CRISPR / Cas9 system: Use of genetic engineering in healing cancer and other genetic diseases.

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INTRODUCTION

Cancer is caused by several changes in the genome and epigenome, culminating mainly in the inactivation of cancer suppressor genes or the activation of oncogenes. With this, the emergence of a new therapy that uses genetic engineering to make an edition of the genome through DNA cleavage promises to revolutionize future treatments for cancer and other genetic 2 diseases. The CRISPR system is associated with a Cas 9 endonuclease guided from an RNA sequence. Most genetic diseases are caused by the loss of global methylation patterns or hypermethylation of specific loci. Thus, when we make this association of the endonuclease Cas 9 with the guide RNA and introduce, in vitro, in other cells, this system, CRISPR / Cas9, targets specific places in the genome, causing breaks in the double strand. After this cleavage, the organism's intrinsic molecular machinery, responsible for correcting errors in the genome, is activated and used to alter the mutated DNA sequence, correcting the error. The CRISPR / Cas9 system, even with a short time of discovery, has become a very efficient tool, capable of repairing and modifying DNA sequences. Although revolutionary and very promising, the technique has been suffering from the so-called off-target effects, where the CRISPR / Cas9 system ends up accidentally attacking stretches of DNA that are not of interest. Its weaknesses and limitations are being studied so that soon, therapies with CRISPR can be used in clinical therapy safely and effectively.

CRISPR IN CLINICAL APPLICATION

The rapid technological development of genome editing needs attention suitable for improving preclinical and clinical trials to assess toxicity, out-of-the-box effects target and other possible side effects. Several attempts have been made to use CRISPRs to correct mutated genes. One of these studies was performed in a mouse cancer model with the Pten and p53 genes mutated. Mice were transfected with a vector carrying CRISPR designated through a tail vein to reach 20% of the hepatocytes to transform through the blood which also successfully corrected the β-catenin gene mutation frequently involved in cancer with CRISPR. Burkitt's lymphoma is a cancer caused by mutations in the cMyc gene and almost all patients suffering from it have Epstein-Barr virus (EBV) infection. Recently, CRISPR/Cas9 system is used against EBV, generating load reduction viral and tumor proliferation. Furthermore, recent studies demonstrate the successful editing of the Trp53 tumor suppressor gene in Arf - / - Eμ Myc lymphomas. Overexpression of the Myc gene is responsible for several types of lymphoma cancer. Subsequent studies show Mll3 as another important suppressor gene disruption of tumor via ex vivo CRISPRs in acute myeloid leukemia. The successful application of TALENs genome editing tool in a one-year-old girl with leukemia provides the basis for the use of more efficient CRISPRs applications in clinics. In addition to cancers, CRISPRs are also used to correct several genetic diseases. An inherited eye disease called Retinitis pigmentosa, which causes cells to break down photoreceptor cells resulting in gradual loss of vision, was recently successfully edited in iPSC for the RPGR gene, which in a healthy individual is responsible for protein production involved in normal vision, giving new hope to blind patients with retinitis pigmentosa. Another X-linked recessive disease, Duchenne muscular dystrophy (DMD), is caused mainly by a frame-shift mutation in the dystrophin protein, essential for the muscle function and very suitable for genome editing, despite its size is too large, 79 exons. It does not require the entire gene to be expressed and with few changes in the sequence that cause the disease, can bring about improvements in muscle functioning. Thus, an exon skipping technology could be applied with proven success in the mouse model of Duchenne muscular dystrophy. The exon skipping technology with CRISPR/Cas9 opens the door to the treatment of various other diseases such as Ataxia telangiectasia, congenital disorder of glycosylation and Niemann-Pick type C caused by splicing errors. A well-known experiment at SUN-YATSEN University in China on embryos humans to treat the gene that causes thalassemia in human embryos shows that only some embryos in 80 received a corrected form of the gene copy. These examples of successful clinical application with CRISPR define the future technology, but they still need to carry out several clinical and pre-clinical trials to determine the side effects on the patient's health, the immunogenic responses to carriers of vectors and possible genome-wide disadvantages as an off-target result.

