Topic: A short review of CRISPR-Cas9

Words: 2308

Name: Mohammed Bilal

1 Summary

In nature, prokaryotes such as Archaea have developed a proficient defence system called CRISPR-Cas9, that protect against invading bacteriophages, based on CRISPR spacer content, and the action of an enzymatic Cas9 endonuclease (Barrangou et al, 2007). Scientists have repurposed CRISPR-Cas9 into a formidable instrument for gene-editing that allows investigators to tinker with DNA sequences and to adjust components of a genome (Memi et al, 2018). CRISPR-Cas9 engineering allows precise modifications of a target sequence of DNA in a genome (Charlesworth, 2019). Already, CRISPR-Cas9 can activate or silence clusters of genes, as has been shown in genetically modified mice (Liu et al, 2016). This technology will edit DNA through somatic cells (body tissues) and introduce new DNA into an organism germline, however, germline editing has dramatically divided opinions (Cribbs and Perera et al, 2017). As research on CRISPR-Cas9 continues to develop and clinical application intensify, unresolved procedural hurdles and ethical concerns need to be addressed before CRISPR-Cas9 applications becomes routine. This review will discuss the emergence of this technology and will focus on its clinical application including its use cases, ethical implications associated with CRISPR-Cas9 and any drawbacks that the technology may possess.

* 1. What is CRISPR-Cas9?

CRISPR are clusters of regularly interspaced short palindromic repeats, broken up by collections of intergenic spacer regions. Spacers are regions of non-coding DNA between genes which represent fragments of defective DNA from former intruders, like bacteriophages (Zhang et al, 2015). Intergenic regions can be transcribed into small sequences of CRISPR RNA (crRNA) and transport endonuclease (Cas9) to sever any viral DNA (Barrangou et al, 2007). CRISPR-linked proteins, mainly Cas9, originate from an inherited natural defence system of prokaryotes which provide memory on former viral infections (bacteriophages) so that they can defend against re-infection (Barrangou et al, 2007). CRISPR is passed on to the following generation of bacteria, creating a future colony that is resistant to the eliminated viral infection. To use this kind of technology, scientists must first pinpoint the position of the gene triggering a fitness concern. They must then generate the precise single-guide RNA (sgRNA/gRNA) that can identify the sequence of nucleotide bases in the DNA. The sgRNA attaches to a Cas9 endonuclease and this compound will be inserted into the target area. This complex will cut DNA once the target sequence is located, and at this point, scientists can modify by either inserting a new sequence or deleting a sequence. This technique transforms CRISPR-Cas9 into a cut and paste instrument for gene therapy. Someday this type of technology will be used in life-saving developments and improvements in treatments of hereditary diseases. CRISPR-Cas9 will be the genetic scissors that “tailor” the human gene pool.

1.2 Mechanism of Action

CRISPR consist of two parts, first, a Cas9 endonuclease that “eat” DNA and second, a short non-coding guide-RNA, that are solitary assembled chimerical RNA which recognise sequences of DNA, to be edited. This gRNA has two further sub-components: (1) a short CRISPR RNA (denoted as crRNA) also (2) a trans-encoded helper RNA (denoted as tracrRNA). CrRNA are 18-20bp sequences that can bind to genomic loci (Pattanayak et al, 2013). A tracrRNA operates as a scaffold for the crRNA-Cas9 interaction. In this circumstance, gRNA forms a binary molecule, with the crRNA and tracrRNA (represented as cr:tracrRNA). This molecule carries the Cas9 endonuclease to a genomic region via base-pairing amongst crRNA, and the target sequence (Pattanayak et al, 2013). Targeted DNA regions, also known as protospacers, contain a five prime protospacer adjacent motif (PAM) region that should match to a gRNA molecule (Marraffini and Sontheimer, 2010). PAM regions help to differentiate amongst self-sequences and invasive sequences (Marraffini and Sontheimer, 2010). Bound to the target sequence, Cas9 can generate DSBs in a large range of genomes (Jinek et al, 2012). DSBs are rebuilt by restoration pathways, through two different processes. Initial process of repair is non-homologous end-joining (NHEJ) of DNA. This chief pathway results in random deletions (indels) and insertions in the mending of the DNA (Rudin et al, 1989). Indel formation results in early stop codons, frameshift mutations and non-sense mediated decay (Zhan et al, 2019). If the experimental objective is to dislocate a gene so no functional protein is made (i.e. a knock-out) then the NHEJ mechanism can be exploited. The next mechanism of restoration is called homology-directed repair (HDR). This results in accurate nucleotide rewrites since the break is mended through endogenous/exogenous DNA repair templates (Hsu et al, 2014). If the experimental objective is to replace the targeted genetic component with a different component (i.e. a knock-in), the cell can be fixed towards the HDR pathway. HDR has many re-writing applications from introducing point mutations to inserting markers. HDR requires additional refinement, researchers previously used this technique to fix a variation, triggering cataracts in mice (Wu et al, 2013) demonstrating proof of concept. CRISPR-Cas9 can assist clinical application through the knock-out of target genes by NHEJ or integration of a corrected sequence into a gene through HDR (Charlesworth et al, 2019).

