# CRISPR-Cas9 Fundamental Uses: Analysis of Human Genome Engineering Through CRISPR/Cas9

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**Abstract.** The invention of CRISPR-Cas9 allowed the wanted genome to be targeted and edited. Clustered regularly interspaced short palindromic repeats (CRISPR) combined with Cas9 protein together can be diverted to a guided location in the genome, editing the DNA strand. This achievement improved the human body's genomes and turned them into cells. CRISPR-Cas9 could be used in cells and animal model generations, and it has been proven that human embryos can be similarly modified. The passage analysis has also included the type II bacterial CRISPR system with custom guide RNA (gRNA) in human cells, which allowed the team to implement sophisticated investigations and experiments: endogenous AAVS1 locus in targeting T cells, K562 cells, and induced pluripotent stem cells. The experiment research provides guidelines for the future development target sites, cleavage efficiency, and off-target activity analysis.

**Keywords:** CRISPR/Cas9 · gene editing · therapeutics · guide RNA · bioethics

## 1 Introduction

Understanding the genomes that cause certain diseases and treating them with the correct mutations requires genome modification. CRISPR-Cas9 can precisely modify specific genome sites, and it is also considered a standard method to clarify gene function, create an animal or plant models of disease, and improve their presentation from microbiological ways [1, 2]. In addition, engineered CRISPR-associated protein 9 provides a more straightforward and economical method for gene-targeted modification. However, though the benefits are tempting, the current state of CRISPR-based programs is still considered in its early stages of development: Cas9 is limited as an in vivo therapy dilemma of ineffective delivery, casual responses, off-target issues, unpredictable harm, and tension of cellular alter [3]. With these problems, the research implemented trial with the hope to alleviate or solve some of these problems. The research topic of this paper is gene editing in the human body with CRISPR-Cas9. The analyzed report applies the engineering of protein and relevant RNA components for the specific bacterial type II CRISPR system in human cells, this sophisticated Cas9 protein corporate with C-terminal SV40 (Effect DNA tumor that could led the tumor to transform) nuclear localization signal, which is cloned into a mammalian genome [4]. The research could


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transform the RNA-guided editing tool into a simpler, dependable, and multiplexable human genome engineering. It may enhance fault tolerance, safety, and efficiency and reduce harm to the human body through improvements [2, 4]. The future development of gene editing could be inferred to improve the fault tolerance rate of editing. Explore and solve gene editing mismatch and unforeseen damage, improve the safety of gene editing, ensuring the technology with higher popularity [5].

## 2 Main Body

### 2.1 Cumulation of Existing Research

Gene editing is a technology developed to produce proteins from other sources in humans and organisms that are usually not synthesized. It could benefit organisms to form a faster and more accurate diagnosis, more targeted treatment, and prevent hereditary diseases. With the development of modern technology to build a new method of genetic engineering, CRISPR is the most successful one with practicability and efficiency in scientific research and technology [6, 7]. The report, RNA-Guided Human Genome Engineering via Cas9, utilized this technology based on a type II CRISPR system in their experimental design. From the report, the team attempted to use the type II CRISPR and customized gRNA for endogenous adeno-associated virus site 1 (AAVS1: locus located on chromosome 19) locus to target 293 T cells (HEK 293 T: mutated SV40 large T antigen), K562 cells (a cell line commonly used for a highly sensitive target), and induced pluripotent stem cells (IPSCs: reprogram mature stem cells to embryonic stem cell for maintaining its original properties). Their findings were to improve the ability of the target genomic site and expedite this process. In addition, from a comprehensive area perspective, this technology has other advantages. For example, type II-C CRISPR-Cas9 is a milestone in the genome editing revolution. Nearly half of Cas9 is found based on the II-C subtype. The system exists in a variety of bacteria and archaea in the environment [8]. Therefore, Cas9 has unique and potentially valuable characteristics under different conditions.

In addition, the type II system requires the original protospacer adjacent motif (PAM) sequence at the target site and cleavage and to consider these unique functions of PAMs to reduce the target density and improve the target positioning accuracy. These factors could result in the synthetical ability to use RNA to program-specific DNA cleavage and be transformed into an editing tool. Combined with the S. pyogenes CRISPR system, it can reconstitute mammalian cells to facilitate efficient genome editing [9]. However, the limitation from NGG PAM (A locus that follows a N and two G nucleobase) on the target space of Cas9 could introduce some inefficiency during the process, because the type II CRISPR has to match the target PAM sequence NGG and the required 8–12 base at the 3′ end of the gRNA site to match the system for it to function properly [4]. The report also shows that the potential chromatin structure, epigenetic status, and chain-breaking activity of target loci may affect the efficiency of eukaryotic genome editing.