EPIGENOME CHANGE WITH CRISPRS / CAS9

Epigenetic modifications consist of DNA methylation and histone modifications providing an essential environment to stimulate gene expression that defines proliferation and differentiation of cellular activity. Histone proteins are responsible for the packaging of all DNA in eukaryotic cells and various epigenetic modifications, including ubiquitination, phosphorylation, SUMOylation (post-translational modification (MPT)) and acetylation. All of these are reversible in nature and are under the control of epigenetically modifying enzymes. These histone epigenetic covalent modifications are of high importance in the repression or activation of gene expression. In addition to histone modifications, chromatin structure is also defined by DNA methylation. The first identified epigenetic modification is carried out by DNA methyltransferase enzymes (DNMTs), which provide an environment to prevent transcription factors from binding and/or bringing repressive protein complexes to DNA. 10 DNA methylation is more stable compared to post-translational histone modifications, but it can still be demethylated by active and passive mechanisms, and is responsible for normal development and differentiation. Currently, several non-coding RNAs (ncRNA), eg micro RNA (miRNA) and small interfering RNA (siRNA) inhibit or activate genes involved in epigenetic regulation of critical biological processes of growth and development. MiRNAs are present in a number of diseases and can be used to target specific gene expression for their regulation, either up and/or down as needed. Recently, CRISPR/Cas9 has been successfully employed to edit genetic changes and many of the miRNAs that are involved in cancer progression and development can be specifically targeted for CRISPR/Cas9 genome editing. Recent advances in genome editing technologies, especially the wonderful results of CRISPR/Cas9, have brought strong hope in how to deal with genetic and deadly factors of cancer diseases. Suppression of gene expression was demonstrated when dCas9 (double mutant) fused with a KRAB associated with the Krüppel repressor, but its genome-wide specificity and heterochromatin were not known until reports of its HS2 binding enhancer having a distinct role in the expression of many genes. of globine. The observation of highly specific H3K9 trimethylation and the limited accessibility of chromatin to the enhancer and promoter suggest that individual enhancement can be successful if modified to control changes in the epigenome. Epigenetic drugs are widely used effectively but have some strong side effects that need alternative platform to specifically modify the epigenome for cancer treatment. Epigenetic drugs have implied severe off-target effects, which trigger several genes including p53, Akt, cMyc and cause dysfunction in several biological pathways, including metabolic and immune pathways. Hilton and colleagues recently demonstrated that a nuclease-null dCas9 fused to the catalytic core of p300 human acetyltransferase co-transfected with multiple gRNAs in HEK297T cells to target endogenous IL1RN, MYOD, and OCT4 promoters, resulted in highly specific gene expression through acetylation. of H3 lysine27 (H3K27) by its promoters and enhancers, thus providing a robust tool for modifications. As most human cancers are due to loss in global patterns of methylation or hypermethylation of specific loci, the present progress and additional discoveries of epigenome editing tools will be critical to reducing cancer.

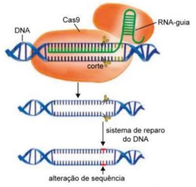
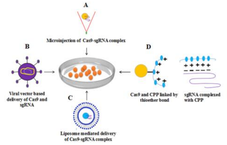


