

CRISPR-Cas Technology, the Tool of the Future

Alejandro Esquivel Álvarez, Elías Quesada Morales, María Paz Villegas Calero, Daniela Matarrita Brenes, María Fernanda Rojas Salas, German Madrigal Redondo, Marianela Chavarría Rojas, Eleaneth Baltodano Viales

ABSTRACT

The CRISPR-Cas system discovered in the eighties consists of a series of RNA proteins that signal and messengers of the immune system in prokaryotic organisms to protect against invading antigens of various sources. After the CRISPR-Cas discovery, many research advances were made in the following decades regarding knowledge, techniques, and applications. This showed the system could edit the DNA in an exact and specific manner, which made it very promising to exploit it in various fields, such as the therapeutic field. New therapies of various diseases, industrial applications, food manufacturing, among others, make its impact quite relevant. The following review will focus on the fundamental understanding and implications of CRISPR-Cas techniques has with an ethical and legal view. In addition, to explore some of the applications present and future in the healthcare department, some methods of drug delivery in gene therapy, and new research that is being developed with the CRISPR-Cas technology.

Keywords: CRISPR-Cas, DNA, gene, pharmacy, RNA, therapy.

Published Online: March 2, 2022

ISSN: 2684-5199

DOI : 10.24018/ejbio.2022.3.2.332

A. Esquivel Álvarez

Instituto de Investigaciones Farmacéuticas (INIFAR), School of Pharmacy, University of Costa Rica, San José, Costa Rica.

(e-mail: aesquivel913@gmail.com)

E. Quesada Morales

Instituto de Investigaciones Farmacéuticas (INIFAR), School of Pharmacy, University of Costa Rica, San José, Costa Rica.

(e-mail: eliqm2695@hotmail.com)

M. P. Villegas Calero

Instituto de Investigaciones Farmacéuticas (INIFAR), School of Pharmacy, University of Costa Rica, San José, Costa Rica.

(e-mail: marivical00@gmail.com)

D. Matarrita Brenes*

Instituto de Investigaciones Farmacéuticas (INIFAR), School of Pharmacy, University of Costa Rica, San José, Costa Rica.

(e-mail: danmatabren@gmail.com)

M. F. Rojas Salas

Instituto de Investigaciones Farmacéuticas (INIFAR), School of Pharmacy, University of Costa Rica, San José, Costa Rica.

(e-mail: maria.rojas_s@ucr.ac.cr)

G. Madrigal Redondo

Instituto de Investigaciones Farmacéuticas (INIFAR), School of Pharmacy, University of Costa Rica, San José, Costa Rica.

(e-mail: generacionlcr96@gmail.com)

M. Chavarría Rojas

Instituto de Investigaciones Farmacéuticas (INIFAR), School of Pharmacy, University of Costa Rica, San José, Costa Rica.

(e-mail: marianela.chavarría@ucr.ac.cr)

E. Baltodano Viales

Instituto de Investigaciones Farmacéuticas (INIFAR), School of Pharmacy, University of Costa Rica, San José, Costa Rica.

(e-mail: eleaneth.baltodano@ucr.ac.cr)

**Corresponding Author*

I. INTRODUCTION

The study of the genome of numerous species has caused great intrigue since its discovery, and has led to the development of multiple techniques to analyze and manipulate it. The present paper aims to delve into the Clustered Regularly Interspaced Short Palindromic Repeats technique (known as CRISPR by its acronym), associated with (Cas) protein systems, known in conjunction CRISPR-Cas, which allow genome editing. These systems consist of three parts: repeated segments separated by unique sequences, called spacers; a leader sequence of the CRISPR sequence with a promoter that forms a single transcription; and a gene set or sequence associated with Cas proteins (Alkhnbashi *et al.*, 2020).

These systems are characterized by RNA proteins that mediate the immune system on prokaryotic cells, constituting a defense against diverse antigens. CRISPR-Cas technology is widely used for genomic editing of diverse organisms such as bacteria, yeasts, mammals, and others (Singh, 2020). It has allowed the construction of libraries filled with information about the genetic material of multiple microorganisms and species, which has resulted in precious inputs for their further study and applications (Shipman *et al.*, 2017).

The CRISPR technique emerged in the 80s and was discovered as part of *Escherichia coli* studies. Several associated functions were found, such as chromosome rearrangement and DNA repairing, among others (Horvath & Barrangou, 2010). Nearing the 2000s, the CRISPR-Cas associated genes system was identified, which started a massively important stretch in the relation between genes and adaptive immunity, especially in microorganisms. Consequently, a more significant application of CRISPR-Cas technology. It is worth noting that its first uses were made at the end of the 90s, but the limitations in the associated mechanisms and involved agents known at the time hindered the full development of the technique (Shipman *et al.*, 2017). Some findings made in this decade include bacterial cells defense mechanisms, acquired immunity against bacteriophages, and interference in plasmid transfer. Near the end of the decade, it was proven that CRISPR-Cas systems could specifically and precisely cut DNA (Singh, 2020).

In the early 2010s, advancements in modern procedures enabled genome editing using guide RNA, which allowed the study of multiple applications in the field throughout the decade. Furthermore, these editing techniques have been applied to the DNA of viruses to treat multiple animal and vegetal diseases, focused on therapies against HIV, hepatitis B virus (HBV), and human papillomavirus (HPV), with increased interest in recent years (Klompe & Sternberg, 2020). Other studied applications include oncologic therapy, blindness, cardiovascular diseases, and other clinical conditions (Singh, 2020).

The timeline for the CRISPR technique development starts in the 80s with the first report of its discovery in *Escherichia coli*. Later in the 90s, the discussion of the potential applications for CRISPR began. In the 2000s, the advancements included findings of a CRISPR system in prokaryotic cells, the identification of Cas genes, the foreign-origin spacers, the first registered trial of the role of CRISPR-Cas applied to adaptive immunity, and spacers that act as single guide RNA (sgRNA) in the presence of crRNAs. More

significant strides were made between 2010-2020, such as discovering that spacer sequences guide Cas9 specifically to DNA action sites. In other words, they act as a guided endonuclease. The application of this novelty began in eukaryotic cells to obtain a 'picture' of the genome. *In vivo* genome editing was then used to modify the RNA; this method has since been used for high sensibility diagnosis. Then human embryos modifications began in 2018 (Singh, 2020).

In short, CRISPR-Cas systems accomplish adaptation, biogenesis, and interference functions (Khan, 2019). Adaptation refers to the incorporation of foreign genetic material as a new spacer. On the other hand, biogenesis consists of the transcription and processing of the CRISPR sequence in RNAs. Furthermore, interference refers to eliminating invasive or foreign genetic elements (Alkhnbashi *et al.*, 2020).

