Paper: **Therapeutic applications of CRISPR/Cas9 system in gene therapy**
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1. What is the significance of this research topic?

The CRISPR/Ca9 technology is one of the most significant breakthroughs in the last 10 years in genome modification. Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Cas system is part of the immune system found in some bacteria and archaea. When a virus invades these organisms, their DNA is "captured" and incorporated in the host DNA as spacers within the CRISPR array, each spacer is separated from the other by repeat units. The next step is transcription of CRISPR repeats and spacers into CRISPR RNA (crRNA). The last step is the activation of the Cas9 protein, which upon conformational change, finds the target DNA which then is followed by the attachment using base pair complementarity of the crRNA to viral RNA; and the cleavage of the foreign RNA complex and its elimination. Cas9 nuclease activity can be easily replaced by a sequence of interest making Cas9 compared to other DNA-binding platforms (meganucleases, zinc fingers (ZF), transcription activator-like effectors (TALEs)) simple and very efficient. Another important advantage of the CRISPR-Cas9 system, is its ability to cleave multiple distinct targets sequences in parallel, inducing multiple mutations in different genes which extend the possibilities for modeling complex diseases when using mice from single gene knockouts without lengthy breeding strategies and with less animal sacrifice. Other reasons to add multiples gRNAs are: 1) using dual nicknases to generate a knockout or edit to reduce off-target activity, and 2) deleting large region of the genome. Since its first application in genome engineering, CRISPR-Cas technology has continued to evolve, leading to many more discoveries:

- Type VI CRISPR systems include enzymes (Cas13) that target RNA without the need for spacer sequence and can be used to reduce RNA levels.
- In Epigenetic modifications (the combined genetic modifications across the genome), a catalytic dead dCas9 protein is fused to a variety of epigenetic modifier; that is the genes which modify the epigenome through DNA methylation, alteration of the structure of the chromatin or its posttranslational modification. Fusing dCas9 to an epigenetic modifier can repress or activate transcription. One major advantage of this combination is its reversibility once the effector is inactivated from the system.
- CRISPR-Cas9 has been used for fluorescent tagging of protein, whereby a fluorescent protein such as GFP is attached to the protein of interest, to provide insight for the protein's function. Also, gRNAs can be fused to protein-interacting RNA aptamers to visualize targeted genomic loci, as well detecting the chromatin dynamics in living cells. The CRISPR tagging system is compatible with fluorescence microscopy; and produces less artifacts attributed to exogeneous overexpression of a protein fused to a fluorescent tag.

Who is working in this area?

2. What methods are used to study the concepts described in the paper?

Among the different proteins involved in the CRISPR complex (Cas-1 to Cas10), Cas9 is the only enzyme within the Cas gene cluster that plays a role in locating and DNA cleavage. The Cas9 protein has 6 domains:

- **Rec 1** and **Rec 2** domain binds the complementary region of the guide RNA. Rec1 role is essential compared to Rec 2as for the binding of repeat/anti-repeat target DNA.
- **Bridge helix (NH)** arginine-based structure which modulates target DNA cleavage and mismatch tolerance.
- **Photospacer Adjacent Motif (PAM)-Interacting (PI)** domain confers PAM specificity, and is responsible for initiating binding to target DNA.
- **HNH and RuvC** domains are nuclease domains that cut single stranded DNA (specifically between the 3rd and 4th nucleotides from the PAM).

A single guide RNA (sgRNA) can be engineered by fusing a crRNA containing the target DNA sequence to a noncoding trans-activating crRNA (tracrRNA) to activate the Cas9 protein. The guide RNA forms a T-shape comprised of one tetraloop and 2 or 3 stem loops, and is constructed to have a 5' end complimentary to the target DNA sequence. One key factor in CRISPR-Cas9 efficiency is the Pam binding step which allows the Cs9 protein to quickly screen for potential target with appropriate PAM before melting. Another key advantage of CRISPR-Cas9 system is its flexibility, several studies have shown that single, mismatches at the 5' end of the sgRNA are well-tolerated, double mismatches can still result in cleavage for some sequences, and beyond this number sgRNA activity is suppressed. CRISPR/Cas9 is used to create double strand breaks (DSBs) to perform desired indels or knock out existing genes in the target DNA strand. Homologous directed-repair (HDR) and non-homologous end joining (NHEJ) are the two major pathways to resolve DSBs. Between the two, HDR is favored as it requires higher sequence homology between the severe and donor strands of DNA and reduce the risks of genomic instability or cell deaths. Strategies have been developed to promote HDR over NHEJ such as inhibiting NHEJ pathway using inhibitors (CYREN)and use HDR factors (e.g., Rad18) to activate HDR. HDR methods are further categorized into conservative methods: double-strand break repair (SDBR), synthesis-dependent strand-annealing (SDSA), break-induced repair (BIR) and non-conservative: single-strand annealing (SSA) pathway. Insertion sites of the gene modification have to be less than 1-bp away from the DSB. Also, HDR template should be designed to prevent the Cas9 enzyme to keep cutting and repairing beyond the targeted sequence.

3. Does the review article lead to new questions or hypotheses in this technical area (by the authors, by other researchers)?

4. What are some practical applications of the research discussed in the article?

6. How does this topic relate to other areas of cell biology, bioengineering, or medicine?

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