CRISPR/Cas9 as an *in-vivo* gene editing technology for human genetic disease

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CRISPR/Cas9 is a gene editing technology derived from a bacterial defence mechanism to fight viral infection. It uses a small RNA as a guide to bring the endonuclease Cas9 to a specific location within the genome, where once it has matched the target DNA sequence, Cas9 induces a double strand break (DSB) within the target and the sequence can be altered. This DSB triggers homology directed repair mechanisms (HDR) to correct the mutation using a donor template. This can be used to correct mutations which are responsible for genetic disease. Many studies have been performed to look at the finer details of this mechanism and to improve on the accuracy in an effort to reduce off-target effects. Various methods to get CRISPR/Cas9 inside cells have been devised and these range from physical to viral vector-based delivery methods. The emergence of such a promising gene editing technology has encountered multiple ethical issues with a lot of attention being put on the protest against inheritable germline mutations and the impact that gene editing will have on the process of evolution. It is safe to say that much more work is required to improve precision and reduce off-target effects before CRISPR/Cas9 is used as an *in-vivo* therapeutic for genetic disease in humans, but when it has been approved for this type of use, it will revolutionise the treatment of genetic diseases, improving the lives of many people.

Mechanism of CRISPR/Cas9

Genetic disorders are caused by mutations in an individual's DNA. These mutations can be at the level of the DNA sequence in the form of single nucleotide polymorphisms (SNPs) or at the level of the chromosome. This review will focus on single gene mutations which cause genetic disease in humans and how they can potentially be corrected with gene editing technology using the CRISPR/Cas9 system. This system is derived from the natural CRISPR/Cas system used in bacterial cells to protect themselves from viral infection (Doudna & Charpentier, 2014). CRISPR is an acronym for clustered regularly interspaced short palindromic repeats and Cas9 is a CRISPR-associated endonuclease (Hsu, Lander & Zhang, 2014). The process makes use of a small RNA molecule, around 20 nucleotides in length, called a single guide RNA (sgRNA) to direct the Cas9 protein to the target sequence in the DNA, this interaction is shown in Figure 1. The sgRNA binds to the target DNA sequence through Watson-Crick base pairing where Cas9 can create a double strand break (DSB) in a precise location (Doudna & Charpentier, 2014; Hsu, Lander & Zhang, 2014). The presence of DSBs triggers the action of DNA repair mechanisms using either error-free homology-directed repair (HDR) or the error-prone process of non-homologous end joining (NHEJ) (Hsu & Zhang, 2014; Chu *et al*., 2015). This can be applied to genetic disease by artificially constructing a sgRNA that is complementary to a disease-causing mutation. Using CRISPR/Cas9, the mutation can be corrected with HDR to incorporate a new DNA sequence which contains the wild-type allele, usually from a donor template (Chu *et al*., 2015). The idea of using CRISPR/Cas9 technology in humans to treat genetic diseases has caused many debates concerning the ethical issues. At present, applications of the CRISPR/Cas9 system have only gone as far as using human cells *in-vitro* with a small number being used *in-vivo*. This article will review the literature surrounding the progression to *in-vivo* use of CRISPR/Cas9 in humans, how scientists intend to make it safer and the ethical issues that must be tackled.

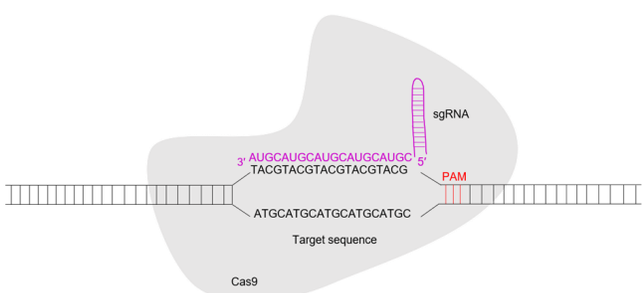

Figure 1. CRISPR/Cas9 System. A simplified diagram of the interaction between the sgRNA, in purple, with the target DNA sequence, in black, and the protospacer adjacent motif, in red, present after the 5′ region of the sgRNA. The Cas9 protein is shown in grey and facilitates this interaction and the DSB which ultimately occurs when there is a match. Adapted from Zhang *et al*., 2015.

How can we get CRISPR/Cas9 into human cells?

There are different methods for delivery of CRISPR/Cas9 into the host cells. The individual components of the process can also be delivered in a range of ways. Both the Cas9 sequence and the sgRNA can be integrated as part of a DNA vector which holds the genetic information for them to be transcribed and, in the case of Cas9, translated. They can also both be delivered as RNA with Cas9 mRNA and sgRNA, or alternatively, in a ribonucleoprotein complex, with Cas9 being delivered as a protein together with the sgRNA (Liu *et al*., 2017; Lino *et al*., 2018). All three options have advantages and disadvantages, and this allows them all to have their specific applications. For instance, using a DNA vector is

more stable than using mRNA but the DNA vector exists longer in the cells than mRNA, potentially allowing for more negative, off-target effects (Liu *et al*., 2017). Arguably, the most efficient method of delivering CRISPR/Cas9 is through the physical process of microinjection (Yang *et al*., 2013). Other delivery methods more suited to *in-vivo* applications include viral vectors, such as adeno-associated virus vectors (AAV), adenovirus or lentivirus vectors, and non-viral vectors, such as lipid nanoparticles (Lino *et al*., 2018). The advantages and disadvantages of each of these methods are outlined in Table 1.

