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IMPROVING THE FUNCTION OF CRISPR-CAS9 FOR GENOME EDITING THERAPY

Meningkatkan Fungsi CRISPR-Cas9 untuk Terapi Pengeditan Gen

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ABSTRAK

Pengeditan gen menjadi mudah dilakukan sejak ditemukannya clustered regularly interspaced short palindromic repeat (CRISPR) *dan* CRISPR-associated protein 9 (Cas9) *sebagai alat untuk menyunting gen suatu organisme. Sebagian besar penyakit genetik tidak dapat disembuhkan secara kausal dengan terapi yang ada, maka pengeditan gen merupakan suatu cara yang prospektif dalam terapi medis di masa depan. Sayangnya, pengeditan gen dengan Cas9 yang ada saat ini masih memiliki banyak kelemahan, yaitu: 1) kurang spesifik, di mana RNA pemandu dapat berikatan dengan beberapa segmen pada genom manusia, sehingga memungkinkan terjadinya salah target; 2) kurang efisien, karena sekalipun telah berhasil memotong utas ganda DNA, kebanyakan penyambungan kembali akan dilakukan secara* non-homology end joining (NHEJ)*, yang justru meningkatkan peluang terjadinya mutasi; 3) sulit disalurkan ke dalam inti sel karena berbagai sawar fisiologis maupun biokimiawi. Tulisan ini akan membahas perkembangan terkini dalam mengatasi ketiga masalah di atas. Untuk meningkatkan spesifisitas, dapat dilakukan modifikasi RNA pemandu dan struktur Cas9. Efisiensi dapat ditingkatkan dengan meningkatkan peluang terjadinya* homology-directed repair *dibandingkan NHEJ, sedangkan untuk meningkatkan distribusi ke dalam sel, dapat digunakan berbagai macam vektor, seperti virus dan nanopartikel. CRISPR-Cas9 merupakan area yang aktif diteliti dalam bidang biosains, dan dalam waktu dekat, diharapkan dapat dimanfaatkan dalam bidang klinik.*

Kata kunci*: CRISPR, Cas9, efektivitas, spesifisitas, terapi gen*

ABSTRACT

Gene editing has become reasonably easy since the discovery of clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (Cas9). Most genetic diseases cannot be treated causally, and currently available therapies are mainly symptom-based. To treat the etiology of genetic diseases, a firm gene editing therapy is necessary to be established. This posits Cas9-facilitated gene editing as a prospective modality to become a clinically approved therapy in the future to treat genetic disorders. However, until recently, Cas9-based genome editing is still facing several hurdles, including low specificity, low effectiveness, and difficult delivery. Currently available Cas9 nucleases are able to bind to non-specific DNA sequence and produce non-specific cleavage. The efficiency has been relatively low due to the preference of non-homologous end-joining (NHEJ) over homology-directed repair (HDR) by the host cell. Furthermore, in order to deliver Cas9 into the nucleus, multiple physiological barriers have to be overcome. This review discussed recent developments in tackling these three hurdles, ranging from designing the guide RNA using multiple bioinformatics tools, modifying Cas9 structure, as well as packaging the nuclease-guide RNA complex into viral vectors and nanoparticles. Considering the active research on this area, it is expected that CRISPR/Cas9 can be utilized as a clinical therapy in the near future.

Keywords: CRISPR, Cas9, effectiveness, specificity, gene therapy

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INTRODUCTION

The human genome consists of ~3 billion base pairs, encoding around 20.500 genes, which can be further translated into 30,057 proteins (Kim et al. 2014). In addition to this complexity, protein post-translational modifications can yield even more functional molecules, thus assigning a single gene with multiple functions across different times and distinct types of cell throughout the human body. Gene mutations have been well recognized as the causal pathology in numerous diseases. For most genetic diseases, unfortunately, treatment options are mostly limited; mainly focusing on maintaining homeostasis and providing supportive care, without being able to tackle the underlying cause. The discovery of genome editing technology, in line with the completion of human genome project, have opened a new window to treat genetic diseases right on their causal root, where in this case genome-editing would become therapeutic (Cox et al. 2015; Meissner et al. 2014).