Fortunately, the system could overcome some restrictions by utilizing different Cas9 enzymes and their additional PAM requirements in microbial diversity. The report stated that CRISPR loci could transcribe into mammalian cells. In general, editing multiple

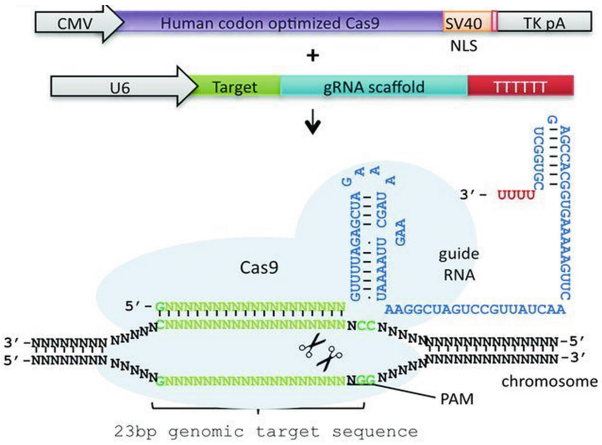


**Fig. 1.** The gene editing process using type II CRISPR system.

genomes in mammalian cells could achieve basic science, biotechnology, and medical discoveries [4].

In the report, the method used for recombinant mammalian cells is to apply the engineered protein and RNA components of this bacterial type II CRISPR system to human cells. The concise version of this process can be summarized as follows: firstly, the human codon at the C end of the SV40 nuclear localization signal was synthesized and cloned into a mammalian expression system with the best version of Cas9 protein. The human U6 polymerase III promoters were transcribed into gRNA to guide the Cas9 cleavage site sequence; this method can avoid using bacterial CRISPR to construct an RNA processor. U6 transcription gives the limitation of starting with a G base pair and requiring PAM sequence NGG with about 20 base-pair on the crRNA target loci. The report concluded that a high degree of variance constitution could target any genomic site of the form GN20GG [4].

On the other hand, the other two strategies, zinc-finger nuclease (ZFNs) and transcription activator-like effector nucleases (TALENs), use endonuclease catalytic domain to a binding protein to induce the targeted DNA double-strand breaks (DSBs) at specific genomic sites. In contrast, Cas9 is a nuclease guided by small RNA through base pairing with the target genome: this shows the system could be designed with ease, simple to predict at non-target sites, and the possibility of modifying multiple genomic sites simultaneously, which is often suitable for high-throughput and multiple gene-editing of various cell types and organisms [1] (Fig. 1).

The research team first synthesized the human cells, which express the Cas9 protein at C-terminal SV40 nuclear localization signal with the gRNAs expressed from the human U6 polymerase III promoters. With the correct PAM sequence presented at the 3′ terminal, the Cas9 will be responsible for unwinding the DNA duplex and segregating the strands when the gRNA recognizes the target sequence. This presented method theoretically can target any genomic sequence that contains GN20GG.

Other related conducted experiments showed similar results. The non-homologous end junction (NHEJ) modifies the ends of broken DNA and connects them, regardless

of homology, resulting in deletion or insertion. In contrast, homologous recombination (HR) used undamaged DNA templates to repair breaks, reconstructing the original sequence. Compared with NHEJ, the process of HR repairing substrate gene replication is prolonged and takes three to six times. Other research results show that the process of NHEJ is much faster than HR, which explains the higher efficiency of NHEJ in mammalian cells [8, 10, 11]. NHEJ is a very effective repair mechanism and is the most active in cells. Due to nucleotide insertion and deletion, it is also prone to frequent mutation errors. However, when the research group requested the original GFP sequence, the use of HR was inevitable for its accuracy.

