Biomaterials 171 (2018) 207–218

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Review

Non-viral delivery systems for CRISPR/Cas9-based genome editing: Challenges and opportunities

Ling Li [a, b], Shuo Hu [a, **], Xiaoyuan Chen [b, *]

[a] Department of PET Center, Xiangya Hospital, Central South University, Changsha, 410008, China
[b] Laboratory of Molecular Imaging and Nanomedicine (LOMIN), National Institute of Biomedical Imaging and Bioengineering (NIBIB), National Institutes of Health (NIH), Bethesda, MD 20892, USA

ARTICLE INFO

Article history:
Received 11 March 2018
Received in revised form
13 April 2018
Accepted 14 April 2018
Available online 18 April 2018

Keywords:
CRISPR/Cas9
Non-viral delivery
Genetic disorder
Cancer
Nanomedicine
Clinical translation

ABSTRACT

In recent years, CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) genome editing systems have become one of the most robust platforms in basic biomedical research and therapeutic applications. To date, efficient *in vivo* delivery of the CRISPR/Cas9 system to the targeted cells remains a challenge. Although viral vectors have been widely used in the delivery of the CRISPR/Cas9 system *in vitro* and *in vivo*, their fundamental shortcomings, such as the risk of carcinogenesis, limited insertion size, immune responses and difficulty in large-scale production, severely limit their further applications. Alternative non-viral delivery systems for CRISPR/Cas9 are urgently needed. With the rapid development of non-viral vectors, lipid- or polymer-based nanocarriers have shown great potential for CRISPR/Cas9 delivery. In this review, we analyze the pros and cons of delivering CRISPR/Cas9 systems in the form of plasmid, mRNA, or protein and then discuss the limitations and challenges of CRISPR/Cas9-based genome editing. Furthermore, current non-viral vectors that have been applied for CRISPR/Cas9 delivery *in vitro* and *in vivo* are outlined in details. Finally, critical obstacles for non-viral delivery of CRISPR/Cas9 system are highlighted and promising strategies to overcome these barriers are proposed.

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1. Introduction

CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) systems are adaptable immune mechanisms of many bacteria and archaea to protect themselves from invading nucleic acids [1–4]. Since first applied in mammalian cells in 2013, CRISPR/Cas system, based on a RNA-guided nuclease, has revolutionized the way that genome editing is performed [5,6]. To date, CRISPR/Cas systems have been categorized into three main types based on the core element content and sequences [7–9]. In Types I CRISPR/Cas systems, CASCADE (CRISPR-associated complex for antiviral defense) complexes containing multiple Cas protein subunits form complexes with crRNA to trigger the recognition and disruption of the target loci. In type III systems, crRNAs are incorporated into a multi-subunit interference complex called Cmr or Csm to detect and degrade invasive RNA. In sharp contrast, only the Cas9 protein is required for DNA interference in the type II systems [10–13]. Actually, the type II CRISPR/Cas system from streptococcus pyogenes (CRISPR/Cas9) has been widely applied in biomedical applications due to its simplicity, versatility, high specificity, and efficiency [14–18]. CRISPR/Cas9 system contains two critical components: Cas9 nuclease and a guide RNA (gRNA) that is a fusion of a crRNA and a constant tracrRNA. Generally, gRNA can be easily replaced by a synthetic chimeric single guide RNA (sgRNA). In the presence of a protospacer-adjacent motif (PAM) (usually 5′-NGG), the Cas9 nuclease can be directed by a sgRNA to any targeted genomic locus based on base pairing and stimulate site-specific double-stranded DNA breaks (DSBs), where two cellular repair mechanisms- non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways- can be exploited to induce error-prone or defined alterations (Fig. 1) [13,19–21]. Meanwhile, the CRISPR interference (CRISPRi) technology, which uses a catalytically dead Cas9 (dCas9) protein lacking endonuclease activity to regulate genes in an RNA-guided manner, has also been developed in recent years [22–24]. Besides, Cas9 nickases ($RuvC^{D10A}$ or HNH^{H840A}), which cut one strand rather than both strands of the target DNA site have also been shown to be useful for genome

* Corresponding author.
** Corresponding author.
E-mail addresses: hushuoxy@csu.edu.cn (S. Hu), shawn.chen@nih.gov (X. Chen).

https://doi.org/10.1016/j.biomaterials.2018.04.031
0142-9612/Published by Elsevier Ltd.

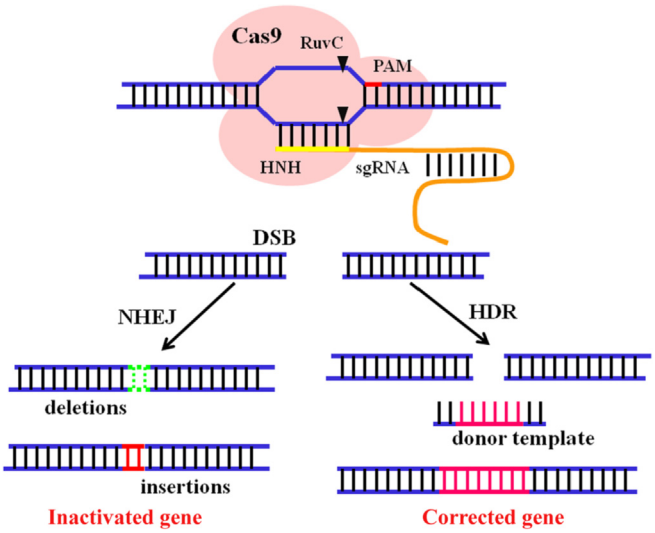

Fig. 1. Schematic illustration of the two different repair mechanisms of CRISPR/Cas9-mediated double stranded breaks (DSBs).

editing [25,26]. This method is applicable for genome editing of any model organism with minimal off-target effects [27,28]. Recently, an alternative CRISPR-based nuclease Cpf1 has been discovered and share many similar advantages and shortcomings of Cas9 [29,30].