* 1. Limitations of CRISPR-Cas9

Despite low cost and superior efficacy of CRISPR-Cas9, compared to other nucleases (Micrococcal Nuclease’s, Transcription Activator-Like Effector Nucleases and Zink Finger Nucleases) limitations still do exist (Memi et al, 2018). Spurious, off-target double-strand breaks represent a barrier that impedes CRISPR-Cas9’s use for therapeutic applications (Newton et al, 2019), data in this paper demonstrated that mechanically altering DNA caused off-target indels at positions across DNA. The authors proposed that the destabilisation of DNA in fundamental processes (like DNA replication, transcription, etc.) are bound to unmask ambiguous off-target indel positions to Cas9 activity, emphasising a requirement for better off-target algorithmic programs for precise predictions. Efforts have been made to truncate the sgRNA sequence to improve the selectivity of sgRNA, without affecting on-target delivery, however consistent results have not been produced (Rusk, 2014). Computational approaches for spotting off-target locations like the programme ‘Digenome-seq’ facilitate discovery of atypical and harmful off-target mutations before pushing towards cell therapy (Zhang et al, 2015). Digenome-seq was described as an unbiased, sensitive and valued approach in detecting off-target consequences of RNA guided endonuclease, along with Cas9 (Kim et al, 2015).

An impairment with CRISPR-Cas9 technology is an inadequacy at restoring double-stranded breaks using HDR in human cells (Chu et al, 2015). This causes high numbers of stochastic (random) editing and mosaicism - cells that are composed of two or more different genetic constitutions (Hershlag and Birstow, 2018). However, silencing of NHEJ vital enzyme ‘DNA ligase IV’, required in preventing apoptosis and mutagenesis, is an effectual method to engineer intended mutations in DNA of mammalian cells and improve HDR efficiency in CRISPR-Cas9 applications (Chu et al, 2015).

A study published by Charlesworth et al (2019) exhibited presence of a pre-existing adaptive immune response in humans that may affect targeted use of CRISPR-Cas9, or trigger harmfulness after inoculating patients with CRISPR-Cas9. For example, if a cytotoxic T lymphocyte was to respond against a foreign Cas9 protein, the consequence would be total obliteration of all cells displaying the exact Cas9 polypeptide at the major histocompatibility complex, eradicating the modified cells leaving the treatment inefficient (Hewitt, 2003). The paper states that an immune response can be a blockade for safe or effective gene therapy. Potential solutions to this include depleting or suppressing the immune system, to avert a cell facilitated response to Cas9. Supplementary Cas9 orthologs from non-infectious bacterial species can be used to bypass the adaptive immune response (Charlesworth et al, 2019).

There is also an abundance of ethical implications to consider when using gene-editing technology such as CRISPR-Cas9. Some ethical questions regarding the application of CRISPR include: “How does the concept of designed babies using CRISPR, go against religious and moral beliefs?”, “Who will have access to genome editing using CRISPR/Cas9?”, “Will the technology be equally accessible on all continents?” and “How will gene-editing technologies further widen social disparities and inequality?” E.g. could we see meta-humans with enhanced intellectual capacity or physical appearance, causing individuals to have advantages over others. There is fear around the safeness of gene-editing technology such as CRISPR-Cas9. The technology holds significant risk toward individuals, key safety concerns like the number of off-target mutations, mosaicism, stochastic editing and epigenetic effects are all questions to consider before CRISPR can be practised (Dai et al, 2016).

