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Journal of Lab Animal Research. 2022; 1(1): 47-51.

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Review Article

CRISPR/Cas9-mediated Genome Editing: *In Vivo* Review

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ARTICLE INFO

Article History:
Received: 17/08/2022
Accepted: 22/09/2022

Keywords:
CRISPR
Disease models
Gene therapy
Genome editing
In vivo

ABSTRACT

The CRISPR/Cas9 system has been a game-changer in genetics and biotechnology. This study aimed to investigate the existing *in vivo* uses and its potential in gene function and biological processes using animal models. With its remarkable precision and accuracy, researchers can now easily edit specific genes within cells and organisms. This technology has opened up new avenues for studying genetic diseases and developing therapies to treat them. One of the most significant advantages of the CRISPR/Cas9 system is its ability to create precise cellular and animal models of human diseases. This allows researchers to investigate the role of genetics in disease development and to develop more effective therapies. For example, the system can correct genetic mutations that cause cystic fibrosis or sickle cell anemia. The therapeutic potential of CRISPR/Cas9 is enormous, especially in gene therapy. By correcting specific genetic mutations, the system can potentially treat human diseases that are currently untreatable with conventional therapies. However, some challenges still need to be addressed before this technology can be used in clinical settings. Despite these challenges, the potential of CRISPR/Cas9 to revolutionize the field of genetics and biotechnology cannot be overstated. Ultimately, this technology has the potential to transform medicine by providing new therapies for a wide range of genetic diseases.

1. Introduction

Genome editing is a type of genetic modification that involves manipulating DNA at the level of individual bases[1]. It has revolutionized biomedical research by holding great potential for treating and preventing various human genetic disorders. However, the most effective genome-editing tool needs to be highly specific in modifying genomic sequences while minimizing off-target effects[2]. Initially, genome-editing techniques involves replacing small genome sections with external donor DNA sequences using the homologous recombination repair pathway in yeast and mammalian cells[3]. Similarly, mouse embryonic stem cells were also used to create mice with specific genotypes[4]. However, these techniques have limitations, such as low editing efficiency and unwanted genome modifications that occur at random sites rather than at the intended location [5].

To overcome these limitations, scientists have developed Meganucleases, which are endonucleases that cut specific DNA sequences to stimulate homology-directed repair (HDR)[6]. This approach introduces site-specific double-stranded breaks (DSBs) into the genomic locus of interest. The DNA donor template with homologous ends is delivered to copy information along the break site using polymerase[7]. However, DSBs can also result in non-homologous end-joining (NHEJ) repair mechanisms, leading to inserting or deleting random nucleotides (indels)[8]. Although NHEJ can effectively generate functional gene knockouts, the creation of indels is an unwanted side

► *Cite this paper as*: Lotfalizadeh N, Sadr S, Ahmadi Simab P, Hajjafari A, Borji H, Bayat Z. CRISPR/Cas9-mediated Genome Editing: *In Vivo* Review. Journal of Lab Animal Research. 2022; 1(1): 47-51.

effect[9]. Therefore, developing site-specific DSBs that trigger HDR while reducing NHEJ activity remains a challenge.

A new approach to editing genomes involves the use of zinc finger nucleases (ZFNs) and transcription-activator-like effector nucleases (TALENs), which follow DNA-protein recognition principles[10]. These are fusion proteins that combine an engineered DNA binding domain with a non-specific nuclease domain from the FokI restriction enzyme[11]. The ZFNs and TALENs can be customized to cut almost any target sequence in the genome with high specificity, which is an advantage over DNA-binding proteins[12]. However, the design, synthesis, and validation of these proteins are challenging in the laboratory.

In contrast, the discovery of the CRISPR/Cas9 system has revolutionized gene editing, making it more accessible. CRISPR/Cas9 employs a small RNA to create a site-specific double-stranded break (DSB), unlike ZFNs and TALENs[13]. The Cas9 endonuclease only requires a 20-nucleotide guide RNA (sgRNA) that attaches to the target DNA and a DNA protospacer-adjacent motif (PAM), a short DNA sequence next to the complementary region that varies depending on the bacterial species of the Cas9 protein used[14,15]. This two-pronged strategy, where the sgRNA guides Cas9 to target any DNA sequence of interest, has replaced the complicated protein design process required by ZFNs and TALENs[16]. The simplicity of the CRISPR/Cas9 technology, along with its unique DNA cleaving mechanism, ability to target multiple regions, and the existence of different type II CRISPR-Cas system variants, has allowed for significant progress using this cost-effective and user-friendly technology to precisely and efficiently modify the genomic DNA of various cells[17,18].

