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Review article

Delivery methods for site-specific nucleases: Achieving the full potential of therapeutic gene editing

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ABSTRACT

The advent of site-specific nucleases, particularly CRISPR/Cas9, provides researchers with the unprecedented ability to manipulate genomic sequences. These nucleases are used to create model cell lines, engineer metabolic pathways, produce transgenic animals and plants, perform genome-wide functional screen and, most importantly, treat human diseases that are difficult to tackle by traditional medications. Considerable efforts have been devoted to improving the efficiency and specificity of nucleases for clinical applications. However, safe and efficient delivery methods remain the major obstacle for therapeutic gene editing. In this review, we summarize the recent progress on nuclease delivery methods, highlight their impact on the outcomes of gene editing and discuss the potential of different delivery approaches for therapeutic gene editing.

Contents

1. Introduction

>3000 human genes are known to be associated with disease-causing mutations [1]. These mutations include nucleotide insertions, deletions and substitutions and chromosomal translocations. Understanding the molecular mechanisms underlying each genetic disease and development of therapeutic strategies are attracting topics in both basic and translational research. Traditional medications achieved very limited success for treating genetic diseases [1], whereas gene therapy has emerged as a legitimate approach [2]. Broadly speaking, gene therapy aims to restore the native or acquire beneficial gene expression patterns. This can be achieved by either introducing *exogenous* copies of functional genes or correcting the *endogenous* defective genes in host cells. Both of these two approaches have been greatly advanced with the emergence of site-specific nucleases.

Site-specific nucleases are customized nucleases that can bind to and cleave designated genomic DNA, leading to double-strand breaks (DSBs). In eukaryotes, DSBs are repaired by error-prone non-homologous end-joining (NHEJ) or homology-directed repair (HDR).

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During NHEJ, nucleotide insertions and deletions (indels) occur at DSB junctions, enabling nuclease-induced gene disruption [3]. HDR is triggered by the presence of homologous DNA template and allows transgene integration or nucleotide substitutions [4]. Recent studies showed that nuclease-induced gene knock-in can be also mediated by homology-independent DNA repair [5,6]. Nuclease-mediated gain-of-function and loss-of-function gene therapy are rationalized *via* four distinct gene-editing outcomes: gene knockout, gene correction, gene addition and gene deletion (Fig. 1). Targeted gene knockout is implemented by frame-shifting nucleotide indels that are introduced during NHEJ repair. Gene correction and addition both rely on HDR-mediated integration of repair template. In the case of gene deletion, pathogenic genomic regions are excised by paired nucleases, leading to recovery of the normal gene functions. While nuclease-induced NHEJ is highly efficient, gene correction or integration *via* HDR is typically of low efficiency in most mammalian systems. Development of strategies overcoming this hurdle is an active field of research for site-specific nucleases.

The most commonly used site-specific nucleases include zinc finger nucleases (ZFNs), transcription activator-like effector nuclease (TALEN), clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 and meganucleases [7]. ZFNs and TALENs are chimeric nucleases consisting of *Fok* I nuclease and Cys_2-His_2 zinc-finger proteins (ZFPs) [8] or transcription activator-like effector (TALE) proteins [9,10]. ZFPs are naturally occurring transcription factors that can be engineered to recognize customized genomic loci. Each ZFP repeat consists of approximately 30 amino acids with a $\beta\beta\alpha$ configuration. The residues on the surface of each α-helix interact with three nucleotides in the major groove of DNA. ZFNs function as dimers, with each monomer recognizing a half site of nine or twelve basepairs in a 3′ to 5′ manner [11]. One major concern of using ZFNs for clinical applications is its potential off-target activity [12,13]. Strategies for improving the specificity of ZFNs include the use of *Fok* I domains with opposite charges that only dimerize upon correct pairing of ZFNs [14,15], and delivery of ZFNs as proteins to reduce the time of exposure of the genomic DNA to nucleases [16,17].

TALE proteins contain invariant N- and C-terminal domains, flanking the central DNA-binding repeats. These DNA binding repeats are typically 34 amino acids in length and are arrayed to recognize DNA from 5′ to 3′. Unlike ZFP repeats that recognize nucleotide triplets, each TALE DNA-binding repeat interacts with a single nucleotide through the amino acids at positions 12 and 13, known as the repeat variable di-residues (RVDs) [9,18]. Similar to ZFNs, dimerization of the *Fok* I domains in TALENs are required for DNA cleavage. TALEN monomers are usually designed to bind to 12 to 20 nucleotides, separated by a 12- to 19-bp spacer sequence. Both ZFNs [19,20] and TALENs [21] can be designed and synthesized in a modular approach, however the assembly of ZFNs is not always straightforward as ZFPs may bind to DNA in a context-dependent manner [11]. For this reason, optimization is sometimes required to generate functional ZFNs. In contrast, modularly assembled TALENs typically have high success rates of gene targeting and thus do not demand extensive engineering or selection. In addition, TALENs have been shown to have improved specificity and reduced cytotoxicity compared with ZFNs [22].