Since its first description for gene-editing in 2012, the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (Cas9) nuclease has made genome editing relatively easy, opening new opportunities to repair the etiology of genetic diseases. CRISPR and Cas9 were initially described in bacteria, where they play a role in their innate immunity towards bacteriophage infection. Although it has been a common assumption to consider CRISPR/Cas9 a single inextricable unit, it is actually the Cas9 enzyme that can be utilized for genome editing application. Cas9, an endonuclease, can induce double strain DNA break in a relatively selective manner, as determined by guide RNA (gRNA) sequence (Doudna and Charpentier, 2014). Compared to its predecessors such as transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs), CRISPR/Cas9 is faster, cheaper, and more reliable (Gaj et al. 2013). This system has made the biomedical researches more time- and cost-effective, thus highlighting its potency to be further translated into clinical therapy.

However, as described by Cox et al. (2015), there are several obstacles need to be overcome in order to translate and apply CRISPR/Cas9 in clinical setting. These include low specificity, low efficiency, as well as low delivery rate of the nuclease (Cox et al. 2015). This current review highlights these three problems, focusing on recent progresses and novel techniques achieved in the last two years. In brief, to overcome specificity problem, Cas9 enzyme and its guide RNA can be structurally modified, taking advantage from open-access bioinformatics tools. The efficiency can be increased substantially by promoting a certain type of DNA repairing mechanism called homology-directed repair (HDR) over the mutagenic non-homologous end joining (NHEJ) mechanism. Strategies to improve Cas9 delivery include the utilization of engineered vector, both viral and non-viral. These modifications are critical and indeed mandatory, in order to firmly establish an efficient, safe, and standardized therapy for humans.

SPECIFICITY

The specificity of Cas9 is highly dependent on the guide RNA (gRNA) sequence and the protospacer adjacent motif (PAM) recognition site. The potency to bind to multiple sequences throughout the genome is high, because gRNA consists of only 20 base pairs. The currently widely used Cas9 can still cleave the DNA when the gRNA does not complement perfectly with the genome target site, thus creating a so-called “off-target” (Fu et al. 2013). When the mismatch is located downstream to the PAM, the occurrence of this off-target will be more robust. Even with as many as six mismatches to the gRNA sequence, cleavage can still occur. The most widely used Cas9 currently is SpCas9, which is isolated from *Streptococcus pyogenes*. Unfortunately, 98.4% of them possess at least one off-target site with three or less mismatches to the gRNA (Bolukbasi et al. 2015; Tsai et al. 2015). Therefore, it is important to increase Cas9 machinery specificity, due to the large size of the genome and high off-target possibility, in order to achieve an efficient and safe therapy before applying it as a therapy to human subjects. Failure to achieve this can lead to unwanted adverse effects such as protein synthesis truncation and malignant transformation.

Technically speaking, increasing specificity is another name for minimizing off-targets. There have been two main approaches taken to achieve a high specificity: (1) properly designing the gRNA, and (2) modifying the Cas9 itself.

Designing a specific gRNA sequence to the desired target is an important element, because this machinery is driven by the gRNA. There are two conditions need to be fulfilled for obtaining a good gRNA sequence. First, the target sequence has to be adjacent to the protospacer adjacent motif (PAM) sequence. Second, gRNA has to be specific, ideally within the whole genome. In order to achieve this, a good knowledge of the desired target sequence and access to the information database are crucial. Since the discovery of CRISPR/Cas9 in modern biology, there has been a huge and collective effort to construct databases and computational tools for recognizing and designing a target-specific gRNA, such as well-known MIT's CRISPR Design, CRISPRseek (Zhu et al. 2014), Benchling, Deskgen (Hodgkins et al. 2015) and more recently released CHOPCHOP v2.0 (Labun et al. 2016), CRISPRdirect (Naito et al. 2015), Guide Picker (Hough et al. 2017) and Off-Spotter (Pliatsika and Rigoutsos 2015). These improved tools, for example, allow the users to evaluate both the sequence and the surrounding regions of the recognized site, thus providing more room to fine-tune the specificity. There have also been improvements in the diversity of both the Cas9 and the target genome species database, reaching over 200 target species (Pliatsika and Rigoutsos 2015), as well as enhancement in user interface and graphical navigation (Hough et al. 2017). Although just an improvement of already existing database, these tools are crucial in designing the gRNA and increasing Cas9 specificity, and therefore their role cannot be undermined.