Given the great diversity of applications for CRISPR-Cas technology, they have been classified into two classes, six types, and eighteen subtypes. Examples of developments of these techniques are CRISPR interference (CRISPRi), which focuses on genetic suppression and activation, and allows for genomic profile, epigenetic modification, and other applications. These techniques have allowed the identification and tracing of genes in chromosomes and their visualization inside the cells. This results in the obtention of valuable information for the development of therapies.

The development of CRISPR-Cas technology is at an all-time high due to its possible applications in the treatment of conditions caused by bacterial infections, hereditary conditions, unhealthy lifestyles, among others. It's because of these advantages and possible applications that CRISPR-Cas has become a focus point of research in the pharmaceutical and industrial fields.

II. ETHIC AND LEGAL IMPLICATIONS

Despite the multiple applications and benefits of discovering CRISPR-Cas, it serves as a genetic modification tool with an expanding horizon of possibilities that involves a particular grade of controversy since its application implies a level of danger. Genetic editing is considered two significant risks: eugenics, or the selection of specific characteristics in human beings, and the possibility of unwanted effects or its utilization for harmful purposes (Capella, 2016).

A series of risks or concerns implied in the CRISPR-Cas technique, including genetic engineering of human babies or embryo manipulation, unexpected mutations, ecosystem disturbance due to the uncontrolled usage of the technique, secondary effects inherited to future generations, unwanted genetic changes in a population, eugenics, and its application for wicked purposes, such as biological weapons (Cárdenas, 2019).

In 2015, Science and Nature published the ethical frontiers for applying CRISPR-Cas; these indicate that it must only be applied in somatic genetic therapy and never in the human germline or, in other words, human embryos. Nevertheless, research of its potential use in the human germline is recommended. That same year, the National Academies (USA), the Royal Society (United Kingdom), and the Chinese

Academy held a symposium to discuss this topic. The most critical remarks of the three derived agreements summarize that; genetic editing can improve human lives but can also be dangerous if misused, scientists must never hide information or rush their work, and human embryos must never be considered for their application (Capella, 2016).

In numerous countries, such as Germany, Spain, and Colombia, patents for human germline modifications are prohibited according to the Directive 98/44/CE of the European Parliament and Council, issued in 1998 (Cárdenas, 2019). The three biotechnological superpowers do not follow the same policies: The United States government does not fund any application on human germlines, but the U.K. does since investigations involving human germlines are allowed as long as they are previously approved by the Human Fertilization and Embryology Authority (HFEA). In contrast, China does not have a regulating authority for this kind of experiment. However, Europe possesses more precise legislation than the United States, including regulations such as the Oviedo Convention (Capella, 2016).

As for bioethics, the different positions referring to the use of human germline can be divided into three groups: total prohibition, regardless of its purpose, prohibition when not used for therapeutic purposes, and total approval of its application under certain conditions (Capella, 2016). In the 2017 "Human Gene Editing: Science, Ethics and Governance" report, human germline editing is considered possible in the future when the public opinion about the topic turns positive (Cárdenas, 2019).

It is necessary to investigate further the adverse effects that CRISPR-Cas therapy might bring when used to determine targets. The high costs of this technology, which imply not every population will get access to it, is another crucial talking point. These authors usually point out that multiple question marks should be answered, such as if the general population would have access to this technology, what kind of cells it can be applied on, or what international laws are necessary for its regulation (Barajas & Hernández, 2020).

III. APPLICATIONS

Due to the fast pace and exponential growth in the CRISPR/Cas techniques and its relatively low cost compared to other techniques in the genome-editing field, the innovations and applications are highly diverse in various fields of human knowledge. The use of these advancements has allowed the development of new models, therapies, studies, raw materials, among others (Quílez, 2017; Fellmann *et al.*, 2017).

IV. DEVELOPMENT OF CELLULAR MODELS

Thanks to its simplicity and low cost within other genome editing tools, CRISPR-Cas has allowed obtaining pluripotent cells from somatic adult cells and the different cell lines obtained from the somatic cells. This advancement has the utmost relevance and importance in developing new models that remove the need to use mother cells from embryos, removing a significant barrier and limitation of prior methods due to ethical conflicts. Another relevant advance in the

development of cellular models is that the technique has made it possible to create cellular models of diseased cells from stem cells, which has made it easier than other methods such as nuclease with zinc fingers (Quílez, 2017; Fellmann *et al.*, 2017; Freiermuth *et al.*, 2018).

Furthermore, CRISPR-Cas allows generating human cellular lineages knockout isogenic in an effortless manner that allows researchers to determine the cells' roles concerning oncogenes, tumor suppressor genes, or other factors in a determined context. Equally, it is possible to knockin the mutant alleles that can be tested upon the effects of a mutation associated with isogenic diseases (Quílez, 2017; Fellmann *et al.*, 2017; Freiermuth *et al.*, 2018; Song *et al.*, 2016).

The use of cellular models allows determining what gene is responsible for causing a disease in a cell, such as the Fabry disease, whose cause is a defect on the gene of the α -galactosidase A (GLA). What that it is possible to obtain lineage cells without the gene and determine if the therapy of human recombinant GLA *in vitro* may better function the cells and eventually propose therapies that can be used in patients (Quílez, 2017; Fellmann *et al.*, 2017; Freiermuth *et al.*, 2018; Song *et al.*, 2016).

A study revealed a method to obtain isogenic cells with α -synuclein mutations used to study Parkinson's disease. To achieve this, FACS-assisted (fluorescence-activated cell sorting) CRISPR-Cas9 editing, which allows to derive biallelic genome-edited populations. This technique is more efficient than other protocols, which require a significant number of clones to obtain an individual without random integrations or other mutations (Arias *et al.*, 2017).

In the field of Parkinson's studies, induced pluripotent stem cells (iPSC) is an important tool that can be obtained through CRISPR-Cas9 technology. For example, a study to determine polymorphisms leading to the disease was conducted, in which fibroblast iPSCs from healthy subjects and Parkinson's patients were sampled. The iPSCs from Parkinson's patients were edited at specific sites suspected to be related to the pathology. If the editing resulted in a phenotype equal to that of healthy cells, the modified genes were confirmed to be related to the disease. This system can also improve reprogramming efficiency from fibroblasts into neurons (Cota *et al.*, 2020).

Despite the applications and usage of cell lineages obtained through CRISPR-Cas, there are limitations known, for example, in the use of cell lineages of mutated cells with direct repair via homology is limited, like the Case of non-mitotic human cells such as neurons. Another limitation is in the Case of the production of genes found in high proportions due to the possible ruptures that might stop the cell cycle (Fellmann *et al.*, 2017).