The introduction of genetically modified species may result in ecological disequilibrium in the ecosystem, due to selective advantages over the wild type (Yunta, E, 2016). Gene drives are genetic engineering technology, that allow biased inheritance of genetic traits so that progeny have a greater chance of inheriting a presented trait. In 2015, gene drives were fashioned in Drosophila melanogaster, capable of driving an alteration in 97% of progeny in two generations. A guide RNA, a Cas9 transgene and a set of homology arms (on both ends of the Cas9-transgene-gRNA cassettes) were inserted into the target locus, producing a loss-of-function mutation, the paper refers to the process as a “mutagenic chain reaction” (Gantz and Bier, 2015). Implanted cassette expressed Cas9-gRNA molecule leading to cleavage and HDR mediated insertions towards the next allele, creating a homozygous transformation (Gantz and Bier, 2015). This paper shows gene drives have the power to spread mutation to the next generation which can include negative traits to associated species.

Another ethical consideration with the CRISPR-Cas9 technology its use for military or terrorist applications. The prospect of CRISPR-Cas9 to manipulate pathogens to make them even more potent, is dangerous for human health, to say the least. The editing of the H5N1 strain of influenza is an example of how scientists increased the potency, transmissibility and virulence factor of this virus, further increasing the threat of pandemic influenza (Herfst et al, 2012). CRISPR-Cas9 raises concerns that somebody with suitable apparatus and experience could re-engineer invasive viruses/species. To overcome these issues community interactions as well as deep ethical thought is required for good decision making for the future of CRISPR. Also, risk assessments must be heightened to ensure effective regulations and anticipatory measures must be taken to oversee sites that use CRISPR technology (Yunta, E, 2016).

1.4 Clinical Trials

Gene therapy transfer have been performed since the '80s, largely ineffective due to problems associated with cell-mediated responses, mutagenesis and viral vectors (Wirth et al, 2013). The primary approved clinical trial was launched in 1990 applying retroviral-facilitated transmission of adenosine deaminase into lymphocytes of females, both sufferers of severe combined immunodeficiency (SCID). As a result of the trial, the total quantity of T-cells from both females normalised, so did several humoral responses (Blease et al, 1995). The research symbolised the first approved scientific trial to insert extrinsic genes into human beings. Furthermore, it also reported on the safety of *in vivo* retroviral-mediated gene transfer to treat inherited disorders.

Initial phase one clinical trial using CRISPR-Cas9 took place on the 28th of October 2016 by oncologist LuYou, at the Sichuan University in China (National Clinic Trial number: NCT02793856). LuYou and his team delivered ‘PD-1’ (programmed death 1) KO engineered T-cells in a patient with metastatic lung malignancy which had advanced despite ordinary treatments failing. PD-1 receptors are located on T-cell surfaces and are responsible for T-cell activation (Zhan et al, 2019). Delivery of knock-out PD-1 was through peripheral blood lymphocytes, which had been taken out and through *ex vivo* gene therapy, PD-1 was knocked out and transfused back into the patient. This trial is proof of concept, implementing *ex-vivo* CRISPR KOs in treatments of cancers (Zhan et al, 2019).

An FDA approved *ex vivo* immunotherapy, comprised of CRISPR-Cas9 derived chimerical antigen receptor (CARs) are being employed to cure acute lymphoblastic leukaemia in mouse models (Eyquem et al, 2017). CAR-T is immunotherapy which reprograms a patient's lymphocytes to facilitate tumour rejection. Recently, gene transfer of CD19 CARs to TRAC (T-cell receptor α chain) fashioned T-lymphocytes of hugely boosted tumour rejection, compared to traditional CAR T therapy using arbitrarily assimilating vectors in treatment of acute lymphoblastic leukaemia (Eyquem et al, 2017).