Another novel technique to inquire the target sequence is by direct identification of the cleavage sites, both on- and off-targets, using a method called "genome-wide global unbiased identification of DSBs enabled by sequencing" (GUIDE-seq) (Tsai et al. 2015). This method is able to detect Cas9-induced DSBs based on the utilization of double-stranded oligodeoxynucleotides to repair the breaks. These DSBs will then be used as a primer for sequencing, and eventually can be used to identify off-targets. Indeed, the researchers found multiple off-sites undetected with existing bioinformatics computational tools or ChIP-seq (Gabriel et al. 2015). Other techniques for direct off-target detection include integration-deficient lentiviral vector, BLESS, HTGTS, and Digenome-seq (reviewed in Tycko et al. 2016).

By combining all knowledge obtained from aforementioned modalities, the selection and designing of gRNA with a unique sequence would be more feasible, and eventually would minimize non-specific targeting.

In addition to engineering the gRNA, the Cas9 itself can also be modified, as demonstrated by Slaymaker et al. (2015). They engineered an enhanced-SpCas9 variant based on energetics calculation, and demonstrated that this variant have lower off-targets. They took advantage from the established crystal structure of SpCas9 (Nishimasu et al. 2014), which has a positive charge in PAM-interacting domain groove of SpCas9, and further neutralized this non-target strand groove. This increased the energy needed to induce base pairing between gRNA and the off-target site, and hence, reduce the cleavage activity at off-target sites. Thus, it seems that the binding strength and interaction of the Cas9 itself with non-target DNA sequence are important in determining its specificity. In line with this, Kleinstiver et al. (2016a) constructed a high-fidelity SpCas9 (SpCas9-HF-1) by mutating certain residues (N497, R661, Q695, Q926) that were known to have formed hydrogen bonds with the phosphate backbones of the target DNA strand. Indeed, by reducing this interaction, the engineered SpCas9-HF-1 possesses the ability to avoid nearly all off-target events, while retaining >85% on-target binding.

Another proposed determinant of Cas9 specificity is the PAM sequence recognized by the Cas9. Muller et al. established a StCas9 protein from *Streptococcus thermophiles*, which recognizes longer PAMS (5′-NNAGAAW) and found lower off targets than wildtype SpCas9 (Müller et al. 2016). Over time, more and more novel Cas9 orthologues from multiple bacteria

species have been identified. Notably, a Cas9 orthologue, Cpf1 nucleases from *Acidaminococcus sp* V3L6 and *Lachnospiraceae bacterium* ND2006 has been characterized, and they show higher specificity (Kleinstiver et al. 2016b).

EFFICIENCY

Genome editing efficiency can be defined as the percentage of clones that had been successfully transformed to achieve the on-target, desired modification. Cas9-induced double strand break (DSB) can either undergo non-homologous end joining (NHEJ, which is non-desired) or homology-directed repair (HDR, desired). Therefore, the efficiency can be defined as the rate of HDR occurring in a Cas9 introducing event. Nevertheless, the term "efficiency" can also mean how much Cas9 machinery can be successfully transported into the cell, since good delivery largely correlates with good efficiency. To avoid confusion, issues in delivering Cas9 into the cells will be discussed separately in the following section; for this section, we will simply define efficiency as how much HDR can be gained over NHEJ.

Most somatic cells will response to DSB by executing NHEJ. NHEJ is more preferable than HDR because based on observation by Rothkamm et al. (2003), HDR occurs almost exclusively in dividing cells—while functional somatic tissues mainly consist of mature, post-mitotic cells. Forcing the cells to undergo mitosis as a payoff to increase HDR is not a rational option. HDR, therefore, remains a limiting step to gain an efficient genome editing. It is now possible to increase HDR rate by applying HDR enhancer agents to increase knock-in efficiency, as shown by Song et al. (2016), where they demonstrated five-fold increase of efficiency by applying a small molecule called RS-1, an HDR enhancer molecule. However, when the authors attempt to inhibit NHEJ using other small molecules, no significant HDR enhancement was observed.