V. DEVELOPMENT ANIMAL MODELS

Another application relevant to CRISPR-Cas technology is to research and develop new drugs by developing animal models that can alter in significant quantity genes through one step, to obtain mutated mice in various genes without the need to cross-breed species. Furthermore, it allows modifying the zygote directly via microinjection or electroporation, making it unnecessary cell growth of embryo mother cells or derives.

In addition, the use of said models via CRISPR may take up to weeks or months, while other methods may take up to years, like breed crossing (Fellmann *et al.*, 2017; Smalley, 2016).

The use of CRISPR-Cas with viral vectors or others based in transposons ("jumping genes") is now applicated to induce somatic mutations in various animal tissue. This can be further researched in the beginning, development, and maintenance of the disease in an autochthonous and immunocompetent environment, which can be listed as another advantage in developing CRISPR models with animals. CRISPR technology also reduces costs and time in developing new models with animals. The time in breeding mice in the said model is substantially shorter, which allows researchers to validate-verify new developments of drugs in a smaller time window and hence develop new therapies (Fellmann *et al.*, 2017; Smalley, 2016; Xue *et al.*, 2014).

Small animals like rodents are not always capable of reproducing the pathologic events found in human beings, which raises importance at the validation of treatments on bigger mammals, such as primates. The subjacent difficulty of utilizing these organisms is the absence of embryonic stem cell lines employed to reproduce diseases caused by endogenous DNA mutations. However, CRISPR-Cas9 has been of great use to genetically modify species dispensing with said cell lines (Tu *et al.*, 2015).

Before the implementation of CRISPR-Cas9 technology, reproduction of multiple generations of offspring was necessary to perform individual or multiple gene knockouts in mammals that required more time and did not secure good results. Nonetheless, thanks to the technology above, double knockout of genes such as Parkin and PINK1 in pigs was achieved, which were inherited as the homozygous form and with no off-target mutagenesis, allowing the study of Parkinson's disease. Triple gene knockout was subsequently achieved in miniature pigs, adding the DJ-1 gene to the list (Luo *et al.*, 2019).

A. Drug Development

With previous models mentioned, CRISPR-Cas has changed drug discovery and development stages due to the innate simplicity, speed, and relatively low cost involved in generating genomic alterations in cellular, animal and human tissue models (Scott, 2018; Enzmann, 2019).

During the process of drug development, researchers have the need to use cell and animal models of human diseases to test possible treatments and determine what works and what doesn't. The development and subsequent use of these models require a great investment in both money and time, but, CRISPR-Cas has enabled researchers to develop cellular and animal models with specific mutations of interest cheaper and faster (Scott, 2018; Enzmann, 2019).

Thanks to the reduced costs in both time and money, researchers can apply the CRISPR-Cas technology to all the steps of the drug development process increasing the number of compounds that can be screened and the number of leads that can be optimized and eventually approved by the regulatory agencies (Scott, 2018; Enzmann, 2019).

One of the other main advantages of this technology is the capability to deliberately activate or inhibit genes, with this, researchers can be led to the genes or proteins that causes or

prevents diseases. Because of this, researchers are currently exploiting CRISPR-Cas ability to knockout specific genes in order to determine what they do and how this can be used to develop novel therapies. Nonetheless, knockout mutations have been used since the early 2000s, the innovation of CRISPR-Cas technology resides in the fact that it can generate complete knockouts of targeted genes, with much more precision than previous methods which helps avoids unwanted effects (Scott, 2018).

Gene editing with CRISPR-Cas also makes easier to identify genes that promote diseases, uncovering in the process some obvious targets for drug development which can lead to the development of a combination of genes or proteins with ease and accuracy to produce a more sophisticated and specific treatment (Scott, 2018).

Other possible application of this technology in drug development is the ability to turn gene activity up or down without making a change to the gene itself, this could help in the investigation of the importance of genes and proteins in a subtler way when compared with the knockout approach (Scott, 2018).

It's important to note that CRISPR-Cas has taken the lead over other techniques regarding drug discovery and development because of its practicality and relative low cost when compared to other gene editing techniques (Fellmann *et al.*, 2017).

B. Optimization and Bioreactor development

In various industries, yeast and bacteria are often used as a source of protein, metabolites, or enzymes with therapeutic properties. However, developing new strains and optimal therapeutic metabolites is highly complex. It requires a significant investment in time until CRISPR-Cas implementation may allow the development of said strains more efficiently and quickly than traditional methods. One method is the SWITCH system, which is used in the bioreactors of *Saccharomyces cerevisiae* capable of producing naringenin, a flavonoid with antioxidant, antiviral, anti-inflammatory, and hypocholesterolemic agents. Furthermore, the SWITCH method allows diminishing the TSC13 expression to diminish the subproduct formation that competes in the naringenin formation. SWITCH is essentially based on integrating Cas genes in specific loci, changes through genetic engineering catalyzed via Cas, replacing Cas for dCas, and altering the expression mediated by dCas (Dominguez *et al.*, 2016; García, *et al.*, 2017).

C. Therapies

One of the main objectives of the CRISPR-Cas system is the therapy of different diseases and conditions for the benefit of the public and human health. Four areas in which the technique has clinical potential: oncology, may be used to identify mutations and repair the sequence, and boost immune response; cardiovascular diseases, CRISPR-Cas may inhibit PCSK9 to control cholesterol levels of LDL; in regards to neurodegenerative diseases, it is possible to apply in the development of syndrome models in the likes of Parkinson and gene therapy when the mutation is known in the specific allele; antimicrobial agents be programmed through vehicles such as bacteriophage vehicles that deliver drug molecules to fight the disease caused by bacteria, that might even be intracellular (Gutiérrez & Salazar, 2017).

CRISPR-Cas may partake in genome modifiers such as insertion of genes via homologous recombination, elimination, and mutagenesis. Furthermore, it is an essential tool to research and study the function of a gene that has been altered. It can be applied in animal models and tools like CRISPRi, activating or inhibiting genes. In more specific functions, like the case of degenerative diseases, it can take a role in the creation of models and genes - cellular therapy. The showcase of therapy in the muscular dystrophy of Duchenne, by correcting the gene DMD that codes for dystrophin and as a cellular therapy for the ischemic heart disease (Lammoglia *et al.*, 2016).

The CRISPR-Cas system has the advantage of allowing the modification of nuclear DNA and mitochondrial DNA. It may be functional in treating related diseases in the mutation of the mitochondrial genome. Research of diseases allows the development of essential tools, like the case of neurodegenerative diseases, that use pluripotent induced somatic cells (iPSC) in the use of models and genome editing, cells may be modified (Araldi *et al.*, 2020).