With the principle of practice, the team developed a green fluorescent protein (GFP) in HEK 293T cells (human embryonic kidney) to test the functional realization of genome engineering. They destroyed the genome integrated GFP coding sequence and a stop codon with 68 gene base pair from the AAVS1 locus. These measures expressed the protein fragment with no fluorescence reaction. Then the HR is applied to rehabilitate the normal GFP sequence functions, including appropriate repair donors, which testifies the GFP+ produced by flow-activated cell sorting (FACS). The team also developed two gRNAs to test the function of stimulated HR: T1 and T2, which both target the mediated AAVS1 fragment to compare them with the TALEN [4, 12]. The result was that all three targeting compounds had completed the HR process. What is noteworthy was that the gene correction rates with T1 and T2 gRNAs were much improved, with 3% on T1 and 8% on T2; in comparison, the TALEN gene correction rate was only about 0.3%. In addition to that, the RNA engineering process was much faster; the developed gRNAs were twice as fast as the TALEN in the engineering process: the first GFP+ cells appeared 20 h after the transfection and 40 h for the AAVS1 TALENs [4]. Also, the team had research on the principle of Cas9D10A mutant, which functions as a nickase. They attempted this part of the study because, in theory, ZFNs and TALENs have shown that only single-stranded DNA breaks can reduce cellular toxicity. As a result, the Cas9D10A mutant achieved similar HR levels [4, 12]. Still, the NHEJ rate was low, and the mutation target site would prevent gRNA from affecting the HR of sequence-specific sites. In addition, the study also conducted experiments on gRNA targeting the GFP gene, DNA methyltransferase 3A (DNMT3a), and DNMT3b gene, which confirmed that gRNA used for targeting human cells was simple to conduct and operate. It also conducted a strong HR at multiple targets.

The research team integrated Figs. 2 and 3 to represent the modified native locus. They tested the practical gRNAs to target the AAVS1 locus in the protein Phosphatase 1 Regulatory Subunit 12C (PPP1R12C: protein coding gene) gene on chromosome 19, targeting 293Ts, human CML K562 cells, and PGP1 iPS cells [4]. The expression of Cas9 protein and one of T1 or T2 gRNAs to compute the next-generation sequence could cause the NHEJ to delete base pairs in 293Ts, K562s, and PGP1 iPS cells [4]. Additionally, the polymerase chain reaction (PCR) sports the transfection combinations to exhibit HR events three days after the transfection; the result shows that the cells that expressed the donor had successfully demonstrated HR events.

The team then practiced editing local loci with the gRNAs previously illustrated to target the chromosome. The targeted cells include 293Ts, human chronic myelogenous leukemia K562 cells, and PGP1 iPS cells. Consistent with the analysis results of previous

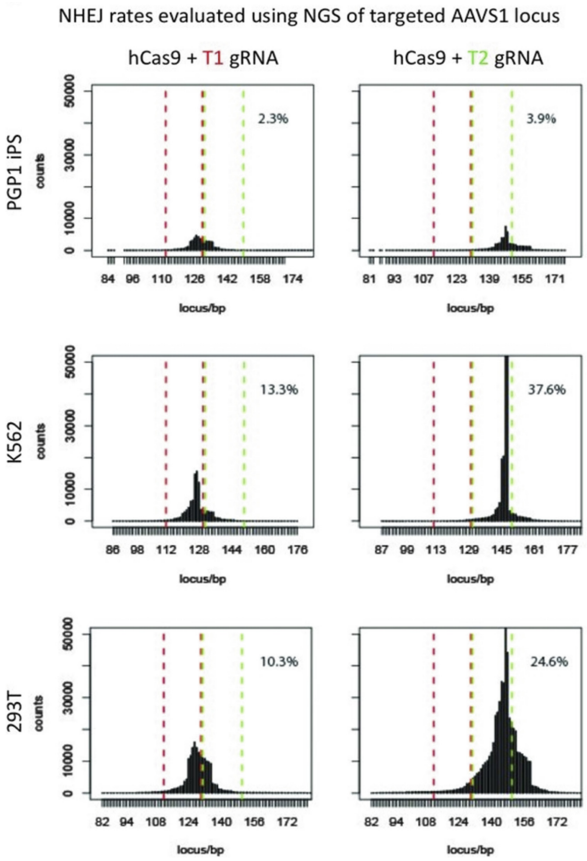


**Fig. 2.** NHEJ frequencies for combined gRNAs and tested locus

GFP reports, there is a large number of NHEJ events at endogenous sites of all cell types: the gRNAs T1 and T2 reported 10% and 25% NHEJ rates in 293Ts, 13% and 38% in K562s, 2% and 4% in PGP1 iPS cells. The NHEJ mediated deletions process for T1 and T2 were most active around the target site, which validated the sequence accuracy during the targeting process [4]. With both T1 and T2 gRNAs intervening, the target site had resulted in the express deletion of the 19-bp fragment, and this verification indicates the multiplexed editing could be accomplished with this method. Lastly, to testify the use of HR to combine a DNA or oligo donor into the AAVS1 locus, PCR and Sanger sequencing was used to confirm integration. It could conclude that this experimental approach can effectively integrate exogenous DNA at endogenous sites of human cells [13, 14] Overall, the agile gRNA genome engineering system could easily modify other genomic sites by changing the required gRNA sequence expression to match the compatible sequence [8, 14].