Contrastingly, *in vivo* gene treatment, by means of CRISPR-Cas9 is less common compared to *ex vivo*, this is due to the obstacles, such as off-target effects, efficiency of HDR, proficiency/fitness of corrected cells, immunogenicity of CRISPR apparatuses and translatability of delivery vehicles (Dai et al, 2016). To date, there is a single PHASE 1 *in vivo* registered clinical trial (not yet enlisting patients) that plans to use CRISPR/Cas9 and TALENS (Transcription Activator-Like Effector Nucleases – manufactured restriction enzymes) to target human papillomavirus: HPV16 and HPV18. CRISPR/Cas9 and TALEN will be delivered by a gel that is applied to the human papillomavirus infected cervix (National Clinic Trial Number: NCT03057912).

In the Second International Summit on Human Genome Editing, a conference took place that discussed ethics of gene editing via germline engineering. Members concluded that altering the germline, at this time, for permanent and inheritable changes was "irresponsible" as risks are too great to warrant clinical trials within the germline (National Academies of Sciences, Engineering, and Medicine, 2019).

1.5 The Future of Genetic Engineering

The future of CRISPR-Cas9 practices rest on cunning expertise to construct Cas9 modifications with no off-target mutations, or minimal effects with safer approaches to expand genetic changes by HDR. Additional developments in viral-related delivery methods will be essential to advance *in vivo* gene therapy using CRISPR (Zhan et al, 2019). Of note, a team at Harvard University, spearheaded by geneticist Dr. George Church, are currently using CRISPR-Cas technology to cut and paste DNA from fully sequenced extinct Woolly Mammoth genomes, into living Asian Elephant’s fibroblast cell, in the hope to generate Mammoth like characteristics, and re-introduce the extinct species back into the ecosystem. Mammoth like transformations have already been engineered into these cells such as thicker hair, subcutaneous fat and altered mammoth cold-resistant haemoglobin. From an ethical standpoint, this is interesting because the research can be seen as being ethically dubious, however, the fact that modifications are not being made to a human being, makes it tolerable or acceptable?

Reference

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A. and Horvath, P. (2007) CRISPR Provides Acquired Resistance against Viruses in Prokaryotes. *Science* 315 (5819), 1709-1712.

Blaese, R. M., Culver, K. W., Miller, A. D., Carter, C. S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., Greenblatt, J. J., Rosenberg, S. A., Klein, H., Berger, M., Mullen, C. A., Ramsey, W. J., Muul, L., Morgan, R. A. and Anderson, W. F. (1995) T Lymphocyte-Directed Gene Therapy for ADA$^-$ SCID: Initial Trial Results After 4 Years. *Science* 270 (5235), 475-480.

Charlesworth, C. T., Deshpande, P. S., Dever, D. P., Camarena, J., Lemgart, V. T., Cromer, M. K., Vakulskas, C. A., Collingwood, M. A., Zhang, L., Bode, N. M., Behlke, M. A., Dejene, B., Cieniewicz, B., Romano, R., Lesch, B. J., Gomez-Ospina, N., Mantri, S., Pavel-Dinu, M., Weinberg, K. I. and Porteus, M. H. (2019) Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nature Medicine* 25 (2), 249-254.

Chu, V. T., Weber, T., Wefers, B., Wurst, W., Sander, S., Rajewsky, K. and Kühn, R. (2015) Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nature Biotechnology* 33, 543.

Cribbs, A. P. and Perera, S. M. W. (2017) Science and Bioethics of CRISPR-Cas9 Gene Editing: An Analysis Towards Separating Facts and Fiction. *The Yale journal of biology and medicine* 90 (4), 625-634.

Dai, W.-J., Zhu, L.-Y., Yan, Z.-Y., Xu, Y., Wang, Q.-L. and Lu, X.-J. (2016) CRISPR-Cas9 for in vivo Gene Therapy: Promise and Hurdles. *Molecular Therapy - Nucleic Acids* 5 (8), e349.

Eyquem, J., Mansilla-Soto, J., Giavridis, T., van der Stegen, S. J. C., Hamieh, M., Cunanan, K. M., Odak, A., Gönen, M. and Sadelain, M. (2017) Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* 543, 113.

