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A Review of the Applications and Mechanisms of CRISPR-Cas9 Systems

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ABSTRACT

Several studies have introduced CRISPR-Cas9 as a prominent genome-editing technology. CRISPR was first established as a bacterial immune system and was later founded as a new genome-editing technology by Jennifer Doudna and colleagues around the turn of the decade. CRISPR is preceded by zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs); however, it improves upon these previous genome-editing designs by using RNA as its binding domain and by already containing nucleases—reducing the need to intentionally engineer these domains into the technology. Complementarity between the different RNAs (tracrRNA and crRNA) and protein aspects (Cas systems) of CRISPR act in conjunction to both efficiently assemble and confer its intended purpose. After extensive use in mammalian cell cultures and various model organisms, the CRISPR-Cas system has subsequently been used to mediate several genetic diseases such as sickle cell anemia and some cancers in humans. CRISPR-Cas9's potential to mediate all other genetic mutations induced by sequence polymorphisms remains promising.

Keywords: CRISPR/Cas-9, gene editing, biotechnology, medicine

INTRODUCTION

The discovery of the CRISPR locus and the later applications of CRISPR-Cas9 as gene-editing technology has started a revolution that has spurred great advances in health care, genomic studies, and biotechnology. These advances will only continue as more is learned about how CRISPR works and how it can be utilized. As CRISPR technology remains an emerging field, continued explorations into new derivative technologies have allowed for the development of new methods, and the continued discovery of potential applications of this technology. This review will provide both a mechanistic analysis and cover some of the new applications of CRISPR-Cas9 mediated gene editing as it specifically pertains to treating diseases normally encountered within healthcare and health sciences. We will also predict some of the potential outcomes of recent trends of study in CRISPR medicine and analyze the potential of these technologies to help remedy many great health challenges. Genome-editing is becoming an important field of study to understand, as it is a rapidly expanding and ever-changing field. CRISPR-Cas9 both has, and will continue to, allow clinicians to make great strides in treating and preventing disease.

MECHANISMS

Before CRISPR-Cas9, other programmable nucleases were used to sever double-stranded DNA molecules to promote homologous recombination. However, these nucleases—zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN)—either lack specificity or precision abilities and were labor-intensive and time-consuming to produce. CRISPR uses a short guide RNA to recognize its complementary DNA target by Watson-Crick base pairing (Hsu et al) (Figure 1). Since CRISPR is a bacteria-derived immune mechanism, its target DNA sequences are normally phage genomes, but the Cas9 domain can be redirected to target a sequence of interest by modifying its guide RNA to compliment the desired DNA target. Further, sequence-agnostic functional effector domains can be fused to CRISPR, allowing "flexible recruitment of desired perturbations," including transcriptional activation at a locus of interest. In theory, any modular enzymatic domain—such as a nickase—may be fused to the CRISPR domain, opening the door to many bioengineering processes.