Figure 1 - The CRISPR/Cas9 engine

CRISPR / CAS9 TRANSPORTERS FOR CELLS

There are difficulties in standardizing and sustaining the transport of the CRISPR system to the cells that need it. Specialized methods have been developed to deliver Cas9 and gRNA to the target site within a cell or tissue claims to aid CRISPR specificity. Nuclease genome editing technology effectively and efficiently alters genome sequences, thus providing an opportunity to correct mutation-causing diseases but require potent methods of delivery to cells. Cas9 can be delivered to cells as mRNA, however, mRNA is unstable and not suitable for long-term gene therapy purposes, however, genome changes remain and are widely used in model organisms including mouse, fish -zebra, Drosophila and C. elegans. The expression of the Cas9 and gRNA complex in cultured mammalian cells is mainly done physically, delivering non-replicating plasmids that express this template. These methods involve electroporation, microinjection, lipofection and nucleofection. The main disadvantage of using plasmid is the random integration of the plasmid or its part at sites outside and inside the target leading to inactivation of gene insertion. To address these deficiencies, several efforts have been made to deliver the Cas9 protein in conjugation with cell-penetrating proteins (CPPs) complexed with guide RNA that form positively charged nanoparticles and show gene disruption. An improved delivery method inspired by DNA nanoclews and DNA nanoparticles was recently reported with the ability to simultaneously deliver Cas9 protein and sgRNA 12 to human cell nuclei and disrupt genes efficiently while maintaining cell viability. Viral vectors such as Adeno-Associated Virus (AAV) and Lentivirus are widely used as a gene delivery tool due to the efficient introduction of an exogenous DNA fragment into the genome by robust HDR. Although the low pathogenicity and oncogenic risk make AAV the best choice, the large size of SpCas9 (4.2 kb in length, ~1400 amino acids) limits the transduction ability of AAV, allowing only a 4.7 kb fragment to be transduced. This issue was addressed by a smaller 'SaCas9' size (3.3 kb). AAV-mediated delivery of this Cas9 variant shows the same efficiencies as native SpCas9 at the same genomic loci without off-target mutation (RAN et.al., 2015). Mainly for gene therapy, ex vivo treatment of cells is carried out, and later transferred to the body. The most stable way to transfer Cas9 and gRNA is with the use of non-viral DNA plasmids. It is of paramount importance to develop tools and methods to efficiently and specifically stimulate or inhibit gene expression to achieve the desired results for cancer therapy. The strategy to control the oncological disease can be done in two ways, removing the tumor tissue or controlling the transgene expression by the tumor-specific promoter like telomerase promoters, often involved in cancers. In vivo delivery of nucleases is addressed by Zuris and colleagues, they conclusively show that protein delivery by cationic lipid is a viable approach to genome editing and is capable of transporting Cre recombinase, TALE, CRISPR/Cas9: transcription factor gRNA and Cas9. Delivery of ribonucleoprotein (RNP) (Cas protein and gRNA) to cells induced site-specific mutation of up to 79% and reduced off-target cleavage associated with plasmid transfection at off-target sites that differ by one or two nucleotides from the target site . The efficiency of delivering RNPs by electroporation transformation is reported twice as compared to plasmid-mediated transformation. Through the administration of RGEN RNP, two endogenous genes (dpy-3 and unc-1) in C.elegans were inherited in a heritable manner, without indel formation. This method has some advantages, as this complex provides an amount of control Cas9 to the cell, followed by rapid degradation. For therapeutic application, the invention of a safe and improved delivery method of Cas9-sgRNA into the cell or organisms is urgently needed. The improvement of the existing method and the invention of a new method as a filler, such as using nanoparticles, can allow efficient and specific editing of the genome.

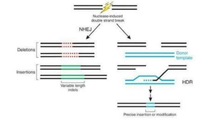
Figure 2 - Methods for delivering the Cas9-sgRNA complex to cell A.microinjection of Cas9-sgRNA viral vector B. Delivery based on (AAV) C. Lipofection D. Cell-penetrating peptide (CPP)-based delivery of the Cas9-sgRNA complex in cells