With these merits, CRISPR/Cas9 systems have shown great potential in studying the function of genetic elements, disease modeling and therapy [31–38]. For example, CRISPR/Cas9 systems have been applied to recapitulate cancer-associated genes both *in vitro* and *in vivo*, creating a convenient and effective platform to investigate cancer-related genetic mutations. Meanwhile, Cas9 nuclease can be easily adjusted to retarget new desired sequences by simply altering the sgRNA sequence and the system can simultaneously target multiple sequences for genome editing [39–41]. In the past decades, generating disease models was an extremely slow and expensive process, which requires complicated embryonic stem cell manipulation, as well as endless mouse husbandry to obtain the desired phenotype and genotype. However, after the emergence of CRISPR/Cas9 systems, novel disease models have been developed with unprecedented speed and precision resulting from the simplicity and flexibility of these systems [42–45]. Furthermore, CRISPR/Cas9 system holds tremendous promise for gene therapy [46–51]. It can correct causal mutations in the original genome and rescue the disease phenotypes of monogenic disorders permanently, which currently represents the most translatable field in CRISPR/Cas9-mediated disease therapy. In addition, CRISPR/Cas9 system has shown potentials in polygenetic diseases, such as cancer, by inhibiting oncogene expression or deactivating oncogenic virus [52–55]. Recently, the first human CRISPR/Cas9-based clinical trial is on the move in China (ClinicalTrials.gov Identifier: NCT02793856) and the first CRISPR clinical trial in the US also received green light from the FDA [56,57]. Both trials are based on editing *ex vivo* T cells from patients with CRISPR/Cas9 system and then transplanting these modified cells back into the patients to help augment cancer therapies.

Despite the aforementioned merits, efficient delivery may likely become the main hurdle in the eventual application and clinical translation of CRISPR/Cas9 system. Currently, the strategies of CRISPR/Cas9 delivery are mainly based on physical approaches (microinjection, electroporation, hydrodynamic injection, *etc.*) and viral vectors (lentivirus, adenovirus (Ad), adeno-associated virus (AAV), *etc.*) [58–60]. Generally, microinjection, electroporation and nucleofection are limited to use in cultured cells (zygotes, embryonic stem cells, T cells, *etc.*) *in vitro* [61–64], while hydrodynamic injection has been employed to deliver CRISPR cassettes to the liver *in vivo* [46,60]. Unfortunately, although physical approaches are often successful in the laboratory, these methods are not very amenable for clinical translation. In recent years, viral vectors have been extensively applied to deliver CRISPR cassettes *in vitro* and *in vivo* largely owing to their high efficiency in gene delivery and long-term stable transgene expression [34,58,65]. Especially, AAV can be flexibly constructed to target specific organs or tissues based on the diverse tissue tropism of AAV serotypes [66,67]. For instance, Yang et al. developed a dual-AAV8 system delivering CRISPR cassettes to correct the point mutation in newborn liver based on the high liver tropism of AAV8 [68]. Similarly, although highly efficient, the intrinsic drawbacks associated with viral vectors, including the risk of carcinogenesis, limitation of insertion size, immune responses and difficulty in large-scale production, severely limited their applications [69–72]. As an alternative, non-viral vectors may offer tantalizing possibility in CRISPR/Cas9 delivery with respect to their low immunogenicity, absence of endogenous virus recombination, less limitation in delivering larger genetic payloads and ease of large-scale production [73–75]. So far, relatively low gene delivery efficiency and transgene expression have been the major barriers in non-viral vectors-based gene therapy [76,77]. However, with the rapid development of novel biomaterials in recent years, efficient delivery of gene payloads to pass the multiple barriers under physiological conditions and promote transgene expression can be achieved.

In fact, there have been a number of excellent reviews concerning different delivery systems for CRISPR-based genome editing [78–81]. Thus, in this review, we do not intend to elaborate on the physical approaches and viral vectors in CRISPR/Cas9 delivery. Instead, we will discuss the limitations and challenges in CRISPR/Cas9-based genome editing, focus on the recent progresses on non-viral vectors for CRISPR/Cas9 delivery, highlight the main extracellular and intracellular barriers of delivery, and finally propose some strategies to overcome these obstacles to fulfill the clinical need of CRISPR/Cas9-based genome editing.

2. Limitations and challenges of CRISPR/Cas9-based genome editing

Before CRISPR-based genome editing can be seriously considered for clinical use, several practical issues and technical challenges must be addressed. At molecular level, there are three main issues that need to be overcome. First, setting and reaching the target site with efficiency and accuracy of both cleavage and repair to improve specificity and reduce off-target probability. Second, understanding how to control various repair pathways-NHEJ or HDR- to facilitate switching based on experimental goals. Third, the CRISPR/Cas9 system needs to be more efficient to have therapeutic efficacy in treating diseases. For example, HDR pathway-mediated precise repair dramatically facilitates many areas of biomedical research. However, the desired recombination efficacy often occurs infrequently, which presents enormous challenges for robust applications [61,82–84]. Although inhibiting the NHEJ pathway with gene silencing or chemical inhibitors can partially increase the efficiency of HDR pathway, the gene repair efficacy still needs to be further improved for disease therapy [82,85]. Collectively, these issues call for the development of more effective CRISPR systems and more powerful predictable tools. For example, homology-independent targeted integration (HITI) strategy has been developed as an alternative approach for CRISPR/Cas9-mediated gene repair in recent studies [86,87]. This approach allows for robust DNA knock-in in both dividing and non-dividing cells *in vitro* and