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PHA 498

Nanocarriers for Effective Gene Editing

Abstract

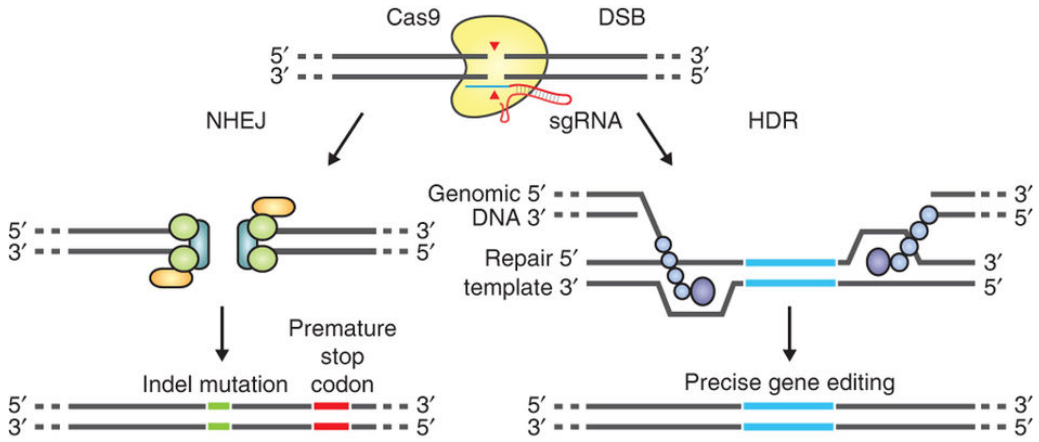
Genetic diseases are largely attributed to mutations in DNA. These can be the result of only a single nucleotide base change, as is the case with point mutations. Infectious diseases are the result of foreign cellular invasion by bacteria and viruses. The past few decades have shown slow growth in discovering cures for the thousands of diseases that affect human life, both intrinsically and extrinsically. However, the recent advancement in CRISPR technology has ramped up the war against disease with ever increasing research in the potential of its application. Combining CRISPR with the endonuclease activity Cas9 has made it possible for precise genome editing. The biggest problem that CRISPR faces, outside of decreasing off-target effects and increasing selectivity, is effective delivery. Recently, scientists explored the use of nanoparticles as a delivery method for the CRISPR/Cas9 payload, which promises the ability to deliver Cas9 and donor DNA and to induce gene editing, simultaneously.

Introduction

Clustered regularly interspaced short palindromic repeats, CRISPR, was first identified in e. coli as a defense mechanism against bacteriophages (Ran, 2013). A virus typically hijacks the cell and uses its machinery to make its own proteins for replication. When the virus initially attacks, CRISPR recognizes the DNA mismatch and creates cellular apoptosis susceptibility (CAS) proteins that subsequently take and cut the DNA apart. It also catalogs the DNA into the CRISPR system to prevent damage from future attacks. Jennifer Duodna, typically credited for the discovery, realized that CRISPR could be used to precisely edit gene sequences through the use of a guide RNA (gRNA), which targets complimentary sequences a few base pairs upstream of the protospacer adjacent motif (PAM) sequence. Cas9 then cuts the sequence and it is repaired through either non-homologous end joining (NHEJ) or homology directed repair (HDR). This

repair is where the gene can be edited through either inserting an appropriate sequence at the breaks or just simple knockout (Ran, 2013). The difference in pathway outcomes is highlighted in "Figure 1" below with NHEJ pathway resulting in an indel mutation and HDR resulting in specific gene editing.

Figure 1: CRISPR Pathways (Ran, 2013)



With any foreign substance that enters the body, which in the therapeutic sense, can come in the form of medicine, the drug has an effect on the body (pharmacodynamics) and the body has an effect on the drug (pharmacokinetics) (BGRG, n.d.). Both share equal importance in the synthesis of medications. Ideally, the pharmacodynamics of a CRISPR-based therapeutic would tell us whether or not the drug had a positive or deleterious effect on the organism of study. While a lot of success has come from this side of research into the efficacy of using CRISPR for gene editing, there has been struggles associated with the pharmacokinetics of the system. Pharmacokinetics is measured by absorption, distribution, metabolism, and excretion of the drug of interest. Absorption is concerned with how fast and how much of a substance is assimilated into the body. Distribution involves the delivery of the drug throughout the body to a targeted region. Metabolism is how the substance breaks down into metabolites. Lastly, excretion occurs when the drug is eliminated from the body (BGRG, n.d.) In particular, absorption and

distribution have shown the most problems because delivering CRISPR effectively involves delivering a rather large payload that is also responsible for genetic editing, without causing an immunogenic response from the body's immune system.

Applying nanomedicine through the use of nanoparticles as a vehicle for delivery of the CRISPR system shows promise for overcoming current problems. Nanomedicine is quite simply the medical application of nanotechnology (Johns Hopkins, n.d.). The use of nanomedicine in therapeutic and even diagnostic medicine has the potential for under the radar, highly-targeted, medicinal delivery. Further, nanomedicine can offer slower delivery, which can effectively limit the frequency of having to take the prescribed medication. While nanomedicine offers a variety of applications, including but not limited to, small molecule delivery, biosensors, and diagnostic tools, the technology has great potential to improve CRISPR delivery (Johns Hopkins, n.d.). Nanoparticles, the proposed vehicle for delivery, can come in many form factors like lipid-based, polymer-based, drug conjugates, inorganic, and even viral.