CRISPR/CAS9 CHALLENGES IN CLINICAL THERAPY

There are some obstacles that limit the commercial therapeutic application of CRISPR. The gene editing capability of Cas9 is of paramount importance. The breakdown of dsDNA by Cas9 is followed by two natural pathways present in the cell; Non-homologous final union (NHEJ) and homology-directed repair (HDR). NHEJ is naturally the preferred pathway for gene correction in nature, but it is error-prone and causes unwanted mutations, so it is not suitable for the application of CRISPR as therapeutic agents. HDR, on the other hand, is accurate and error-free, but it is not the naturally preferred path for correcting DSBs, so it requires finding more ways to make HDR favored over NHEJ in a natural environment, to efficiently translate the benefits of CRISPRs to clinics. NHEJ is predominant in G1, S, and G2 phases, while HDR in late S and G2 phases. These two pathways were manipulated by researchers for genome editing using CRISPR in mammalian cells for the first time. The editing frequency achieved by the NHEJ ranges from 2% to 25%, which causes high-efficiency deletion of the intermediate sequence. The HDR pathway uses donor DNA as a template to repair DSB in a 'copy and paste' manner using the homologous recombination mechanism. By providing engineered donor DNA, precise modifications, small or large, can be made to the genome. The CRISPR Cas9 genome editing capability using the HDR pathway is not fully developed. As the NHEJ pathway is imperfect, causing InDels to form at the cleaved site, it can lead to a weft shift mutation, resulting in malfunctioning proteins or nonsense mutation-mediated transcript decays, thus causing gene disruption. The specificity rate increases with the increase in HDR-mediated repair of DSB induced by CRISPR Cas9. Therefore, using HDR is more favored. However, NHEJ competes with HDR to rectify DSBs. The use of the DNA ligase IV antagonist Scr7 inhibitor (a key enzyme involved in the NHEJ repair pathway) increased genome editing efficiency by up to 19-fold, preventing NHEJ. G2 nuclease cell cycle synchronization has been shown to increase HDR efficiency while reducing unwanted NHEJ events. Likewise, replacement of normal Cas9 with Cas9 nickase (Cas9n) activates HDR with low off-target potentials. However, the recombination efficiency is low (1 in 106 - 109 cells), limiting the large-scale applications of HDR in the gene targeting assay. The specificity and precision of the gene editing process following site-specific genomic cleavage by RGENs depends on the nature of the donor DNA. If the foreign DNA has greater than 400 bp homology to the target, it will lead to more efficient introduction of precise nucleotide substitutions or deletions, endogenous gene tagging and targeted transgenes. The specificity of CRISPRs is also in question and many clinical laboratories are concerned about their off-target effects and ways they can minimize these off-targets and develop clinical trials to measure them. Several advances are made in the delivery of nucleases to target cells ex vivo and in vivo, but there is still a need to improve delivery systems to realize the CRISPR therapeutics dream. In addition to specificity, a possible key to success is the isolation of mutant cells (with DNA of interest) from a diverse population of cells. The evolution of the host-pathogen interaction is an ongoing process. The natural development of CRISPRs in bacteria and archaea as adaptive immune responses took hundreds of years to combat invading pathogens. Recently, some study groups have identified anti-CRISPR proteins in viruses that can destroy bacterial CRISPRs with all their memory records of invading viruses and therefore exposing them to the greatest threat of virus attacks. Such findings emphasize the need to develop more accurate and more effective clinical trials for therapeutic CRISPRs.

Figure 3 - The two DNA repair pathways. The NHEJ is naturally favoured, while the via HDR has therapeutic application in the correction of several mutated genes.



CONCLUSION

The CRISPR/Cas9 system, even with a short time of discovery, has become a very efficient tool, capable of repairing and modifying DNA sequences. In the field of genetic engineering, the ability to identify a specific location in DNA and modify it has always been a challenge, but this system has made it possible to manipulate genes to create a diversity of mutations in different organisms and cells. It is also worth mentioning its use in the discovery of possible targets for the treatment of pathologies and correction of phenotypes. CRISPR/Cas9 holds great promise for the development of diverse biotechnological therapies, with its easy genetic manipulation capability, efficiency and wide application. However, we must not forget the potential weaknesses or limitations of this system until it becomes accepted by medical practice, and thus, its application for the benefit of society is possible.