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Advances in CRISPR/Cas9 Gene Editing Methods Based on In Vitro Assembled Ribonucleoprotein: A Postprint

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Date: 2018-10-09T00:00:00+00:00

Abstract

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) system is a novel gene editing technology that has emerged in recent years and found extensive applications in the biomedical field. The CRISPR/Cas9 system requires gRNA to realize site-specific genome editing through the Cas9 protein, typically delivering Cas9 and gRNA via lentiviral infection or plasmid transfection. However, these methods are prone to inducing immune responses and uncontrollable gene fragment insertion, which poses certain risks and limits the application of CRISPR/Cas9 technology in vivo. The strategy of transducing ribonucleoprotein (RNP) assembled in vitro, developed in recent years, has garnered considerable attention owing to its advantages of rapidity, safety, and low off-target editing rates. This review summarizes the delivery methods and applications of Cas9 RNP and discusses the current challenges, with the aim of providing a foundation for further development of CRISPR/Cas9 technology and expanding its applications.

Full Text

Progress in CRISPR/Cas9 Gene Editing Methods Based on In Vitro Assembly of Ribonucleoprotein

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Abstract

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) system is a novel gene editing technology

developed in recent years that has found widespread application in biomedical research. The CRISPR/Cas9 system requires the Cas9 protein and guide RNA (gRNA) to achieve site-specific genome editing. Conventionally, Cas9 and gRNA are delivered via lentiviral infection or plasmid transfection. However, these approaches can trigger immune responses and cause uncontrollable gene fragment insertion, posing certain risks that limit the clinical application of CRISPR/Cas9 technology. Recently, a strategy based on the transduction of in vitro-assembled ribonucleoprotein (RNP) complexes has attracted considerable attention due to its advantages of rapid action, enhanced safety, and reduced off-target effects. This article summarizes the delivery methods and applications of Cas9 RNP, discusses current challenges, and aims to provide a foundation for further development and expanded application of CRISPR/Cas9 technology.

Keywords: CRISPR/Cas9; Ribonucleoprotein; CPP-RNP

1. The CRISPR/Cas9 Gene Editing System

CRISPR/Cas9 is a novel gene editing system that has garnered tremendous attention since its inception. This system represents an RNA-mediated, heritable adaptive immune system widely present in bacteria and archaea [1, 2]. To date, three types of CRISPR/Cas systems have been identified [3], with the Type II system being the most extensively studied and applied. The Type II system consists of a single guide RNA (sgRNA) that provides targeting specificity and the Cas9 protein that executes DNA cleavage. The Cas9-sgRNA gene targeting system has been successfully implemented for gene editing in diverse organisms, including human cells, mice, zebrafish, yeast, bacteria, fruit flies, nematodes, and crops such as rice [4-8]. Compared with other gene editing technologies such as zinc finger nucleases (ZFNs) [9, 10] and transcription activator-like effector nucleases (TALENs) [11, 12], CRISPR/Cas9 offers simpler operation and higher gene targeting efficiency.

2. Delivery Methods for Cas9/gRNA and Advantages of RNP

The efficiency of CRISPR/Cas9-mediated gene editing is directly influenced by the expression levels of Cas9 and gRNA within cells. These components can be introduced through various methods, including plasmid transfection, viral infection, direct transfection of in vitro-transcribed Cas9 mRNA and gRNA, and delivery of pre-assembled Cas9 protein-gRNA complexes (RNP). Among these approaches, plasmid transfection and viral infection are most commonly used. However, both methods carry risks of uncontrollable exogenous gene insertion [13] and can elicit immune responses [14], posing potential safety concerns. While direct transfection of Cas9 mRNA and gRNA avoids insertion events associated with exogenous vectors, this approach suffers from RNA degradation and uncertain activity [15].

RNP technology represents an emerging method for delivering Cas9 and gRNA that has been developed in recent years. As illustrated in Figure 1 [Figure 1: see original paper], the principle involves incubating Cas9 protein with in vitro-transcribed gRNA, which assemble into complexes through electrostatic interactions and structural complementarity [16]. Upon delivery into target cells, these RNP complexes can mediate non-homologous end joining (NHEJ). In the presence of donor DNA, they can also facilitate precise gene repair and insertion.