The technology of CRISPR-Cas may be applied to any molecule containing DNA, including viruses. HIV treated with the mutation of essential infectious sites for the infection of the host and removing the proviral DNA in infected cells or the genome of JCV (Virus of the Central Nervous System) that is susceptible to removal with CRISPR-Cas (Soppe & Lebbink, 2018). Also, the Epstein-Barr virus that causes herpes may be removed from infected cells using CRISPR-Cas. Furthermore, it can limit the infection provoked by the virus of hepatitis B and interfere in the development of cancer caused by the human papillomavirus (de Buhr & Lebbink, 2018).

One of the major concerns in recent times is how to act to the new super bacteria resistant to antibiotics. The Cas element has been discovered to induce chromosome cuts in bacteria, which are lethal. The technique involved also has a high specificity, that it can solely act in a bacteria strain without affecting other bacteria, like gut bacteria. Possible vehicles have been researched for CRISPR-Cas as the target, and a consistent and reliable vehicle is through phagemids (Chávez, 2018).

The hyper-IgM syndrome linked to the chromosome X (XHIM) is an immunodeficiency caused by mutations in the CD40L gene that is expressed on active T cells and results in the absence of IgG, IgA, and IgE due to a change in immunoglobulin. Patients with XHIM are more susceptible to infectious disease and autoimmune pathologies. Gene therapies have been researched to treat said condition, and in mice, the results with CRISPR-Cas were positive, with a high rate of recovery in the CD40L function. Furthermore, the technique did not show activity outside the target with a deficient proportion of treated cells; it is enough to treat the condition and improve quality of life (Zhang *et al.*, 2020).

Applications of CRISPR-Cas in lung cancer; go in four pillars or objectives: oncogenes, cancer suppressor genes, genes resistant to drugs, and T cells. The CRISPR-Cas system may deactivate one unique oncogene and, with it, the tumor growth. Oncogenes researched are EGFR, NESTIN, FAK, CTNND2, RSF1, and IGF1R. It is possible to restore the activity of suppressor genes of tumors and direct them towards the inhibition of tumorigenesis. Furthermore,

resistant genes can be modified to be more susceptible to chemotherapy to enhance activity and efficiency. The last objective is to modify the patient's T cells to enhance the microenvironment to promote a more anti-tumor environment and destroy cancer cells (Jiang *et al.*, 2019).

In a study, CRISPR-Cas9 was used to confine resistance in immune cells, to modify the receptor of chemokines CCR5, essential for the HIV-1 virus to infect. Results showed that it was viable to apply the technology in human iPSC, which allowed to study the role of CCR5 in the infection that forms *in vitro* and creates cells resistant to HIV, with possible therapeutic applications (Kang *et al.*, 2015).

The use of iPSC and CRISPR-Cas to treat β -thalidomide. A disease that originates in the mutation of the subunit β of the hemoglobin (HBB) resulting in inefficient oxygen transport, making it challenging to meet the required levels. The objective was to create iPSC with the HBB gene-corrected due to the high impact in the treatment since nowadays it is the only treatment available. It is a transplant allogeneic of somatic cells, which complicates due to the low chance of finding an adequate donor (Hu, 2016).

A mice essay demonstrated that CRISPR-Cas9 could be applied with simple guide RNA directed to noncoding gene regions, reducing the huntingtin gene's expression level related to Huntington's disease. This study evaluated mesenchymal stem cells extracted from the bone marrow of mice, which showed a significant decrease in HTT mRNA expression and improvements to motor deficits. This evidences the system is a potential therapy for this disease that minimizes off-target effects (Ekman *et al.*, 2019).

Microglia represents a viable therapeutic target for CRISPR-Cas9 since it can interact with components such as the glia maturation factor. Microglia plays an essential role in the immune response to neurodegenerative, inflammatory, and infectious pathologies. Its activation functions as a marker for the presence of these kinds of disorders and results in a possible treatment for diseases (Pahan, 2019).

A study on mice evaluated the possibility of using soluble receptors for advanced glycation end (sRAGE) products as a possible treatment for Parkinson's disease. CRISPR-Cas9 editing was used to generate Umbilical Cord Blood-derived Mesenchymal Stem Cells (UCB-MSC), which secrete sRAGE; UCB-MSCs were transplanted to the Corpus Striatum of animals with Parkinson's, and tests were performed to determine their effect. Results showed that neuronal cell death on the Substantia Nigra and Corpus Striatum was dramatically reduced, and movement improved due to RAGE inhibition, resulting in a potential treatment for Parkinson's disease (Lee *et al.*, 2019).

The application of CRISPR-Cas9 to correct a dominant, autosomal mutation of the PSEN2 gene has been studied as a possible treatment for Alzheimer's disease. Mutations of this gene increase beta-amyloid substances that, presumably when accumulated in the central nervous system, triggers Alzheimer's, which makes these genes important therapeutic targets. This investigation was carried out in iPSC-derived neurons using carrier cell lines and control over PSEN2 mutation. The correction of the mutation resulted in electrophysiological deficits reversal (Ortiz *et al.*, 2017).

D. CRISPR-Cas vectors

The main concern with CRISPR/Cas is the safety and efficiency of the therapy through intravenous administration. Due to the concern, it is most important to determine which is the best vector in the particular case, in the gene therapy, to be only towards the target to avoid non-target activity. Ideally, vectors should act only in the target and create low cytotoxicity, and CRISPR be quickly eliminated after the genetic modification; yet, no vehicles comply with all the ideal requirements (Doudna & Charpentier, 2014; Wilbie *et al.*, 2019).

Different methods of delivery exist that can deliver genetic modifiers to affected cells. One of the direct deliveries of genetic information is using CRISPR-Cas components by *ex vivo* in cells obtained from the patient and like T CAR cells. There is a diverse array of methods to make such procedures *ex vivo*, like microinjections in cells of rapid growth electroporation mechanisms. Recently, two novel techniques have been researched TRIAMF and iTOP (Wilbie *et al.*, 2019; D'Astolfo *et al.*, 2015; Foss *et al.*, 2019; Xu, 2019; Yen *et al.*, 2018).

TRIAMF is a technique in which cells are obtained through the membrane due to the pores being smaller than the cellular diameter, which induces the formation of pores through the cellular membrane. This method presents efficiency similar to electroporation techniques with lower cytotoxicity. Regarding the iTOP method, cells are laid in a hypertonic medium with propane-betaine; both components create the formation of endosomes through macropinocytosis that allows that option to absorb proteins and eventually release once the endosomal membrane is disturbed (D'Astolfo *et al.*, 2015; Yen *et al.*, 2018).