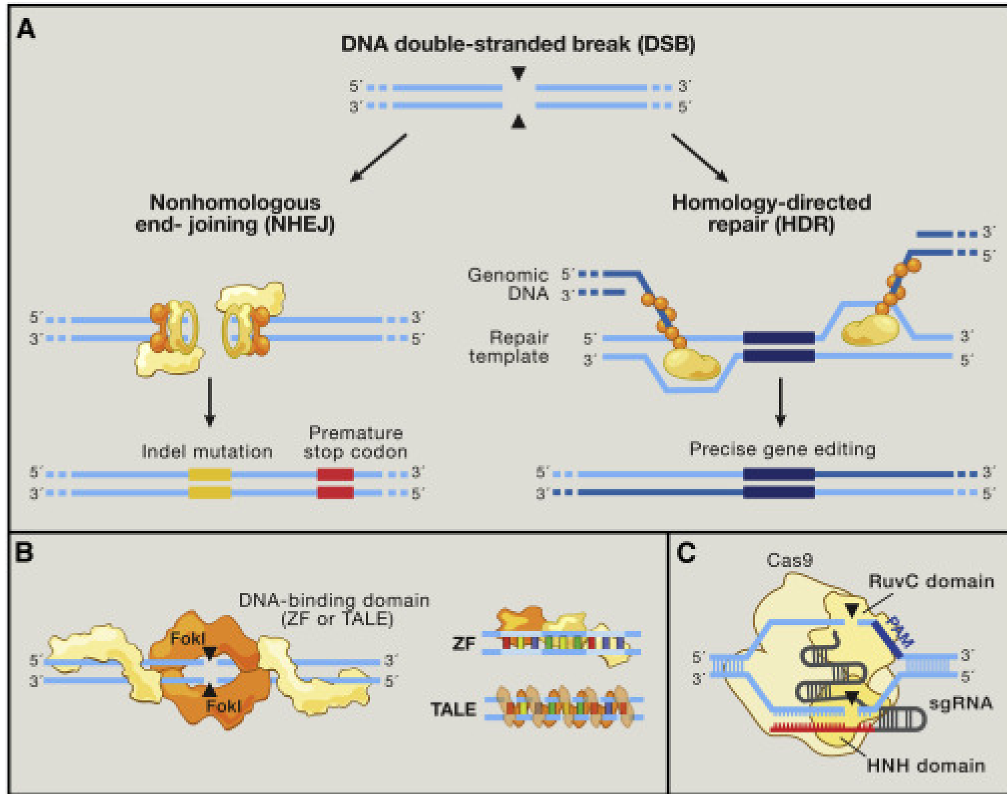

***Figure 1**: Editing technologies using native DNA repair mechanisms (Hsu et al)*

CRISPR is defined as a locus in a bacterial genome that contains DNA sequences, called protospacers, that were recovered from previous viral infections (van der Oost et al). Once foreign DNA protospacers are detected by the prokaryote, CRISPR adaptation causes the incorporation of the recovered DNA sequence into the CRISPR locus. These protospacers are now called spacers and are then flanked by repetitive sequences in the prokaryotic genome called direct repeats. Then the CRISPR locus is transcribed to poly-spacer precursor crRNA (pre-crRNA), which contains continuously alternating spacers and direct repeats. Separately, but concurrently, the upstream Cas9 gene is transcribed and translated to protein. Jiang and Doudna (2017) detail the process of crRNA maturation, where Cas9 complexes bind and process the pre-crRNA by cleaving the DNA so that one spacer next to one direct repeat is incorporated into

each Cas9 complex—which is now referred to as a crRNA. Further concurrently, a second gene is separately transcribed to a *trans*-activating CRISPR RNA, called tracrRNA. The tracrRNA is incorporated into Cas9 due to its homology to the direct repeat portion of the crRNA. Then, due to the complementarity between the tracrRNA and the direct repeat portion of the crRNA, the mature CRISPR-Cas9 complex can be recruited to the sequence within the viral genome for which it is complementary. Then, the complementarity between the spacer portion of the crRNA and its homologous sequence in the invading viral genome activates the Cas9 nuclease domains to make a double-stranded break in the viral genome—rendering its infection null. Since Cas9 is a multidomain nuclease, it cleaves the viral DNA with an HNH-like nuclease, which cleaves the DNA strand complementary to the gRNA, and a RuvC-like nuclease that cleaves the strand opposite of this.

The versatility that CRISPR-Cas9 brings to genome editing has been displayed in manipulations of mammalian cells, (Allen et al)). CRISPR-Cas9 is a class 2 type II system, where the crRNA, tracrRNA, and multiple domains comprise a ribonucleoprotein (RNP) capable of introducing site-specific double-strand breaks in DNA. These breaks are facilitated by the creation of an R loop, where the annealing of the crRNA to an unhybridized complementary DNA sequence creates an A-form helix. One stipulation of complementary base pairing between the crRNA and the target DNA is that the target DNA must be adjacent to a PAM site. In Cas9, the PAM (protospacer adjacent motif) is 5'-NGG-3' and must be properly paired with in order for the nucleases to cut the target DNA. Other Cas proteins contain different PAMs, and thus can be used in replacement of Cas9 in the event that complementarity to 5'-NGG-3' cannot be found in the target DNA sequence. In mammalian cells, CRISPR-Cas9 has been used to knock-out genes by taking advantage of an endogenous cellular DNA repair mechanism called

non-homologous end joining (NHEJ). The cell has an innate DNA repair mechanism that mediates double-stranded breaks endogenously. However, this mechanism is often repaired imperfectly, where it includes or excludes the correct sequence base pairs. Insertions or deletions of this nature—termed Indels—often cause frameshifts, and can subsequently inactivate or greatly perturb the gene in which they occur. CRISPR-Cas9 was used to create a double stranded break and took advantage of a second endogenous DNA repair mechanism to "knock-in" a gene of interest (Allen et al). This second DNA repair mechanism is called homologous recombination repair, where the cell uses its homologous chromosome as a template to resynthesize the damaged DNA strand. Once the double-stranded break is created, exogenous DNA—normally in the form of an engineered plasmid—containing the gene of interest is added into the edited cell. The plasmid is engineered to flank the gene of interest with sequences of DNA that are homologous to the sequences within the region of DNA that is being knocked-in. Therein, the double-strand break is repaired by hijacking an endogenous cellular DNA repair mechanism, but where the exogenous DNA is utilized as the template to repair the break—effectively adding the gene of interest into the target genome. This is referred to as homology-directed recombination.