Compared with other delivery strategies, RNP-based editing offers several key advantages (Table 1):

1. Similar to mRNA transfection, RNP delivery avoids the risk of exogenous gene insertion [14] and is less likely to trigger immune responses. Moreover, the Cas9 protein is more stable than mRNA, and complex formation with gRNA enhances gRNA stability [17], facilitating clinical applications and genetic modification of plants and animals.
2. Unlike plasmid transfection or viral infection, which result in sustained Cas9 expression, Cas9 protein delivered as RNP degrades over time, thereby reducing off-target effects [18].
3. The method is simple and rapid. Cas9 protein and gRNA are mixed in vitro, and gene editing can proceed immediately after transduction. Eliminating the need for transcription and translation enables instant and efficient genome editing.

Figure 1. The assembly and mechanism of Cas9 RNP

Table 1. Comparison of gene editing in different formats of CRISPR/Cas9 (plasmid/virus, in vitro-transcribed Cas9 mRNA and gRNA, component expression time, component stability) [15][17]

3. Delivery Methods and Applications of Cas9 RNP

RNP technology has opened a new chapter in CRISPR/Cas9 applications. However, the assembled Cas9/gRNA complexes must be efficiently delivered into cells to exert their gene editing function. Traditional RNP delivery methods can be categorized as physical or chemical approaches, and more recently, nanocarriers and cell-penetrating peptides (CPPs) have been employed to mediate RNP entry into cells.

3.1 Physical Methods

Physical methods primarily include electroporation, nucleofection, and microinjection, which utilize specialized electrical equipment or microscopic instruments to deliver RNP complexes directly into the cytoplasm or nucleus. These approaches are broadly applicable and have been widely used in plant and animal

genetic engineering. For example, Kathrin Schumann et al. [20] used electroporation to deliver RNP into T cells, successfully generating cxcr4-low-expressing T cells and demonstrating the potential of RNP technology for human primary T cell therapy. Angela Meccariello et al. [21] employed microinjection to knockout pigmentation genes in the invasive agricultural pest *Ceratitis capitata*, inducing segmental mutations in the corneal limbus of late embryos and larvae, thereby providing new strategies for pest control. In 2017, Brett T. Staahl et al. [22] utilized RNP microinjection for intracranial gene knockout in mice, demonstrating the specificity and safety of precise RNP delivery to neuronal cells in adult animals, which suggests potential applications in tissue-specific editing and treatment of neurological diseases. Nucleofection is a highly efficient non-viral transfection method that combines specific instrument programs with proprietary transfection solutions. Steven Lin et al. [23] applied nucleofection to deliver RNP for homology-directed repair (HDR) and significantly improved HDR efficiency when combined with cell cycle-modulating drugs.

3.2 Chemical Methods

Although physical transfection methods are relatively straightforward and can be applied to difficult-to-transfect cells, they often cause physiological damage [24], posing significant challenges for in vivo therapeutic applications. Chemical methods, by contrast, cause less cellular damage and offer broader applicability, with liposome- and polymer-based transfection being most commonly used. RNP complexes can bind to liposomes through electrostatic interactions and enter cells via membrane fusion or endocytosis. In 2015, Zuris et al. [25] and Liang et al. [26] demonstrated the feasibility of using Lipofectamine 2000 and Lipofectamine 3000 for RNP-mediated gene editing in vitro and in vivo. Je Wook Woo [13] used polyethylene glycol (PEG) to deliver RNP into plant protoplasts of *Arabidopsis*, tobacco, lettuce, and rice, achieving targeted mutagenesis rates as high as 46% in regenerated plants. Since no exogenous genes were introduced, this approach reduces restrictions associated with genetically modified organism (GMO) regulations [27], offering possibilities for broad application of CRISPR/Cas9 in plant biotechnology and agriculture. With advances in chemical materials, polymer-mediated transfection has also been widely applied for RNP delivery due to lower immunogenicity and broader applicability. Huahua Yue et al. [28] constructed a polyethyleneimine (PEI)-graphene hybrid polymer that delivered RNP into human cells with editing efficiencies up to 39% and demonstrated high stability. Other polymeric delivery systems, including chitosan, poly(L-lysine) (PLL), and dendrimers [29] and their modified forms, have also been employed for RNP delivery. Although chemical methods cause less direct cellular damage than physical approaches, most transfection reagents show limited efficacy in difficult-to-transfect cells such as tumor cells, and cytotoxicity remains a limiting factor for broader application [24].

