigan KM, Al-Dahhak R, Gastier-Foster J, *et al*. Evidence-based path to newborn screening for Duchenne muscular dystrophy. Ann Neurol. 2012;71(3):304-13. doi: 10.1002/ana.23528, PMID 22451200.
33. Paulsen JS, Nehl C, Hoth KF, Kanz JE, Benjamin M, Conybeare R, *et al*. Depression and stages of Huntington's disease. J Neuropsychiatry Clin Neurosci. 2005;17(4):496-502. doi: 10.1176/jnp.17.4.496, PMID 16387989.
34. Langbehn DR, Brinkman RR, Falush D, Paulsen JS, Hayden MR, International Huntington's Disease Collaborative Group. A new model for prediction of the age of onset and penetrance for Huntington's disease based on CAG length. Clin Genet. 2004;65(4):267-77. doi: 10.1111/j.1399-0004.2004.00241.x, PMID 15025718.
35. Gaj T, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. 2013;31(7):397-405. doi: 10.1016/j.tibtech.2013.04.004, PMID 23664777.
36. Boudreau RL, McBride JL, Martins I, Shen S, Xing Y, Carter BJ, *et al*. Nonallele-specific silencing of mutant and wild-type huntingtin demonstrates therapeutic efficacy in Huntington's disease mice. Mol Ther. 2009;17(6):1053-63. doi: 10.1038/mt.2009.17, PMID 19240687.
37. Drouet V, Perrin V, Hassig R, Dufour N, Auregan G, Alves S, *et al*. Sustained effects of nonallele-specific huntingtin silencing. Ann Neurol. 2009;65(3):276-85. doi: 10.1002/ana.21569, PMID 19334076.
38. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, *et al*. Cancer statistics for the year 2020: An overview. Int J Cancer. 2021;149(4):778-89. doi: 10.1002/ijc.33588, PMID 33818764.
39. Li H, Yang Y, Hong W, Huang M, Wu M, Zhao X. Applications of genome editing technology in the targeted therapy of human diseases: Mechanisms, advances and prospects. Signal Transduct Target Ther. 2020;5(1):1. doi: 10.1038/s41392-019-0089-y, PMID 32296011.
40. BeltCappellino A, Majerciak V, Lobanov A, Lack J, Cam M, Zheng ZM. CRISPR/Cas9-mediated knockout and *in situ* inversion of the ORF57 gene from all copies of the Kaposi's sarcoma-associated herpesvirus genome in BCBL-1 cells. J Virol. 2019;93(21). doi: 10.1128/JVI.00628-19, PMID 31413125.
41. Yau EH, Kummetha IR, Lichinchi G, Tang R, Zhang Y, Rana TM. Genome-wide CRISPR screen for essential cell growth mediators in mutant KRAS colorectal cancers. Cancer Res. 2017;77(22):6330-9. doi: 10.1158/0008-5472.CAN-17-2043, PMID 28954733.
42. Uhlig HH, Powrie F. Translating immunology into therapeutic concepts for inflammatory bowel disease. Annu Rev Immunol. 2018;36:755-81. doi: 10.1146/annurev-immunol-042617-053055, PMID 29677472.
43. LaFleur MW, Nguyen TH, Coxe MA, Yates KB, Trombley JD, Weiss SA, *et al*. A CRISPR-Cas9 delivery system for *in vivo* screening of genes in the immune system. Nat Commun. 2019;10(1):1668. doi: 10.1038/s41467-019-09656-2, PMID 30971695.
44. Limanskiy V, Vyas A, Chaturvedi LS, Vyas D. Harnessing the potential of gene editing technology using CRISPR in inflammatory bowel disease. World J Gastroenterol. 2019;25(18):2177-87. doi: 10.3748/wjg.v25.i18.2177, PMID 31143069.
45. Friedman JM, Gutmann DH, MacCollin M, Riccardi VM. Neurofibromatosis: Phenotype, natural history, and pathogenesis. Am J Hum Genet. 2000;67(1):264. PMCID PMC1287094.
46. Kallionpää RA, Uusitalo E, Leppävirta J, Pöyhönen M, Peltonen S, Peltonen J. Prevalence of neurofibromatosis type 1 in the Finnish population. Genet Med. 2018;20(9):1082-6. doi: 10.1038/gim.2017.215, PMID 29215653.
47. Buratti E, Baralle D. Exon skipping mutations in neurofibromatosis. Methods Mol Biol. 2012;867:65-76. doi: 10.1007/978-1-61779-767-5_5, PMID 22454055.
48. Scherer A, Stephens VR, McGivney GR, Gutierrez WR, Laverty EA, Knepper-Adrian V, *et al*. Distinct tumor microenvironments are a defining feature of strain-specific CRISPR/Cas9-Induced MPNSTs. Genes. 2020;11(5):583. doi: 10.3390/genes11050583, PMID 32456131.

Article History: Received: 09-03-2022; Revised: 18-05-2022; Accepted: 06-06-2022.
Cite this article: Haranath C, Jonnala R, Bhargav E, Ahad HA, Chintamaneni PK. Clustered Regularly Interspaced Short Palindromic Repeats and its Associated Protein- 9: Drug Delivery and Therapeutic Applications. J Young Pharm. 2022;14(3):268-72.