E. Viral Vectors

Viral vectors have been used as the first choice to resolve delivery issues with CRISPR-Cas. The most researched vectors are the adenoviral A, adeno-associated and lentiviral. The adeno-associated viruses have shown low immunogenicity with the first injection and significant gene expression, but storage capacity is quite restrictive. Consequently, regulatory elements are limited, such as promoters (Banaszynski *et al.*, 2006; Senturk *et al.*, 2017; Zetsche *et al.*, 2015).

Adenoviral vectors may easily contain all the necessary components for gene edition due to storage capacity, Cas proteins' expression, and one or more ARNs of one unique vector (Senturk *et al.*, 2017; Shin *et al.*, 2018; Wang *et al.*, 2015).

Lentiviral vectors in recent times are the most used vehicles in gene therapy since they produce the most extended gene expression. Advantages of this vector are its relative safety in the gene inclusion and capacity to transduce cells in constant cell division and those that do not divide as frequently or fast. Even though this advantage gives capacity for some time, it can be a downside in gene edition due to non-target events that are more likely to happen (Wilbie *et al.*, 2019; Banaszynski *et al.*, 2006; Senturk *et al.*, 2017).

The main problem with this kind of vector is the immunogenic effects that may cause a grave immune response that may result in issues and damages in the patient or destroy the target, altogether avoiding the desired

therapeutic effect in target cells (Wilbie *et al.*, 2019).

F. Non-viral vectors

Due to the disadvantages in viral vectors, such as the immunogenic and limited storage capacity, other vehicles (vectors) have been developed from synthetic materials that have less risk of activating immune responses after the first dose. Identically, particles incorporate inert components to evade the immune system, such as polyethylene glycol (Wilbie *et al.*, 2019).

Amongst the most used non-viral vectors that exist, there is a conjugation of molecule-excipient with the active molecule, lipidic materials highly known which are part of the formulaic in nanoparticle systems, and more recently, the development of lipidic nanoparticles. Other vehicles are based on polymer particles that may be used similar to lipids, such as cationic polymers such as polyethylamine (Finn *et al.*, 2018; Patel *et al.*, 2017; Zhang *et al.*, 2019).

Recently, studies have found that inorganic materials to encapsulate components of CRISPR-Cas such as red zinc would release components in the cell's cytosol (Alsaiani *et al.*, 2018; Yin *et al.*, 2016).

At last, research has been done in the mixture of vectors with virals and non-viral vehicles such as lipidic nanoparticles with adenovirus associates, which have shown successful delivery. Alas, the disadvantage is that it requires absorption of both vectors in the same tissue at the same time to ensure genetic repair or modification of any type within the target cell (Wilbie *et al.*, 2019; Yin *et al.*, 2016).

VI. NEW INVESTIGATIONS WITH CRISPR-CAS

In the healthcare field, efforts are generally focused on treatment, diagnostics, or improving patients' quality of life. In the case of the CRISPR-Cas technology, it is no exception, and the efforts to develop new applications in the health field aims to improve patients' quality of life.

One of the problems that modern medicine and investigation face is that some pathogens, especially microorganisms, can genetically vary at a very high rate, which means a great variety of strains of the same microorganism (Raschmanová *et al.*, 2018). Furthermore, the irrational use of drugs has generated significant resistance, especially antibiotics. Therefore, CRISPR-Cas technology has been a solution, and new research tries to fix these problems (Sapranaukas *et al.*, 2011; Ruiz, 2018).

Part of this solution is how this technology allows researchers to take an "Image" of the DNA or the genome to differentiate between infecting strains in patients. This is also important during differential diagnoses when different viruses like dengue and Zika cause similar symptoms. On the other hand, part of the response to bacterial resistance prevents resistance genes from coupling to the infecting microorganisms or developing acquired immunity in patients through CRISPR-Cas (Jaitin *et al.*, 2016).

Along the same lines of acquired immunity, the development of *in vivo* models has achieved the study of complicated diseases like HIV, human papillomavirus, herpes, among others, that allow researchers to understand better what is going on inside the patients. This same idea can be used to study different types of cancer, as the study

realized on breast cancer which made it possible to determine the proliferation marker in healthy tissue and thereby determine the risk of suffering this disease (Ruiz; 2018).

For example, a treatment was developed for type 3 familial hypercalcemia in genetic diseases. It has also allowed the study and observation of mutations in various populations worldwide and at a regional level. However, it should be noted that they must always have the ethics and responsibility guidelines at hand and the purpose of the studies. With these investigations, it is possible to determine the potential of hereditary diseases and prevent or treat them, which would be an actual dream. These efforts continue to be directed in this area because the more is known about it, the greater the possibility of designing new and novel treatments such as the familial amyloidosis case. Only some advances are mentioned; many more are bound to be found due to the speed of this field (Ruiz; 2018).

An important aspect that should be mentioned is that there are several systems according to their application, such as CRISPR-Cas9, CRISPR-Cas13a, and CRISPR-Cas3, among others, of therapeutic importance (Yao *et al.*, 2016). It should be noted that with the diversity of CRISPR-Cas systems, it is possible to determine surrounding viral oncogenes, genes related to the evasion of the immune responses, genes of tumors in microenvironments, therapeutic targets, or pathological targets. This information and the knowledge of vectors allow the development of new treatments and the continuous study of diverse pathologies that a patient may suffer (Ruiz; 2018).

CRISPR technology has been a unique tool for creating new drugs, even in complicated areas, such as the eyes, where new treatments mediated by CRISPR-Cas are being studied, particularly for a hereditary disease that generated problems in the retina. As soon as the active principle is known to work, it is only a matter of optimizing the formulation (Yu & Wu, 2020).

One of the inherent conflicts in any medication or treatment is the adverse or unwanted effects known as unwanted interactions. In the case of CRISPR-Cas, although its capacity is optimized for it to have a highly effective response, there may be unwanted effects, so the use of bioinformatics software has been used to predict these adverse reactions, as well as the use of other tools. Nonetheless, it is highly complicated to determine these interactions, even with clinical trials. Therefore, control variables, sensibilities, and other possible parameters are essential (Bonini *et al.*, 2021; Wilbie *et al.*, 2019). With such complex tasks and the need to guarantee safe and effective therapies, this area continues to be worked. Thus new trials are being developed where variables are more accessible to control and develop conditions for researchers to obtain comparable results (Wilbie *et al.*, 2019; Yao *et al.*, 2016).