Cas9 nuclease functions differently from ZFNs and TALENs in that it recognizes target DNA through a target-specific CRISPR RNA (crRNA) and a target-independent *trans*-activating crRNA (tracrRNA) [23]. crRNA and tracrRNA can be merged into a single guide RNA (sgRNA) [24]. With this modification, gene editing using CRISPR/Cas9 only requires two components: an invariant Cas9 protein and a custom-designed sgRNA with a 20-bp sequence complementary to the target sites. These features render CRISPR/Cas9 the most convenient platform for genome engineering applications. The only restriction of gene targeting by CRISPR/Cas9 is that a conserved protospacer-adjacent motif (PAM), typically 5′-NGG-3′, must follow the 20-bp targeted genomic sequence. The PAM recognition by Cas9 proteins can be reprogrammed to facilitate flexible gene targeting [25]. In addition, Cas9 analogues from different bacterial species with altered PAM recognition patterns have been reported [26–29]. Alternative RNA-guided endonuclease systems have also been discovered and adopted for targeted gene editing [30]. Despite the ease to use, CRISPR/Cas9 is found to be prone to off-target cleavage [31,32]. Considerable efforts have since been devoted into improving the specificity of CRISPR/Cas9. Truncated sgRNA was found to be more sensitive to mismatches at the off-target sites [33]. Improved specificity can be achieved by reducing the time of exposure of genomic DNA to nucleases, such as restricting the dosage of Cas9 protein or sgRNA within cells [34], delivery of Cas9 as short-lived proteins [35,36] or the use of chemically inducible Cas9 expression system [37,38]. Cas9 nickases [39,40] and inactivated Cas9 with *Fok* I fusion [41,42] can also reduce the chance of off-target cleavage since they require adjacent nicking events or paired nucleases to create DSBs. In addition, engineering Cas9 to incorporate specific point mutations [43,44] or to fuse with DNA-binding domains [45] have both proven effective to improve Cas9 specificities.

Meganucleases, or homing endonucleases, are naturally occurring endonucleases that recognize 12 to 40 nucleotide sequences with high specificity [46]. This specificity arises from the complex interactions between meganucleases and targeted DNA that nevertheless make the reprogramming of meganucleases particularly challenging, thereby hampering the broad applications of meganucleases. Recently, TALE DNA binding domain was fused to meganucleases to generate highly specific chimeric nucleases, known as megaTALs [47,48]. These megaTALs have been used for gene integration in primary and stem cells [49,50], indicating their great potential for clinical applications.

In order for site-specific nucleases to edit genomic sequences, they must be delivered into the nucleus of host cells, passing through cell membrane, endosome and cytoplasm. The exact processes along this path are still elusive [2]. Particularly, certain cell types such as primary and stem cells are resistant to many delivery methods, impeding the use of nucleases for regenerative medicine. Moreover, cell- or tissue-specific delivery remains the major obstacle for the *in vivo* applications of nucleases. These limitations promote researchers to explore and expand on the myriad existing gene transfer methods, develop novel

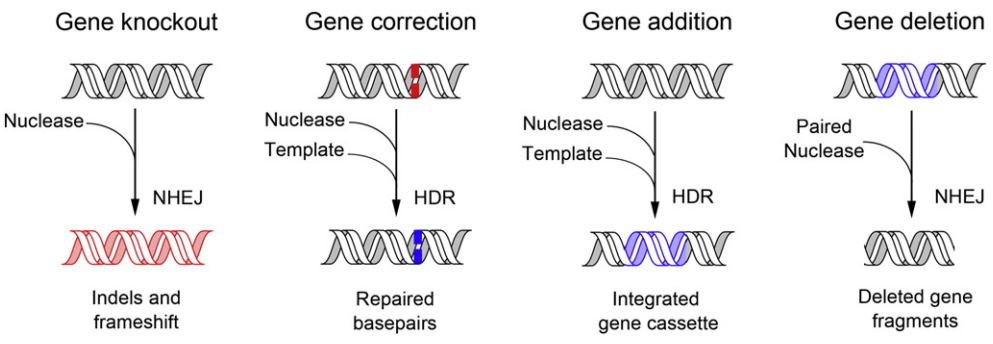

Fig. 1. Outcome of nuclease-mediated gene editing.