APPLICATIONS

CRISPR-Cas9 has shown promise in treating genetic disorders by genome editing. However, light was shed on a drawback of the supposed human application of CRISPR, where off-target effects remain a concern in the edited genome due to the ability of gRNA to base pair with only partially complementary DNA sequences (Rodriguez-Rodriguez et al). This effect is compounded by the fact that different PAM sites can confer greater specificity for their target DNA by being longer and less accepting of variability. Different bioinformatics software programs have been able to determine criteria by which CRISPR can be made most specific for its target, such as the domain of organism, specific PAM or Cas protein used, and length of target DNA. One promising technique involves engineering the nuclease domains within Cas9 to be inducible, so that their expression, and thus activity, is only temporary. Another method being used to mediate off-target effects is to use two separate Cas mutants that each only cut one strand, which is done by engineering each gRNA to base pair with opposite strands of DNA that are at least within 100 nucleotides of each other. Once off-target effects can be mediated, the potential of CRISPR in disease treatment can be explored using disease models. Specifically, single nucleotide polymorphism (SNPs) can be treated by performing gene knock-out, by creating protective mutations, or by introducing therapeutic transgenes. The ΔF508 mutation characteristic of Cystic fibrosis, in particular, has been corrected using CRISPR, as demonstrated in edited stem cells that were experimentally differentiated into intestinal organoids. Further, significant headway has been made regarding hematologic diseases such as β-hemoglobinopathies sickle-cell disease and β-thalassemia using homology-directed repair.

Although gene knock-in or knock-out are popular options when treating certain diseases, it has been shown that gene knock-down can also be a useful treatment technique by controlling cellular transcript levels (Newsom et al). This can result in a similar effect as pure knock-in or knock-out by themselves but creates a novel method by which to treat a disease. CRISPR knock-down is done by using catalytically active CRISPR domains that contain gRNAs specific to mRNA transcripts in order to target and cleave, and thus silence, transcripts that would otherwise lead to gene products. Further, another method of gene knock-down using CRISPR interference is termed CRISPRi and shares similarities with RNAi (Larson et al). However, where normally RNAi targets and silences already present mRNA transcripts—as does the method described by Newsom and colleagues—CRISPRi silences transcription itself, preventing any mRNA from being transcribed in the first place. This is achieved by engineering both a catalytically-inactive Cas domain and a gRNA that targets the promoter region of the gene in question, which effectively prevents transcription by sterically hindering RNA polymerase from initiation transcription. The advantage of knock-down, as opposed to knock-in or knock-out, is that it's inducible and reversible. These methods show promise in mediating diseases that propagate through genetic mutations such as single-nucleotide polymorphisms (SNPs), serial repeats, and base-pair deletions.

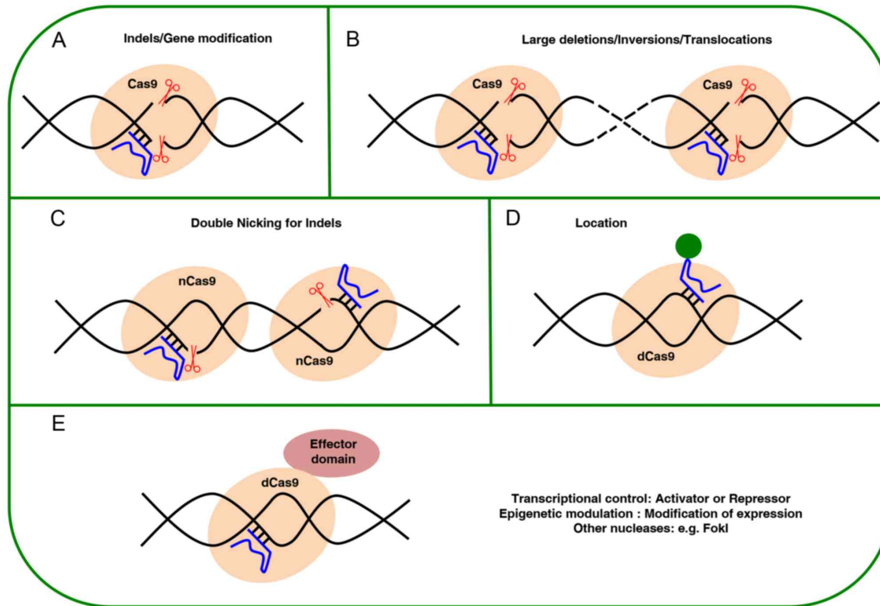

Figure 2*: - Applications of Cas9. (Rodriguez-Rodriguez et al)*

CONCLUSION

The field of CRISPR-Cas9 mediated gene editing has allowed for advances in therapeutic treatment for a whole host of different diseases, such as hereditary eye diseases, Alzheimer's and Huntington's disorders, some cancers, and HIV. While treatment for these conditions has been in the production pipeline in the clinical trials stage for quite a while, this seems to only be the beginning when it comes to the development of treatments that are sure to come as this technology advances. Though there are many benefits that come along with the advances in CRISPR technology, some concerns have been raised about the ethics of gene editing, especially when it comes to germline edits and possible cosmetic medical uses. As a result, the field of bioethics has grown in tangent with the increased use of CRISPR-Cas9 editing technology. The future of CRISPR research looks to be promising, and it has the potential to revolutionize medicine for generations to come.

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