VII. OTHER APPLICATIONS

CRISPR-Cas technology can be leveraged in other fields beyond healthcare. In general terms, this technique is often used to modify bacteria, yeasts, algae, and other types of fungi and thus develop processes to obtain products such as biofuels, organic acid, polyhydroxyalkanoate, phytochemicals, and amino acids (Abdelaal, 2020).

In the production of biofuels, this system can modify the genome of the microorganisms used in the process to minimize the metabolic load. Thus the efficiency of the fermentation process is maximized and can be applied to produce biofuels such as bioethanol (Shanmugam, *et al.*, 2020). It is also helpful to genetically modify insects such as *Aedes aegypti* for pest control (Hoy, 2019). The genetic manipulation of bacterial systems has a broad industrial utility, such as obtaining active metabolites, whether biochemical or biofuel products or precursors (Cho *et al.*, 2018).

VIII. PATENT

A patent describes a method for modeling diseases associated with genetic loci in eukaryotic systems. This includes vectors and vector systems that encode one or more components of the CRISPR complex (with the method for its design and use) and methods for directing the formation of the CRISPR complex in specific eukaryotic cells (Cong *et al.*, 2016).

Another patent utilizes the *Neisseria meningitidis* CRISPR-Cas9 system, it describes the Cas9 protein, the sgRNA, a coding sequence carrying the Cas9 and a sgRNA coding sequence vector. This invention can be applied in genome editing of the CCR5 receptor, which functions to prevent infection of cells by HIV (Zhang, & Qin, 2016).

Xenobiotic organ transplants can lead to the patient's immune system recognizing certain antigens from the donor animal and end up rejecting the transplant. A patented method provides a way to deactivate the swine SLA-1 gene using CRISPR-Cas9, which prevents the immune system from recognizing the SLA/peptide receptor and subsequently rejecting a transplant of pig organs (Cai *et al.*, 2016).

A patent describes a method that employs the CRISPR-Cas9 system to knockout the human apoCIII gene; it includes a sgRNA sequence directed to a region of the gene and a vector with the sequence. The method can be used to prevent and treat hyperlipidemia and other cardiovascular conditions (Jian *et al.*, 2016).

IX. CONCLUSIONS

This review allowed us to delve into the origin, applications, and ethical characteristics of the CRISPR-Cas technique since its discovery. It was possible to delve into the history of the technique and the principal applications it has had during the years since its discovery, like the development of cellular and animal models the development of new therapies to tackle complex diseases like cancer or HIV.

This review also delved into the development of ideal vectors for the delivery of CRISPR-Cas therapies which are of utmost importance because the use of adequate vectors diminishes the risk of adverse reactions, and, since these therapies work at a genetic level, it's important that the treatments only tackle specific cells to have the best outcomes and the least adverse effects possible.

Finally, it is concluded that although CRISPR-Cas's technology has come a long way since its discovery, its use still has important limitations, mainly due to the ethical

concerns of these therapies in humans. Nonetheless, this technique has proven promising in many fields beyond healthcare, and its future applications for food, fuel, and other materials production are promising. Therefore, continuous research and development of this technology are vital to achieve the best possible results while maintaining maximum safety.

ACKNOWLEDGMENT

The authors would like to express their sincere gratitude to the INIFAR, the School of Pharmacy and the University of Costa Rica for encouraging this research.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