Prospectively, the identification and characterization of novel HDR enhancer molecules provides a promising research direction in order to increasing genome editing efficiency.

In order to undergo HDR, it is necessary to provide donor homologous sequences as a template to repair the break. Providing these donor sequences thus will facilitate HDR process and eventually increase its occurrence. A recently discovered clever method is to supply the donor strand flanked by gRNA-PAM sequences. This manipulation maintained to increase HDR efficiency two- to five-fold. More strikingly, combining this technique with the application of cell cycle regulators, 97% HDR was observed, leaving only 3% of NHEJ event (Zhang et al. 2017). Richardson et al. (2016) provided an asymmetric single-stranded DNA donor of the optimal length complementary to the strand and have successfully increased HDR rate up to 60%, which more recently optimized by Liang et al. (2017) by attaching phosphorothioate compund to the asymmetric donor, resulting in 65% efficiency.

Suzuki et al. (2016) invented a technique called homology-independent targeted integration (HITI). This basically exploits NHEJ—which takes place in both dividing and post-mitotic cells—for an efficient targeted gene integration. In this protocol, the donor sequence is specially customized to guarantee that the integration will only take place in the forward direction. When reverse integration occurs, the DNA will be cleaved repetitively until the desired forward integration is achieved, thus forcing the system to be correct. This system has yielded a high efficiency even in post-mitotic cells, such as the retina and the central nervous system. With these recently invented techniques, genome editing with relatively high efficiency in post-mitotic cells is now possible, providing a realistic direction to the clinical application.

DELIVERY

In eukaryotes, including human cells, the genome lies in a protected environment inside the nucleus. In addition to this, Cas9 itself is not an endogenous protein encoded by mammals' genome; it has to be provided from elsewhere. Therefore, to exert its effect, the introduced Cas9 machinery system has to be able to overcome anatomical and physiological barriers and ultimately reach the desired cell's nucleus.

Currently, there are two main strategies to deliver Cas9 and other gene-therapy

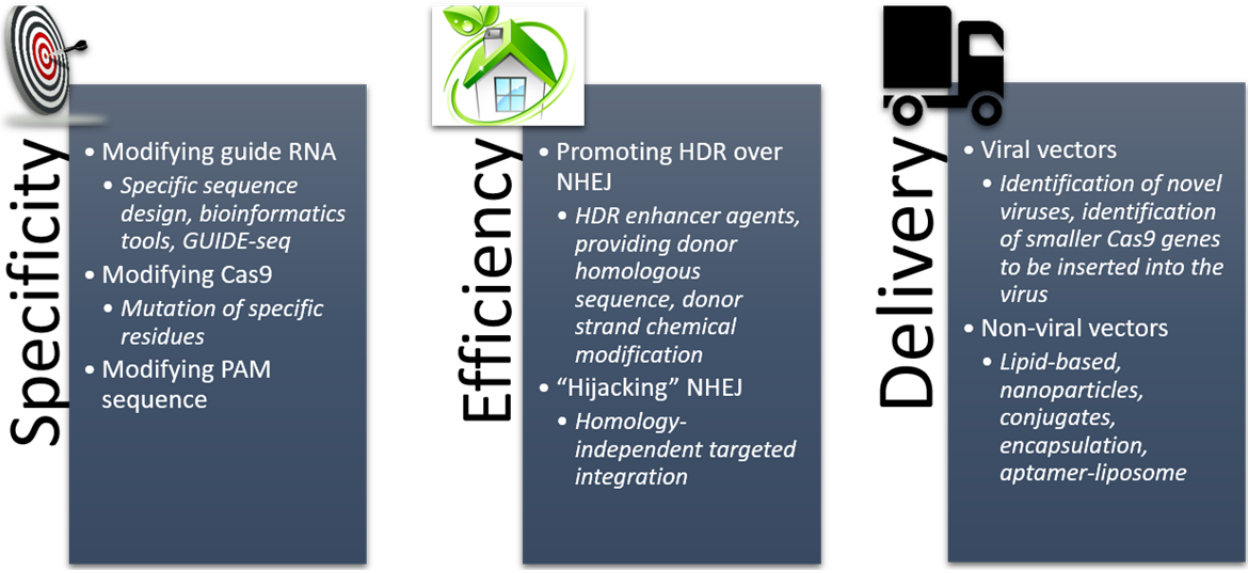

Figure 1. The limitations of CRISPR/Cas9 and the progresses that have been achieved to overcome the limitations (adapted from He et al. (2017) and Cox et al. (2015))