Using guide RNAs to program sequence-specific DNA cleavage sites expands a new method of genome engineering. The report has shown that it can use the S. pyogenes type

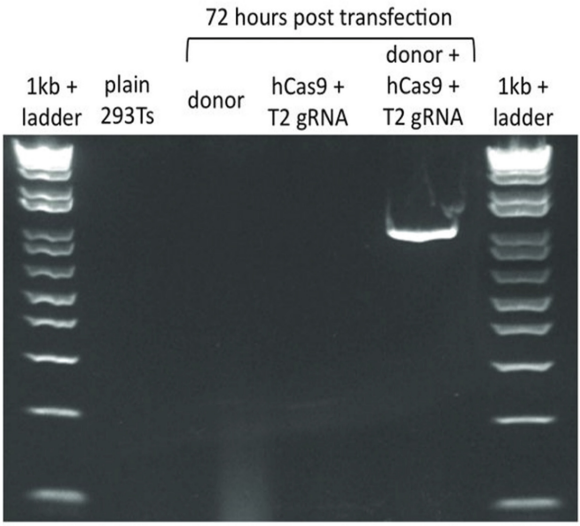


**Fig. 3.** The PCR model to represent 3 days after transfection

II CRISPR system to reconstitute mammalian cells to facilitate improved efficiency for genome editing. When ZFNs and TALENs are used and applied with higher efficiency, the improvement of targeting to modify genome sequences dramatically reduces the difficulty [12, 15]. Other studies have also confirmed the efficiency of CRISPR mediated mammalian cells, which will lead to the following hypothesis: gRNA genome targeting will have a broad range of effects on synthetic biology, direct and multiple interferences of gene networks, and In vivo and in vitro gene therapy [4, 7, 15]. The benefits of human genome editing would cure disease with an accurate diagnosis, targeted treatments, and prevention of genetic disorders [12, 15]. These have specific requirements for gene editing ability, which raised the importance of safety for gene editing in the field of genome modification and genome therapy. The frequency and off-target nuclease activity in CRISPR, ZFN, and TALEN is essential for gene medications to work; any potential risk may be heritable, which will change the genome of human embryos and may be passed on to future generations, thus changing the aspects of future generations [15]. One way to address these potential risks is to improve CRISPR specificity. Evaluating Cas9 homologs identified by bioinformatics and improve these nucleases to higher specificity would be mitigated to some extent [4, 5, 16].

## 2.2 Author's Viewpoints

### 2.2.1 Viewpoint 1

The potential of gene editing is theoretically unlimited, which makes the author believe that gene editing has the potential to change people's future lifestyles and reform. The CRISPR-Cas system has the characteristics of diversity, modularity, and effectiveness, which is anticipated to be a new revolution by people. The combination of gRNA and CRISPR-Cas can delete, insert, or change the target RNA or DNA [9]. This technology

has been used to speed up the process of research, which present an improved method for future treatment. More specifically, the simplicity and adaptability of CRISPR-Cas9 had opened a new channel for the discovery and development of gene therapy, which change the way scientists deal with gene aberrations by fixing a non-functional gene. In addition, the CRISPR-Cas9 technique has been used to modify a variety of plant species to improve their performance [5, 17]. The CRISPR system can induce accurate sequence mutations, which evaluate genes functions and could be used to enhance specific traits. Cas9 can be transformed into a DNA binding protein by mutation of the nuclease domain, which allows CRISPR-Cas to modify specific epigenetic changes or gene expression regulation. CRISPR-Cas9 is also considered the most effective for transgenic essential crops. Compared with other transgenic technologies, it has high efficiency, low cost, and convenient use. CRISPR-Cas9 has begun to bring revolutionary changes to biological research and agricultural development [18]. CRISPR-Cas9 will be the preferred method to target specific genomic sequences of simple and complex organisms in the future. Although some factors limit CRISPR-Cas9 system from its application, current and future developments have be used to improve its efficiency in editing human, animal, and plant cells.

### 2.2.2 Viewpoint 2

The contender of human ethics has always been an inseparable topic in gene editing. People hold different opinions about gene editing. Some people believe that gene editing is to help humans get rid of diseases, while others believe that technology will have unpredictable consequences for future generations. Although the development of CRISPR–Cas9 allows easy genome editing and has several benefits, the importance of ethics and biosafety contains issues [16, 19].