REFERENCES

- Abdelaal, A. S., & Yazdani, S. S. (2020). Development and use of CRISPR in industrial applications. *En Genome Engineering via CRISPR-Cas9 System*, 177-197.
- Alkhnbashi, O. S., Meier, T., Mitrofanov, A., Backofen, R., & Voß, B. (2020). CRISPR-Cas bioinformatics. *Methods*, 172, 3-11.
- Alsaiahi, S. K., Patil, S., Alyami, M., Alamoudi, K. O., Aleisa, F. A., Merzaban, J. S., et al. (2018). Endosomal Escape and Delivery of CRISPR/Cas9 Genome Editing Machinery Enabled by Nanoscale Zeolitic Imidazolate Framework. *Journal of the American Chemical Society*, 140(1), 143-146.
- Araldi, R. P., Khalil, C., Grignet, P. H., Teixeira, M. R., de Melo, T. C., Módolo, D. G., et al. (2020). Medical applications of clustered regularly interspaced short palindromic repeats (CRISPR/Cas) tool: A comprehensive overview. *Gene*, 745, 144636.
- Arias-Fuenzalida, J., Jarazo, J., Qing, X., Walter, J., Gomez-Giro, G., Nickels, S. L., et al. (2017). FACS-Assisted CRISPR-Cas9 Genome Editing Facilitates Parkinson's Disease Modeling. *Stem Cell Reports*, 9(5), 1423-1431.
- Banaszynski, L. A., Chen, L.-C., Maynard-Smith, L. A., Ooi, A. G. L., & Wandless, T. J. (2006). A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell*, 126(5), 995-1004.
- Barajas, A. & Hernández, M. (2020). La bioética del sistema CRISPR-Cas9 como terapia génica en enfermedades de importancia mundial. *Ciencia Huasteca Boletín Científico de la Escuela Superior de Huejutla*, 8(16), 29-33.
- Bonini, A., Poma, N., Vivaldi, F., Kirchhain, A., Salvo, P., Bottai, D., et al. (2021). Advances in biosensing: The CRISPR/Cas system as a new powerful tool for the detection of nucleic acids. *Journal of Pharmaceutical and Biomedical Analysis*, 192, 113645.
- Capella, V. B. (2016). La revolución de la edición genética mediante CRISPR-Cas9 y los desafíos éticos y regulatorios que comporta. *Cuadernos de Bioética*, 19(2), 223-239.
- Cárdenas, R. (2019). El Derecho ante la técnica de edición genética CRISPR. *Acta bioethica*, 25(2), 187-197.
- Chávez, V. M. (2018). El sistema de edición genética CRISPR/Cas y su uso como antimicrobiano específico. *Revista especializada en ciencias químico-biológicas*, 21(2), 8. (Spanish).
- Cho, S., Shin, J., & Cho, B.-K. (2018). Applications of CRISPR/Cas System to Bacterial Metabolic Engineering. *International Journal of Molecular Sciences*, 19(4), 1089.
- Cong, L., Cox, D. B. T., Heidenreich, M., Platt, R. J., SWIECH, L., & Zhang, F. (2016). Delivery, use and therapeutic applications of the crispr-cas systems and compositions for genome editing. European Union Patent N.º EP3079725A1. Available from: <https://patents.google.com/patent/EP3079725A1/en?q=crispr+cas>
- Cota J, Sandoval S, Gaytan Y, Diaz N, Vega B, Padilla E, Díaz N.E. (2020). Nuevos modelos transgénicos para el estudio de la enfermedad de Parkinson basados en sistemas de edición con nucleasas. *Neurología*; 35(7), 486-499. (Spanish).
- D'Astolfo, D. S., Pagliero, R. J., Pras, A., Karthaus, W. R., Clevers, H., Prasad, V., et al. (2015). Efficient intracellular delivery of native proteins. *Cell*, 161(3), 674-690.
- De Buhr, H., & Lebbink, R. J. (2018). Harnessing CRISPR to combat human viral infections. *Current Opinion in Immunology*, 54, 123-129.
- Dominguez, A. A., Lim, W. A., & Qi, L. S. (2016). Beyond editing: Repurposing CRISPR-Cas9 for precision genome regulation and interrogation. *Nature Reviews. Molecular Cell Biology*, 17(1), 5-15.
- Doudna, J. A. & Charpentier, E. (2014) The new frontier of genome engineering with CRISPR-Cas9. *Science*, 346(6213).
- Ekman F, Ojala D, Adil M, Lopez P, Schaffer D, Gaj T. (2019) CRISPR-Cas9-Mediated Genome Editing Increases Lifespan and Improves Motor Deficits in Huntington's Disease Mouse Model. *Molecular Therapy - Nucleic Acids*, 17;829-839.
- Enzmann, B. (2019). How CRISPR Is Accelerating Drug Discovery. *GEN - Genetic Engineering and Biotechnology News*, 39(1).
- Fellmann, C., Gowen, B. G., Lin, P.-C., Doudna, J. A., & Corn, J. E. (2017). Cornerstones of CRISPR-Cas in drug discovery and therapy. *Nature Reviews. Drug Discovery*, 16(2), 89-100.
- Finn, J. D., Smith, A. R., Patel, M. C., Shaw, L., Youniss, M. R., van Heteren, J., et al. (2018). A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing. *Cell Reports*, 22(9), 2227-2235.
- Foss, D. V., Hochstrasser, M. L., & Wilson, R. C. (2019). Clinical applications of CRISPR-based genome editing and diagnostics. *Transfusion*, 59(4), 1389-1399.
- Freiermuth, J. L., Powell-Castilla, I. J., & Gallicano, G. I. (2018). Toward a CRISPR Picture: Use of CRISPR/Cas9 to Model Diseases in Human Stem Cells In Vitro. *Journal of Cellular Biochemistry*, 119(1), 62-68.
- Garcia, K., Lehka, B. J., & Mortensen, U. H. (2017). SWITCH: A dynamic CRISPR tool for genome engineering and metabolic pathway control for cell factory construction in *Saccharomyces cerevisiae*. *Microbial Cell Factories*, 16(1), 25.
- Gutiérrez, D. & Salazar, L. (2017) Tema 2017: CRISPR-Cas: Utilidad clínica de la edición genómica como opción terapéutica. *Revista Clínica de la Escuela de Medicina UCR-HSJD*, 7(6). (Spanish).
- Horvath, P., & Barrangou, R. (2010). CRISPR/Cas, the Immune System of Bacteria and Archaea. *Science*, 327(5962), 167-170.
- Hoy, M. A. (2019). CRISPR-Cas Genome Editing: Another Revolution in Molecular Biology. *En Insect Molecular Genetics*, 345-361.
- Hu, X. (2016). CRISPR/Cas9 system and its applications in human hematopoietic cells. *Blood Cells, Molecules, and Diseases*, 62, 6-12.
- Jaitin, D. A., Weiner, A., Yofe, I., Lara-Astiaso, D., Keren-Shaul, H., David, E., et al. (2016). Dissecting Immune Circuits by Linking CRISPR-Pooled Screens with Single-Cell RNA-Seq. *Cell*, 167(7), 1883-1896.e15.
- Jiang, C., Lin, X., & Zhao, Z. (2019). Applications of CRISPR/Cas9 Technology in the Treatment of Lung Cancer. *Trends in Molecular Medicine*, 25(11), 1039-1049.
- Kang, H., Minder, P., Park, M. A., Mesquitta, W.-T., Torbett, B. E., & Slukvin, I. I. (2015). CCR5 Disruption in Induced Pluripotent Stem Cells Using CRISPR/Cas9 Provides Selective Resistance of Immune Cells to CCR5-tropic HIV-1 Virus. *Molecular Therapy - Nucleic Acids*, 4, e268.
- Khan, S. H. (2019). Genome-Editing Technologies: Concept, Pros, and Cons of Various Genome-Editing Techniques and Bioethical Concerns for Clinical Application. *Molecular Therapy - Nucleic Acids*, 16, 326-334.
- Klompe, S. E., & Sternberg, S. H. (2020). CRISPR-Cas immune systems and genome engineering. *Rosenberg's Molecular and Genetic Basis of Neurological and Psychiatric Disease*, 157-177.
- Lammoglia, M. F., Lozano, R., García, C. D., Avilez, C. M., Trejo, V., Muñoz, R. B., et al. (2016). La revolución en ingeniería genética: Sistema CRISPR/Cas. 13.
- Lee, J., Bayarsaikhan, D., Arivazhagan, R., Park, H., Lim, B., Gwak, P., Lee, B. (2019). CRISPR-Cas9 Edited sRAGE-MSCs Protect Neuronal Death in Parkinson's Disease Model. *International Journal of Stem Cells*, 12(1), 114-24.
- Luo, J., Padhi, P., Jin, H., Anantharam, V., Zenitsky, G., Wang, Q., Kanthasamy, A. G. (2019). Utilization of the CRISPR-Cas9 Gene Editing System to Dissect Neuroinflammatory and Neuropharmacological Mechanisms in Parkinson's Disease. *Journal of Neuroimmune Pharmacology*, 14(4), 595-607.
- Ortiz-Virumbrales M, Moreno C, Kruglikov I, Marazuela P, Sproul A, Jacob S., et al. (2017) CRISPR/Cas9-Correctable mutation-related molecular and physiological phenotypes in iPSC-derived Alzheimer's PSEN2 N141I neurons. *Acta Neuropathologica Communications*, 5(1).
- Pahan K. (2019). A Broad Application of CRISPR Cas9 in Infectious, Inflammatory and Neurodegenerative Diseases. *Journal of Neuroimmune Pharmacology*, 14(4), 534-6.

