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Review article

Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications

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ABSTRACT

The CRISPR-Cas9 genome-editing system is a part of the adaptive immune system in archaea and bacteria to defend against invasive nucleic acids from phages and plasmids. The single guide RNA (sgRNA) of the system recognizes its target sequence in the genome, and the Cas9 nuclease of the system acts as a pair of scissors to cleave the double strands of DNA. Since its discovery, CRISPR-Cas9 has become the most robust platform for genome engineering in eukaryotic cells. Recently, the CRISPR-Cas9 system has triggered enormous interest in therapeutic applications. CRISPR-Cas9 can be applied to correct disease-causing gene mutations or engineer T cells for cancer immunotherapy. The first clinical trial using the CRISPR-Cas9 technology was conducted in 2016. Despite the great promise of the CRISPR-Cas9 technology, several challenges remain to be tackled before its successful applications for human patients. The greatest challenge is the safe and efficient delivery of the CRISPR-Cas9 genome-editing system to target cells in human body. In this review, we will introduce the molecular mechanism and different strategies to edit genes using the CRISPR-Cas9 system. We will then highlight the current systems that have been developed to deliver CRISPR-Cas9 *in vitro* and *in vivo* for various therapeutic purposes.

1. Introduction

The clustered regularly interspaced short palindromic repeats (CRISPRs) are repeated segments of DNA originally discovered in prokaryotic organisms. CRISPR and CRISPR-associated (Cas) proteins are a part of the adaptive immune system in archaea and bacteria to defend against invasive nucleic acids from plasmids and phages. The CRISPR structure was first reported by Ishino in 1987 [1], and the acronym CRISPR was proposed by Jansen in 2002 after several similar structures were identified in different bacteria and archaea [2,3]. A milestone happened in 2005 when hyper-variable spacers with sequence homology to foreign plasmids and viruses were discovered. Mojica and colleagues thereafter speculated that the CRISPR structure and its related protein might possess immune defense functions and play significant roles in protecting against transmissible genetic elements [4].

Since then, more details about the CRISPR system have been elucidated at an accelerated pace. Charpentier, Doudna and Zhang are the three crucial contributors to this field. Charpentier was the first to elucidate the mechanism of the CRISPR-associated protein 9 (Cas9) genome-editing system [5]. In addition, Charpentier and Doudna reported the biochemical characterization of Cas9-mediated gene editing and optimized the system [6]. Zhang was the first to adopt the CRISPR-Cas9 system in eukaryotic cells for genome editing [7]. In 2015, CRISPR-Cas9 was named as the "*Breakthrough of The Year*" by the *Science* magazine [8].

Cas proteins are endonucleases that use a single guide RNA (sgRNA) to form complementary base pairs with target DNA and then cleave the DNA at specific sites. Among the different types of Cas proteins, Cas9 is the most widely used type because of its simplicity, high efficacy, and ease to use. The Cas9/sgRNA two-component system is highly efficient and specific in gene-editing. sgRNA recognizes a specific sequence in the genome, and the Cas9 protein subsequently acts as a pair of scissors to cleave the DNA sequence. Theoretically, the system can be exploited to engineer almost any DNA sequences in the genome, thus making the CRISPR-Cas9 system the most powerful gene-editing tool so far. One important application of this technology is to quickly generate knockout cell lines or animal models. The CRISPR-Cas9 system has therefore triggered a global research boom in both academia and industry. As shown in Fig. 1, the number of publications about CRISPR-Cas9 has been exponentially increased over the past few years. Its applications have been extended to a great variety of fields, including biological research [9], human medicine [10], biotechnology [11] and agriculture [12].