Moreover, due to the great potential and capabilities, CRISPR-Cas9 brings the risk of being used for non-legal purposes. To the point that these risks do not exceed their potential benefits, the health consequences of exposing individuals to interventions with potentially harmful effects are problematic. There are concerns about the health consequences of individual exposure to interventions that may have detrimental effects. The magnitude of the potential risk of deviation or unintended consequences in human germ cell genome editing has not been determined yet. Therefore, safeguards against erroneous or premature attempts of such interventions should at least rely on existing mechanisms that guide the clinical use of other reproductive therapies [20].

On the contrary, effective gene-editing technology has the potential to treat diseases. It contains the potential to target cofactors or viral genomes to reduce infection and eliminate viruses and induce transcriptional activation of latent viruses in latent virus libraries to eliminate viruses. This multifunctional gene-editing technology can be applied to prevent viruses in human cells.

In conclusion, gene editing is still an immature technology at the current scientific and technological level. Many scientists deem that gene editing might still be a double-edged sword to humanity's development: it can bring convenience and safety to human life, but it can also cause unpredictable harm to the human body [3].

### 2.2.3 Viewpoint 3

CRISPR-Cas gene editing system can bring significant progress to humankind. One of the tremendous advances of this century is the cooperation with Cas genes. This technology still has many unsolved problems and risks due to its immaturity—one of the significant limitations in producing off-target effects in host cells [10]. The limitation of non-targeted effects and chromosome translocation due to non-targeted cleavage had suppressed the sgRNA and Cas9 protein used in genome editing, which failed the gene-editing process. Selecting a more specific sequences on the genome and optimizing sgRNA and Cas9 could reduce the off-target mutation of RNA guided endonuclease; however, it will not be easy to edit complex genomes. For example, humans have a much higher chance of off-target conversions than lower organisms [17]. In addition, the number of mutations superposition as generations passes, which makes more challenging to control the dispersion of gene. More importantly, the destruction of gene-driven target groups may seriously impact the balance of ecosystems.

Another problem is that genes could transfer to other species in the environment. However, with the progress of technology and the realization of accurate sequencing technology, more precise detection technology will continue to innovate to this prevention [10, 15]. With the deepening of the research on miss impact factors, reduction strategies, and vacancy of detection technology, the CRISPR-Cas9 system will be applied to a broader range of fields to benefit humankind in the future.

## 3 Conclusion

The rapid development of the CRISPR system has created a variety of significantly beneficial results in modern science. It allows for transcriptional regulation, efficient genome modifications, and epigenetic editing with simplicity. The application field of this technology also seems to be unlimited. With its highly accurate genome editing tool, the CRISPR-Cas system could improve the quality of life. A new class of genomic engineering tools is defined; the CRISPR system can heterologous recombine cells to promote effective genome editing. However, the development is still immature [3]. The in vivo therapeutic drug utilizing Cas9 is limited, including ineffective delivery, accidental response, unforeseen damage, and tension of cell changes. To alleviate some current problems, the team conducted research on gene editing to improve the ease of use of genome engineering significantly. The analyzed report applies the engineering of protein and RNA components of this bacterial type II CRISPR system in human cells. Specifically, the engineered type II bacterial CRISPR system with the specific design of gRNA matching human cells could show the reliance on CRISPR components, also the introduction of multiple gRNAs can establish multiplex editing of target loci. These developments establish an RNA-guided editing tool with the compatibility of editing the human genome. The research could transform the RNA-guided editing tool into a more uncomplicated, dependable, and multiplexable human genome engineering. It may enhance fault tolerance, safety, and efficiency and reduce harm to the human body through improvements. Even with this progress, concerns about potential off-target effects may lead to severe gene mutations and chromosome translocations.

In clinical application, reducing off-target is always the most important. Although researchers have demonstrated that Cas9 off-target is more limited than other nucleases. A noticeable deviation from the target is detected. The desirable means at current development is to direct delivery of Cas9 RNPs instead of plasmid expression would reduce non-targeted effects. Cas9 RNPs (ribonucleoprotein: method to resolve difficult transfect) do not require exogenous DNA, and Cas9 gRNA RNP will degrade over time. The use of RNPs may limit the potential off-target effect. As stated above, limiting off-target rates is a fundamental factor to consider when applying treatments to humans. CRISPR/Cas9 provides the necessity to avoid immeasurable mutations for later generations, which means innovative strategies are required to avoid off-target effects.

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