CRISPR/Cas9

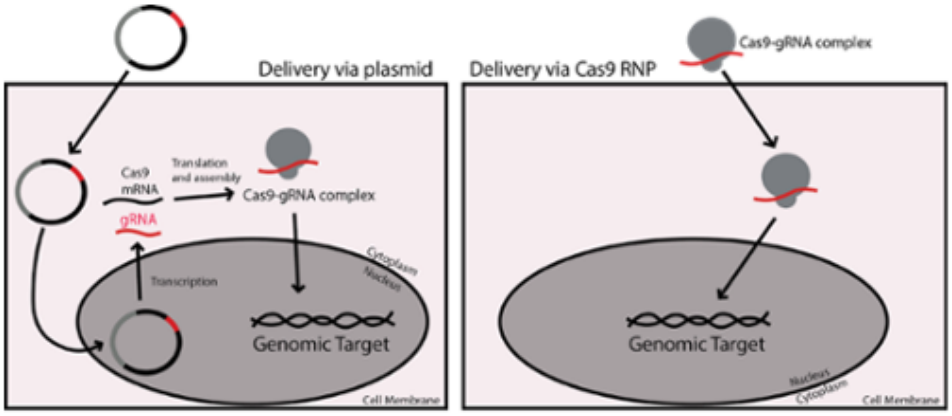
There are several ways to perform genetic engineering besides CRISPR, but most are lacking in ease of use (Guha, 2017). These exist in the forms of small interfering RNA (siRNA), zinc finger nuclease (ZFN), and Transcription Activator-Like Effector Nucleases (TALEN). siRNA are short duplex RNA molecules that effectively silence genes by targeting mRNAs (Nemudryi, 2014). siRNAs do not actually edit the gene, they just alter their expression levels. Another editing tool, ZFNs, are composed of a zinc finger protein domain that is coupled with a site-specific nuclease for cutting DNA. The cutting is extremely site-specific and is overall very complex. ZFNs are also burdened with the high cost of protein domain construction and inaccurate cleavage. The third tool, TALENs, rely on their pathogenic origin's ability to secrete

transcription activator-like effectors to their host cell's cytoplasm. They are also able to bind to DNA and suppress target genes by mimicry of eukaryotic transcription factors. However, they are still relatively costly to use and are tricky to design (Guha, 2017).

CRISPR/Cas9 has great potential because it is very cost-efficient, easy to use, offers direct-targeting, and has low off-target effects. Even the slight possibility for off-target effects is concerning for those against genomic engineering, however, the system is constantly being improved to further decrease the chance for deleterious effects and improve targeting. Recent adaptations to the Cas9 nuclease include improvements in specificity through the use of eSpCas9 and a high-fidelity version, SpCas9-HF1 (University of California - Berkeley, 2017).

The CRISPR system was originally delivered to be transcriptionally or translationally manufactured within the cell upon entry using plasmid or viral delivery of complex Cas9 and the gRNA. However, the system can be pre-complexed in vitro in the form of a Cas9/gRNA Ribonucleoprotein (Cas9 RNP) and delivered by electroporation or transfection and offer immediate detection and quick clearing from the cell for better results (McDade, 2016). The difference in delivery between regular Cas9 (plasmid) and Cas9 RNP is illustrated below in "Figure 2."

Figure 2: Plasmid vs Cas9 RNP Delivery (McDade, 2016)



Methods of Delivery

Researchers are utilizing different vehicles for delivery of the CRISPR/Cas9 system. These vehicles exist in non-viral and viral form factors. Viral vectors can exist in the forms of adenoviral, adeno-associated virus (AAV), and lentiviral vectors. These vectors deliver Cas9 system-encoding cassettes. Packaging in adeno-associated virus vectors are the current, most commonly used methods of delivery (Ling, 2015). While AAVs offer high efficiency in gene delivery and expression, there is a cause for concern with packaging, the potential of carcinogenesis (cancer causing), immunogenicity (immune response upregulation), and large delivery number of viruses (shelled) to the body. Fiscally speaking, there also exists difficulties of large-scale vector production. Thus, it is worth considering the use of non-viral vectors for delivery in the forms of nano- and micro-carriers. These vehicles are capable of direct modification to improve efficacy and limit error(s). They also have low immunogenicity because their size allows them to slip past the body's defense mechanisms. Non-viral vectors also void the possibility of endogenous virus recombination. Further, their cost of production is significantly less than with viral vectors. The most commonly used non-viral vectors are cationic nanocarriers and are made up of lipids or polymers (cationic liposomes, polymers, dendrimers, peptides). Electrical interaction properties allow easy loading and condensing of nucleic acids (Ling, 2015).