Gantz, V. M. and Bier, E. (2015) Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science (New York, N.Y.)* 348 (6233), 442-444.

Herfst, S., Schrauwen, E. J. A., Linster, M., Chutinimitkul, S., de Wit, E., Munster, V. J., Sorrell, E. M., Bestebroer, T. M., Burke, D. F., Smith, D. J., Rimmelzwaan, G. F., Osterhaus, A. D. M. E. and Fouchier, R. A. M. (2012) Airborne Transmission of Influenza A/H5N1 Virus Between Ferrets. *Science* 336 (6088), 1534.

Hershlag, A. and Bristow, S. L. (2018) Editing the human genome: where ART and science intersect. *Journal of assisted reproduction and genetics* 35 (8), 1367-1370.

Hewitt, E. W. (2003) The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology* 110 (2), 163-169.

Hsu, Patrick D., Lander, Eric S. and Zhang, F. (2014) Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell* 157 (6), 1262-1278.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., Charpentier, E., Medicinska, f., Institutionen för, m., Molekylär Infektionsmedicin, S., Umeå Centre for Microbial, R. and Umeå, u. (2012) A Programmable Dual-RNA—Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* 337 (6096), 816-821.

Kim, D., Bae, S., Park, J., Kim, E., Kim, S., Yu, H. R., Hwang, J., Kim, J.-I. and Kim, J.-S. (2015) Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nature Methods* 12, 237.

Liu, X. S., Wu, H., Ji, X., Stelzer, Y., Wu, X., Czauderna, S., Shu, J., Dadon, D., Young, R. A. and Jaenisch, R. (2016) Editing DNA Methylation in the Mammalian Genome. *Cell* 167 (1), 233-247.e17.

Marraffini, L. A. and Sontheimer, E. J. (2010) Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* 463 (7280), 568-571.

Memi, F., Ntokou, A. and Papangeli, I. (2018) CRISPR/Cas9 gene-editing: Research technologies, clinical applications and ethical considerations. *Seminars in Perinatology* 42 (8), 487-500.

National Academies of Sciences, E. a. M. (2019) *Second International Summit on Human Genome Editing: Continuing the Global Discussion: Proceedings of a Workshop–in Brief.* Washington, DC: The National Academies Press.

Newton, M. D., Taylor, B. J., Driessen, R. P. C., Roos, L., Cvetesic, N., Allyjaun, S., Lenhard, B., Cuomo, M. E. and Rueda, D. S. (2019) DNA stretching induces Cas9 off-target activity. *Nature Structural & Molecular Biology* 26 (3), 185-192.

Pattanayak, V., Lin, S., Guilinger, J. P., Ma, E., Doudna, J. A. and Liu, D. R. (2013) High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature Biotechnology* 31 (9), 839-843.

Rudin, N., Sugarman, E. and Haber, J. E. (1989) Genetic and Physical Analysis of Double-Strand Break Repair and Recombination in Saccharomyces cerevisiae. *Genetics* 122 (3), 519-534.

Rusk, N. (2014) Next-generation CRISPRs. *Nature Methods* 12, 36.

Wirth, T., Parker, N. and Ylä-Herttuala, S. (2013) History of gene therapy. *Gene* 525 (2), 162-169.

Wu, Y., Liang, D., Wang, Y., Bai, M., Tang, W., Bao, S., Yan, Z., Li, D. and Li, J. (2013) Correction of a Genetic Disease in Mouse via Use of CRISPR-Cas9. *Cell Stem Cell* 13 (6), 659-662.

Yunta, E. (2016) Ethical Issues in Genome Editing using Crispr/Cas9 System. *Journal of Clinical Research & Bioethics* 07.

Zhan, T., Rindtorff, N., Betge, J., Ebert, M. P. and Boutros, M. (2019) CRISPR/Cas9 for cancer research and therapy. *Seminars in Cancer Biology* 55, 106-119.

Zhang, X.-H., Tee, L. Y., Wang, X.-G., Huang, Q.-S. and Yang, S.-H. (2015) Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Molecular therapy. Nucleic acids* 4 (11), e264-e264.