Although the CRISPR-Cas9 system is a newly developed gene-

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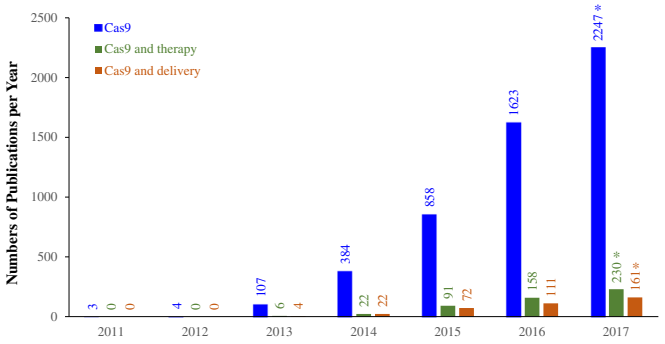

Fig. 1. PubMed search of "Cas9", "Cas9 and therapy", and "Cas9 and delivery" in title/abstract for each year. The numbers in 2017 are projected based on the numbers of publications from the first eight months.

editing tool, its simplicity, ease of use and potent gene-editing capability have quickly attracted the most attention from scientists in different areas and initiated a race to harness CRISPR-Cas9 for therapeutic applications in humans. Importantly, the CRISPR-Cas9 system holds high promise for human gene therapy. For example, it has been successfully exploited to correct gene mutations that drive the development of cancers. It has also been used to create oncolytic viruses to selectively transduce and kill tumor cells [13]. Moreover, the CRISPR-Cas9 system was used to replace specific genome sequences of human T cells with modified sequences [14]. In 2016, a Chinese group initiated the first clinical study of CRISPR-Cas9 by injecting Cas9-engineered T cells to a patient with metastatic non-small cell lung cancer (NSCLC). The result was promising, and a second injection was planned [15].

Despite the great promise of the CRISPR-Cas9 genome-editing system, several challenges remain to be tackled before its successful applications for human patients. The biggest challenge is the safe and efficient delivery of the system to target cells in human body. In this review, we will introduce the mechanism and different strategies of the CRISPR-Cas9 system to edit gene sequences. We will then highlight the current systems that have been developed to deliver the CRISPR-Cas9 system *in vitro* and *in vivo* for various therapeutic purposes.

2. Mechanism of the CRISPR-Cas9 gene-editing system

CRISPR-Cas systems are divided into two classes. The class 1 system contains types I, III and IV, and the class 2 system contains types II, V, and VI [16]. The class 1 CRISPR-Cas system uses a complex of several Cas proteins, whereas the class 2 system only uses a single Cas protein with multiple domains. The class 2 CRISPR-Cas system is therefore preferable for gene-engineering applications because of its simplicity and ease of use [17]. Among different types of the class 2 CRISPR-Cas systems, the type II CRISPR-Cas9 is the most widely used and studied system. In this system, CRISPR spacers direct the system to the target, and the Cas9 protein controls spacer acquisition and defense. Natural CRISPR systems act in three stages, including adaptation, expression and interference [17–21].

In the adaptation stage, foreign DNA fragments (approximately 30–45 nucleotides, also named protospacers) from invading plasmids or viruses are incorporated as new spacers into CRISPR arrays. The selection of protospacers from the foreign DNA is based on the protospacer-adjacent motif (PAM). New spacers then provide sequence-specific memory against their corresponding invading plasmids or viruses [22,23]. In the expression stage, the CRISPR array is transcribed to pre-CRISPR RNA (pre-crRNA), which is further processed to mature CRISPR RNA (crRNA). Each crRNA contains a conserved repeat sequence and a transcribed spacer, which is complementary to the foreign DNA. A pool of crRNAs can target multiple gene elements because each crRNA corresponds to an invasion sequence [24]. In the interference stage, crRNAs act as a guide to specifically target the PAM, and Cas9 cleaves the matched DNA. In the type II CRISPR-Cas9 system, the sgRNA-Cas9 complex binds to its target DNA to ensure that the Cas9 cuts both strands of the DNA, thus blocking the propagation of foreign DNA [25].