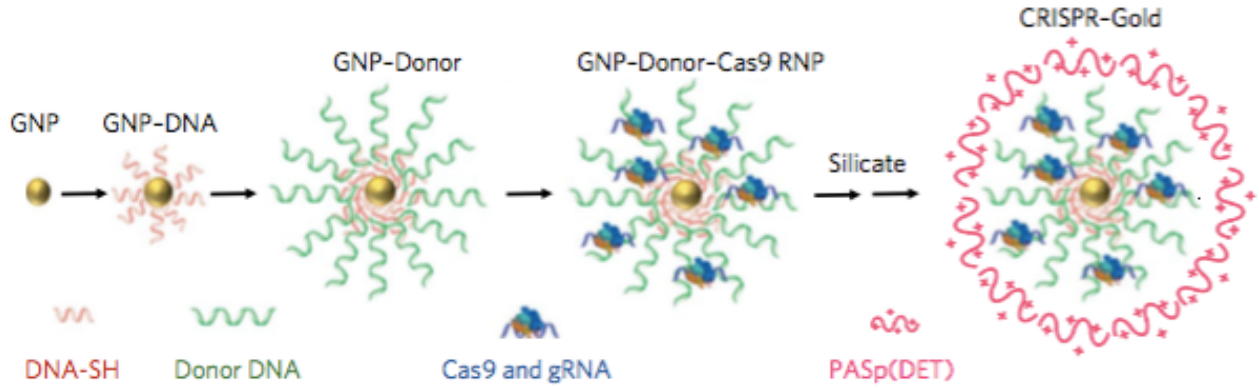
Lipid-based vectors such as liposomes and solid lipid nanoparticles (LPN), were studied very early in gene therapy and rely on lipid-based gene transfection of cationic lipids. These cationic lipids consist of three domains including, the cationic head group, hydrophobic tail, and the link between the two domains (Ling, 2015). Liposomes and LPNs offer different advantages like relatively low immunogenicity and good storage stability, respectively. However, they have

generally poor stability and transfection efficacy. Yet, improvements to these vectors can be achieved by structural modification through replacing ammonium groups with phosphonium and arsonium (Ling, 2015). Polymeric vectors such as dendrimers, are another type of nanocarrier and these have lower immunogenicity and allow for easy, large-scale production. Another polymeric vector, polyethylenimine (PEI), is commonly used for gene delivery (Ling, 2015). PEIs have high transfection efficacy and can be divided into two subcategories, linear (l-PEI) and branched (b-PEI). They have high efficacies, but have varying levels of cytotoxicity due to their molecular weight. PEGylation of PEIs greatly improves cytotoxicity by creating a hydrophilic shell. Another type of non-viral vector that is biodegradable and very biocompatible is the naturally-derived, Chitosan (chitin). Unfortunately, this vector suffers from low transfection efficiency (Ling, 2015). Nanocarriers have great future potential for delivery of most any type of payload because of their flexibility in design and modifications through size, surface interactions, targeting, and toxicity.

Gold Nanoparticles for CRISPR Delivery

In a recent study conducted by Lee et al., researchers were able to deliver Cas9 RNP in gold nanoparticles to mice with Duchenne muscular dystrophy (DMD) (2017). The team successfully synthesized the complex (CRISPR-Gold) by making a reaction with thiol-terminated DNA and gold nanoparticles (GNPs). Then, this was hybridized with donor DNA. After, they exploited the binding affinity between the Cas9 RNP and the DNA to absorb the Cas9 RNP onto the GNPs. Then, they added a layer of silica onto the nanoparticles, which subsequently increased the negative charge density. Lastly, they complexed it with cationic endosomal disruptive polymer (PAsp(DET)) (Lee, 2017). A visual guide to the synthesis is provided below in “Figure 3.”

Figure 3: CRISPR-Gold Synthesis (Lee, 2017)



The treatment, compared to non-gold particle delivery led to increased efficiency and effectively corrected the mutated gene responsible for DMD to its wild-type form. Mice treated with CRISPR-Gold had a two-fold increase in the hanging time (compared to control-Cas9 RNP and donor DNA without particles) muscular test. They also found higher numbers of cells CD45+ and DC11b+ after injection, which are common in muscle tissue that has inflammation and is undergoing muscle regeneration. There was no acute up-regulation of inflammatory cytokines in plasma, which is promising due to low chance of immunogenicity. Further, multiple injections did not cause the mice to lose weight or show up-regulation of inflammatory cytokines (Lee, 2017).

Conclusion

CRISPR has really opened the door for genetic modification, correction of genetic disorders, elimination of persistent-bacterial infections, and has even made waves against certain cancers and viral infections. Recent advances have overcome limitations in minimizing off-target effects and improving overall efficacy. Another hurdle that has been overcome is the effective delivery with heavy reliance on viral vectors that suffer from immunogenicity and cost of

production. The use of nanocarriers as an alternative to AAVs has shown significant improvement in the application of the system and will likely make the FDA more tolerable to implementation into the next generation of medicine. The combination of these two biotechnical achievements might very well lead to medicine that is cheaper, more effective, and less time consuming with single injection treatment.

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