2. CRISPR/CAS9: History and Mechanism

In 1987, Ishino and colleagues were studying *Escherichia coli* when they observed the presence of a cluster of DNA sequences that were repetitive and separated by variable spacer regions[19]. The significance of these sequences was unknown at the time. Mojica et al. identified similar sequences in many other bacteria and archaea and named them Clustered Regularly Interspaced Palindromic Repeats or CRISPR[20].

In 2007, Barrangou and colleagues experimented with a well-characterized phage-sensitive *S. thermophilus* strain and two bacteriophages, showing experimentally that CRISPR provides adaptive immunity, confirming the hypothesis that CRISPR might be an adaptive immunity system[21]. In 2008, it was discovered that CRISPR RNAs (crRNAs) serve as guides in a complex with Cas proteins to promote phage resistance[22]. Additionally, it was observed that the CRISPR/Cas system could be repurposed as a programmable restriction enzyme to target DNA. This finding was significant because it suggested that CRISPR could potentially be used for genome editing in heterologous systems. In the same year, Marraffini and Sontheimer explicitly predicted that CRISPR might be repurposed for genome editing in heterologous systems, opening up new possibilities for applying CRISPR[23]. In recent years, researchers from various groups have identified the components of the recombinant CRISPR/Cas9 system and have demonstrated its functionality in mammalian cells[24-26]. This work has paved the way for the development of CRISPR as a powerful tool for genome editing, opening up new possibilities for research and innovation in biotechnology and medicine. Extensive research has been conducted on the CRISPR mechanism, which is categorized into different types and subtypes[27]. There are two primary categories: class 1 and class 2, which have different effector protein complex organizations. Class 1 comprises 15 subtypes, further subdivided into three types (I, III, and IV), while class 2 is defined by a single-protein effector module and is divided into types II, V, and VI[28]. The type II CRISPR mechanism is unique in that it uses only one Cas protein (Cas9) for gene silencing[29].

The CRISPR mechanism involves the integration of DNA from past viral or plasmid infections into a CRISPR locus with short repetitive sequences separated by spacer sequences[30]. The locus is transcribed, and precursor CRISPR RNAs (pre-crRNAs) are processed to generate small crRNAs with the help of trans-activating CRISPR RNA (tracrRNA) that complements the CRISPR repeat sequence[31,32]. The mature crRNAs serve as guides for Cas nucleases to recognize and cut invading DNA based on sequence complementarity. The Cas9 protein has two nuclease domains that cleave both DNA strands matching the 20-nucleotide target sequence, resulting in double-stranded breaks (DSBs)[33]. To produce precise gene editing in a therapeutic context, Cas9 requires a short-conserved sequence (2-5 nucleotides) called protospacer adjacent motif (PAM) immediately downstream of the 3´ crRNA [34]. Cas9 can cut the non-complementary DNA strand and produce DSBs within 3 bp to 8 bp upstream of the PAM.

The CRISPR/Cas9 system is a straightforward and adaptable technology that requires only a customized single guide RNA (sgRNA) to generate DSBs at nearly any DNA target site, making it widely used for genome editing in various cell types and organisms [35]. In 2012, a simplified two-component CRISPR/Cas9 system was developed by combining tracRNA and crRNA into a single guide RNA, which is as effective as Cas9 programmed with separate tracRNA and crRNA in guiding targeted gene alterations[36].

3. CRISPR/CAS9 as an efficient tool for genome editing in mammalian cells

3.1. Application of the CRISPR/Cas9 system in the rapid generation of animal models

The CRISPR/Cas9 technology is a revolutionary tool in genetic engineering, providing researchers with a powerful method for generating genetically modified animal models with incredible efficiency and speed[37]. CRISPR/Cas9 has eliminated many of the time-consuming and laborious steps previously required to create animal models with specific genetic mutations[38]. Before the development of this technology, researchers would need to manipulate

embryonic stem cells, which were difficult to grow and maintain, and then screen offspring for germline transmission of the desired mutation. This process was often inefficient, labor-intensive, and expensive, presenting a significant hurdle in developing genetically engineered animal models[39,40].