Type II CRISPR-Cas9 is the most routinely used CRISPR gene-editing system and is usually refer to as CRISPR. Scientists have demonstrated how to successfully engineer type II CRISPR system to edit genome in mammalian cells. The Cas9 protein is an endonuclease containing two nuclease domains, RuvC and HNH. The RuvC domain cleaves non-complementary DNA strands, while the HNH domain cleaves complementary DNA strands (Fig. 2A). The sgRNA is composed of the trans-activating crRNA (tracrRNA) and crRNA (Fig. 2B). The crRNA contains a 20-nt protospacer element and an additional sequence that is complementary to the tracrRNA. The tracrRNA hybridizes to the crRNA and binds the Cas9 protein, forming the CRISPR-Cas9/sgRNA complex to create double-stranded breaks (DSBs) at target sites in the genome. The dual-tracrRNA:crRNA is normally engineered as a single-strand sgRNA containing two crucial segments: a duplex RNA structure at the 3′ end to bind Cas9 and a guide sequence at the 5′ end to bind target DNA sequence. As shown in Fig. 2, this two-component system is simple but powerful. sgRNA recognizes a specific sequence in the genome, and Cas9 acts as a pair of scissors to cleave the DNA sequence.

The molecular mechanisms of the CRISPR-Cas9 system-mediated genome-editing are illustrated in Fig. 3. Cas9 protein cuts 3–4 nt upstream of the PAM site. After DSBs are formed, either the Non-Homologous End Joining (NHEJ) repair pathway or the Homology Directed Repair (HDR) pathway will be initiated. The NHEJ repair pathway often leads to the generation of insertion/deletion (InDel), leading to frameshifts and/or premature stop codons within the open reading frames (ORFs) of target genes. By contrast, a donor DNA template is needed to repair the DSBs in the HDR pathway. Correct DNA sequences are precisely inserted into the target site using a donor DNA template. The HDR pathway is substantially less effective than the NHEJ pathway because gene replacement or knock-in is usually less efficient than gene knock-out.

CRISPR-Cas9 holds great promise in the therapy of genetic disorders by directly editing disease-related mutations. Tremendous efforts have been devoted to improve the specificity, gene-editing efficacy, and delivery efficiency of the CRISPR-Cas9 system. As a result, CRISPR-Cas9 has become a revolutionary genome-editing tool for a wide variety of therapeutic applications.

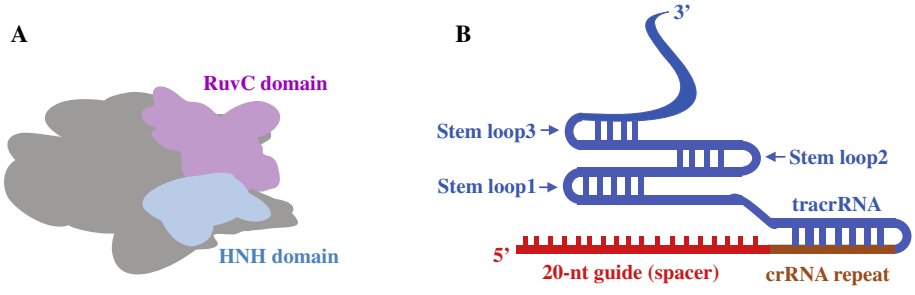

Fig. 2. Schematic diagram of the Cas9 protein (A) and sgRNA (B). Cas9 protein contains two nuclease domains, the RuvC domain and the HNH domain. The RuvC domain cleaves non-complementary DNA strands, and the HNH domain cleaves complementary DNA strands. The sgRNA is composed of the trans-activating crRNA (tracrRNA) and crRNA. The crRNA contains a 20-nt protospacer element and an additional nucleotides that are complementary to the tracrRNA. The tracrRNA hybridizes to the crRNA and binds to the Cas9 protein, forming the CRISPR-CAs9/sgRNA complex to edit genome sequences.