3.3 Nanocarrier-Mediated RNP Delivery

In recent years, nanoparticle carriers have proven to be highly efficient tools for delivering genes and proteins [30-33], offering further improvements in RNP-mediated gene editing efficiency. Liposome- and polymer-based nanodelivery systems are most commonly used. Sun et al. [34] encapsulated RNP within DNA nanoclews (NC) and further assembled them with cationic PEI polymers, achieving approximately 40% knockout of an EGFP reporter gene. In 2017, *ACS Nano* reported another application of polymeric nanoparticles for RNP delivery [35], where cationic arginine gold nanoparticles (ArgNPs) served as modular building blocks. Cas9 protein was fused with a glutamic acid peptide, and through electrostatic interactions with ArgNPs, self-assembled RNP-ArgNP nanocomplexes were formed that entered cells via direct penetration for highly efficient gene editing. Kunwoo Lee et al. [36] used gold nanoparticles to deliver RNP together with template DNA, successfully editing multiple cell types and effectively correcting DNA mutations causing Duchenne muscular dystrophy in mice. Despite these advances, nanocarriers face challenges such as complex preparation and limited stability [37], and their practical utility in combination with CRISPR/Cas9 systems requires further investigation.

In 2014, Ramakrishna et al. [38] chemically conjugated the cell-penetrating peptide (CPP) m9R to Cas9, while gRNA formed nanoparticles through electrostatic interaction with CPPs. This approach mediated the delivery of Cas9 and gRNA into cells and achieved knockout of endogenous genes. Although this study did not assemble CPP-RNP complexes in vitro, it provided important insights for CPP-mediated RNP delivery. Compared with physical and chemical methods, CPPs typically utilize electrostatic interactions with negatively charged cell surface components to cross the cell membrane and deliver macromolecules into cells without requiring transfection reagents or specialized equipment. This offers better biocompatibility [39] and reduced cellular and organismal damage (Table 2). However, in chemical conjugation, CPPs randomly react with amino or carboxyl groups on the Cas9 surface, potentially affecting Cas9 activity and reducing editing efficiency. Fusion expression, by contrast, allows optimization of CPP position and linker sequences to maximize Cas9 activity. After fusing CPP with Cas9, the protein is assembled with in vitro-transcribed gRNA to form CPP-RNP complexes that can be delivered into cells. Guided by nuclear localization signals (NLS), the RNP is released from endosomes and enters the nucleus to execute gene editing (Figure 2 [Figure 2: see original paper]).

Table 2. Comparison of different transduction delivery systems for RNP (physical transduction, chemical transduction, nanocarrier, cell-penetrating peptide-mediated; active targeting ability; transfection reagent requirement; serum tolerance; cell compatibility) [24][40] [19][40][41] [19][31]

Figure 2. The diagram of gene editing by CPP-Cas9 RNP complex

CRISPR/Cas9 gene editing technology holds broad application prospects due

to its operational simplicity and high efficiency. However, the efficiency and safety of Cas9/sgRNA delivery have limited its broader application. The RNP approach overcomes issues associated with traditional plasmid and viral vectors, such as the risk of exogenous gene insertion, enabling rapid and effective gene editing with reduced off-target effects. Compared with physical, chemical, and nanocarrier methods, CPPs offer superior biocompatibility and provide a novel strategy for CRISPR/Cas9 gene editing. Nevertheless, CPP-mediated delivery of macromolecules still requires improvement in terms of cellular uptake efficiency and targeting specificity. There remains considerable potential for research in screening CPPs with enhanced membrane penetration or specificity and improving endosomal escape efficiency. Further advances in CPP research will help enhance the efficiency of RNP-based CRISPR/Cas9 gene editing and promote the application of CRISPR/Cas9 systems.

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