agents into the cell: using viral and non-viral vectors. There is also the option whether to deliver Cas9 gene or Cas9 protein by using compatible transporter. To deliver Cas9 gene, viruses are the vector-of-choice, due to their innate properties to deliver nucleic acids into the host cells. Adenovirus, lentivirus, adeno-associated virus (AAV), and retrovirus, have been well known and widely utilized to deliver most therapeutic gene sequences (Gori et al. 2015). However, several drawbacks exist in utilizing most viral vectors. They include the limited packaging capacity, immune response towards the viral antigen, ability to produce malignant transformation, as well as broad tropism (Yin et al. 2014). To solve packaging problem, smaller Cas9 orthologues from a wide range of bacteria have been isolated and characterized, such as *Staphylococcus aureus* Cas9 (Friedland et al. 2015; Ran et al. 2015), *Neisseria meningitides* Cas9 (Lee et al. 2016), and more recently, *Campylobacter jejuni Cas9* (CjCas9; Kim et al. 2017). CjCas9 consists of only 984 amino-acid residues (2.95 kbp), currently the smallest Cas9 characterized. Smaller size would facilitate a better and easier packaging—together with the gRNA sequence—into widely used viral vectors such as adeno-associated virus (AAV), which can only load ~4 kbp of DNA.

Combining more than one virus (Gong et al. 2017), as well as inventing new viral vectors also contribute in increasing delivery rate. Park et al. (2016) described a novel RNA virus called Sendai virus, as a delivery vector for efficient editing (75-98%). Manipulation of existing viral vectors also can improve delivery, for example by generating different serotypes of AAV, such as AAV2, 6, 8, and 7m8 (reviewed in He et al. 2017).

The intact, pre-synthesized Cas9 protein can be directly delivered into the cell using lipid-based vectors, in which the Cas9 and gRNA can be encapsulated, thus facilitating cellular endocytosis. Zuris et al. (2015) have demonstrated that this technique resulted in 80% successful gene editing of mouse ear hair cells. Other modalities, include cationic polymer-based vector, nanoparticle conjugation/encapsulation, and combination between viral and non-viral delivery are also promising (Wang et al. 2016). Mout and colleagues (2017) utilized gold nanoparticles co-assembled with Cas9 protein and demonstrated that this nanoassembly can be uptaken directly and efficiently by the cells, resulting in ~90% delivery efficiency. More recently, by using zwitterionic amino lipids to co-deliver Cas9-mRNA and gRNA, a ~95% efficiency can be reached, both in-vivo and in-vitro (Miller et al. 2017).

In translating CRISPR/Cas9 into clinics, a targetable delivery to certain type of cell or organ is critical. Zhen et al. (2017) demonstrated a novel targeted delivery using

aptamer-liposome-Cas9 chimera to increase cell-binding specificity and efficiency, where upon intravenous administration can selectively bind to the desired cell type compared to only liposome-Cas9, free Cas9, or vehicle solution. Eventually, as also mentioned by Ginn et al. (2013), Cas9 and its vectors, both viral and non-viral, will have to align up with existing gene-therapy delivery machineries, which have been successfully progressing into clinical trials and approval.

SUMMARY

The specificity, efficiency, and delivery of Cas9 in genome editing therapy is undergoing progressive optimization (Figure 1). Progresses have been made in genome editing therapy, and the CRISPR/Cas9 system has been to date a most promising tool to be clinically translated, due to its increasing efficiency, specificity, safety, and cost-effectiveness. Although until currently, there has not been any ongoing clinical trial for genome-editing therapy, considering the rapid development on this field, one can be optimists that in the future genome-editing can be a state-of-the-art therapy for incurable genetic diseases.

While patients suffering from diseases out there are waiting for science to deliver a cure, scientists can contribute sincerely by editing, manipulating, and improving genome-editing machinery, in the quest to bring a better life for others.

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