However, with the CRISPR/Cas9 system, researchers can now introduce mutations directly into the genome of a zygote by injecting the Cas9 protein and a guide RNA (gRNA) into the fertilized egg[41]. This approach has eliminated the need to manipulate embryonic stem cells, streamlining the process of introducing mutations into the genome. Moreover, the CRISPR/Cas9 system is highly efficient, enabling researchers to generate mice with mutations in multiple genes in a single editing step[42,43]. This capability has opened up the possibility of creating animal models with complex genetic mutations that are more representative of human diseases like cancer, which often involve multiple genes and pathways[44]. In addition, the CRISPR/Cas9 technology allows researchers to introduce additional mutations in pre-existing animal models of disease without the need for embryonic stem cell derivation or complex genetic crosses[45]. This capability makes it easier to create animal models of diseases that accurately reflect the genetic heterogeneity of human diseases, which can facilitate the development of novel treatments and therapies. With the CRISPR/Cas9 system, researchers can now create animal models that are more complex and accurately represent human disease, accelerating the development of new treatments and therapies for various diseases [46].

Overall, the CRISPR/Cas9 technology has significantly reduced the time and cost required to generate genetically modified animal disease models, making it more accessible to researchers. This technology has the potential to accelerate the development of new treatments and therapies for various diseases, representing a significant breakthrough in genetic engineering. The use of CRISPR/Cas9 has opened up new avenues for research in fields such as oncology, immunology, and neuroscience, offering the potential to unlock new insights into complex diseases and their treatment.

3.2. Application of the CRISPR/Cas9 system in animal models for the treatment of human diseases

The CRISPR/Cas9 technology has transformed the field of genetics research by providing a revolutionary gene-editing approach that offers new possibilities for developing effective therapies for once-incurable genetic conditions. This technology has been extensively used to evaluate the efficacy of treatments for genetic disorders through genetically modified animal models.

In 2013, Wu et al. conducted one of the first studies to use the CRISPR/Cas9 system to efficiently correct a genetic disease in mice[47]. The study demonstrated how the CRISPR/Cas9 system could correct a dominant cataract-causing mutation in the Crygc gene. This breakthrough paved the way for further research exploring the therapeutic potential of this technology. Another study used the mdx mouse model of Duchenne muscular dystrophy, a rare disorder caused by mutations in the gene that encodes dystrophin[48]. The study demonstrated that the CRISPR/Cas9 system could potentially provide an effective treatment for this debilitating genetic disease by generating genetically mosaic progeny with varying degrees of muscle phenotypic rescue. In 2014, a groundbreaking study demonstrated that the CRISPR/Cas9 system could successfully correct a mutation in post-natal animals[49]. The study corrected the fumarylacetoacetate hydrolase (Fah) gene's homozygous point mutation in the Fah59815B mouse model, leading to severe liver damage[49]. This breakthrough demonstrated that the CRISPR/Cas9 system could be used to develop gene therapies for genetic diseases. Other studies have shown that the CRISPR/Cas9 technology can be used to treat genetic disorders in adult mice by delivering CRISPR/Cas9 machinery to the mdx mouse model of Duchenne muscular dystrophy to restore dystrophin expression in skeletal and cardiac muscle cells[50,51].

These groundbreaking *in vivo* studies have made significant progress toward developing new therapies for genetic diseases. Human genetic disorders are currently incurable, significantly impacting patients' quality of life and life expectancy. The significant progress made in the CRISPR/Cas9 technology highlights the potential of this gene therapy approach to cure human genetic diseases, offering a promising outlook for the future of medicine.

4. Conclusion

The revolutionary CRISPR/Cas9 system, which utilizes RNA to guide DNA editing, has significantly impacted genome editing and research. The latest advancements in CRISPR/Cas9 tools have made it more accessible for researchers worldwide to study human diseases, with increased selectivity, high DNA specificity, and minimal by-product formation. Using *in vivo* animal models, researchers can generate disease models in just a few weeks, paving the way for the potential treatment of genetic disorders using CRISPR/Cas9 technology. However, the technology's efficiency, specificity, and delivery still require improvement to make it widely available in clinical settings. The potential for misuse of CRISPR/Cas9 is also a major concern, with significant implications for medical practice. The discovery of the CRISPR/Cas9 mechanism offers new possibilities for medical treatments that have the potential to revolutionize the approach to genetic disorders.

Declarations

Competing interests

The authors have declared no conflicts of interest.

Authors' contributions

Narges Lotfalizadeh, Soheil Sadr, Pouria Ahmadi Simab,

Ashkan Hajjafari, and Hassan Borji wrote the draft of the manuscript. Zeynab Bayat revised the final draft of the manuscript. All authors have read and approved the final version of the manuscript for publication in the present journal.

Funding

No funding was received for conducting this study.

Ethical considerations

The authors declare that this manuscript is original and has not been submitted elsewhere for possible publication. The authors also declare that the data used/presented in this manuscript has not been fabricated.

Availability of data and materials

The authors will provide the data from the present study in case of request.

Acknowledgments

None.

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