- Patel, S., Ashwanikumar, N., Robinson, E., DuRoss, A., Sun, C., Murphy-Benenato, K. E., et al. (2017). Boosting Intracellular Delivery of Lipid Nanoparticle-Encapsulated mRNA. *Nano Letters*, 17(9), 5711-5718.
- Quílez, Á. (2017). Aplicaciones de CRISPR en la generación y síntesis de nuevos fármacos. *MoleQla: revista de Ciencias de la Universidad Pablo de Olavide*, 27, 10. (Spanish).
- Raschmanová, H., Weninger, A., Glieder, A., Kovar, K., & Vogl, T. (2018). Implementing CRISPR-Cas technologies in conventional and non-conventional yeasts: Current state and future prospects. *Biotechnology Advances*, 36(3), 641-665.
- Ruiz, O. H. (2018). El sistema CRISPR-Cas y su aplicación en las enfermedades infecciosas. *Hechos Microbiológicos*, 9(1-2), 9-11.
- Sapranaukas, R., Gasiunas, G., Fremaux, C., Barrangou, R., Horvath, P., & Siksnys, V. (2011). The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Research*, 39(21), 9275-9282.
- Scott, A. (2018). A CRISPR path to drug discovery. *Nature*, 555.
- Senturk, S., Shirole, N. H., Nowak, D. G., Corbo, V., Pal, D., Vaughan, A., Tuveson, D. A., Trotman, L. C., Kinney, J. B., & Sordella, R. (2017). Rapid and tunable method to temporally control gene editing based on conditional Cas9 stabilization. *Nature Communications*, 8, 14370.
- Shanmugam, S., Ngo, H.-H., & Wu, Y.-R. (2020). Advanced CRISPR/Cas-based genome editing tools for microbial biofuels production: A review. *Renewable Energy*, 149(C), 1107-1119.
- Shin, J., Lee, N., Cho, S., & Cho, B.-K. (2018). Targeted Genome Editing Using DNA-Free RNA-Guided Cas9 Ribonucleoprotein for CHO Cell Engineering. *Methods in Molecular Biology (Clifton, N.J.)*, 1772, 151-169.
- Shipman, S. L., Nivala, J., Macklis, J. D., & Church, G. M. (2017). CRISPR-Cas encoding of a digital movie into the genomes of a population of living bacteria. *Nature*, 547(7663), 345-349.
- Singh, V. (2020). An introduction to genome editing CRISPR-Cas systems. *Genome Engineering via CRISPR-Cas9 System*, 1-13.
- Smalley, E. (2016). CRISPR mouse model boom, rat model renaissance. *Nature Biotechnology*, 34(9), 893-894.
- Song, H.-Y., Chiang, H.-C., Tseng, W.-L., Wu, P., Chien, C.-S., Leu, H.-B., et al. (2016). Using CRISPR/Cas9-Mediated GLA Gene Knockout as an In Vitro Drug Screening Model for Fabry Disease. *International Journal of Molecular Sciences*, 17(12), 2089.
- Soppe, J. A., & Lebbink, R. J. (2017). Antiviral Goes Viral: Harnessing CRISPR/Cas9 to Combat Viruses in Humans. *Trends in Microbiology*, 25(10), 833-850.
- Tu, Z., Yang, W., Yan, S., Guo, X., & Li, X.-J. (2015). CRISPR/Cas9: A powerful genetic engineering tool for establishing large animal models of neurodegenerative diseases. *Molecular Neurodegeneration*, 10(1), 35.
- Wang, D., Mou, H., Li, S., Li, Y., Hough, S., Tran, K., et al. (2015). Adenovirus-Mediated Somatic Genome Editing of Pten by CRISPR/Cas9 in Mouse Liver in Spite of Cas9-Specific Immune Responses. *Human Gene Therapy*, 26(7), 432-442.
- Wilbie, D., Walther, J., & Mastrobattista, E. (2019). Delivery Aspects of CRISPR/Cas for in Vivo Genome Editing. *Accounts of Chemical Research*, 52(6), 1555-1564.
- Xu, W. (2019). Microinjection and Micromanipulation: A Historical Perspective. *Methods in Molecular Biology (Clifton, N.J.)*, 1874, 1-16.
- Xue, W., Chen, S., Yin, H., Tammela, T., Papagiannakopoulos, T., Joshi, N. S., et al. (2014). CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature*, 514(7522), 380-384.
- Yao, R., Liu, D., Jia, X., Zheng, Y., Liu, W., & Xiao, Y. (2018). CRISPR-Cas9/Cas12a biotechnology and application in bacteria. *Synthetic and Systems Biotechnology*, 3(3), 135-149.
- Yen, J., Fiorino, M., Liu, Y., Paula, S., Clarkson, S., Quinn, L., et al. (2018). TRIAMF: A New Method for Delivery of Cas9 Ribonucleoprotein Complex to Human Hematopoietic Stem Cells. *Scientific Reports*, 8(1), 16304.
- Yin, H., Song, C.-Q., Dorkin, J. R., Zhu, L. J., Li, Y., Wu, Q., et al. (2016). Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nature Biotechnology*, 34(3), 328-333.
- Yu, W., & Wu, Z. (2020). Ocular delivery of CRISPR/Cas genome editing components for treatment of eye diseases. *Advanced Drug Delivery Reviews*, S0169409X20300570.
- Zetsche, B., Volz, S. E., & Zhang, F. (2015). A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nature Biotechnology*, 33(2), 139-142.
- Zhang, Z., Wan, T., Chen, Y., Chen, Y., Sun, H., et al. (2019). Cationic Polymer-Mediated CRISPR/Cas9 Plasmid Delivery for Genome Editing. *Macromolecular Rapid Communications*, 40(5), e1800068.
- Zhang, Z.-Y., Thrasher, A. J., & Zhang, F. (2020). Gene therapy and genome editing for primary immunodeficiency diseases. *Genes & Diseases*, 7(1), 38-51.
- Jian, C., Wei, J., & Yue, P. (2016). A CRISPR-Cas9 system targeting apoCIII and its application. China Patent N.º CN105462968A. <https://patents.google.com/patent/CN105462968A/zh?q=crispr+cas9>
- Zhang, J. & Qin X. (2016). Target sequence and sgRNA of human CCR5 gene recognized by CRISPR-Cas9 system in *Neisseria meningitidis* and its application. China Patent N.º CN105331609A. <https://patents.google.com/patent/CN105331609A/en?q=crispr+cas9>
- Cai, Z., Mou, L., Chen, P., Xie, C., Zhang, J., Gao, H. et al. (2016). CRISPR-Cas9 specific knockout method of pig SLA-1 gene and sgRNA for specific targeting of SLA-1 gene. World Intellectual Property Organization Patent N.º WO2016197359A1. <https://patents.google.com/patent/WO2016197359A1/en?q=crispr+cas9>