Delivery method	Advantages	Disadvantages
Adeno-associated virus vector	Low toxicity in host. Many serotypes for wide application.	Can integrate into host genome.
Lentivirus vector	Efficient at infecting cells	Can integrate into host genome. Cannot control quantity of
Adenovirus vector	Does not integrate into host genome. Many serotypes for wide application.	High immunogenicity. Cannot control quantity of
Microinjection	Extremely efficient. Reliable to get a known quantity into	Targets one cell at a time. Limited *in-vivo* application.
Lipid nanoparticles	Protects DNA/RNA from degradation. Easy entry to the cell.	Easily broken down by endosome. No direct delivery to the nucleus.

Table 1. A brief description of the advantages and disadvantages of the different delivery methods to introduce CRISPR/Cas9 into host cells. Information was obtained and adapted from (Liu *et al*., 2017; Lino *et al*., 2018).

When using a gene editing technology such as CRISPR/Cas9 to treat genetic disease in humans, it is important to consider methods of targeting the correct cells, especially when the disease is not systemic. For example, junctional epidermolysis bullosa (JEB) is a genetic skin disease that mainly occurs due to mutations in the laminin 332 protein which plays a vital role in adhesion of the epidermis and the dermis through the basement membrane (Benati *et al*., 2018). To treat this disease, the laminin mutations specifically in defective skin cells must be edited, and not the cells in the rest of the body. This is proving to be a considerable obstacle in the process of applying CRISPR/Cas9 systems to targeted cell types both *in-vitro* and *in-vivo*. One promising way to target cells is by adding selective components into the vector construct which cause it to be expressed only in the correct conditions, particularly in the presence of a molecule or mutation which only occurs in the affected cells and not any others. This was demonstrated in the treatment of a fusion oncogene in a human Ewing sarcoma cell line where the CRISPR/Cas9 DSBs are only induced when a detrimental chromosome rearrangement has occurred, bringing two genes into close proximity to form a fusion oncogene (Martinez-Lage *et al*., 2020). This allows for CRISPR/Cas9 targeted gene editing in specific cell types which contain the mutation of interest while remaining inactive in the wild-type cells. This could be applied to the example of JEB where the DNA vector construct only becomes active in keratinocytes that contain a skin-specific promoter.

How can off-target effects be minimised?

One of the main concerns with using CRISPR/Cas9 is the inaccuracy of the sgRNA to bind only to the target sequence and not anywhere outside this region to other similar sequences with high homology. These are known as off-target effects and have been investigated extensively. An additional component of the CRISPR/Cas9 system as yet unmentioned is a short nucleotide sequence which comes after the sgRNA target and is known as a protospacer adjacent motif (PAM) (Zhang *et al*., 2015) which is shown in Figure 1. A well documented Cas9 protein is that of SpCas9, derived from bacterial species Streptococcus pyogenes and the PAM sequence for this variant is NGG, but this sequence varies between each species of Cas9 (Hsu, Lander & Zhang, 2014). It has been proposed that the Cas9 protein first recognises the PAM sequence and then identifies whether or not the preceding sequence matches the sgRNA (Szczelkun *et al*., 2014), indicating that the PAM plays a very important role in CRISPR/Cas9 binding to the correct target sequence. Sequence mis-matches between the sgRNA and the target DNA are better tolerated when they are closer to the PAM, which means any deviations from the 3′ end of the sgRNA could lead to a DSB being made in the wrong place in the genome (Zhang *et al*., 2015).

One method which has been used to reduce the off-target effects of CRISPR/Cas9 is to use a Cas9 protein which induces a single stranded break (SSB) rather than a DSB at the target sequence. These are known as nickase enzymes and would require two to work at the same time, generating a SSB on both the forward and the reverse strands containing the target sequence (Lino *et al*., 2018). Using co-operative nickase forms of Cas9 would still generate the DSB needed for HR to correct the mutation, but any off-target binding, and strand breakage would only cause a SSB which is much less detrimental, and easier to resolve, than off-target DSBs (Lino *et al*., 2018). Using genome databases, it is possible to identify off-target sequences of a sgRNA and predict where they may occur (Zhang *et al*., 2015). In this case, it may be possible one day to determine the consequences of such off-target effects and evaluate the impact they may have on an individual. It is plausible that some off-target effects may be much less drastic than the symptoms of a genetic disease such as severe JEB, and therefore this would possibly alleviate the disease.

Ethical Concerns

The advancement of gene editing technologies has been met with many concerns about the moralities and practicalities surrounding application in humans. One of the major worries is that a vector used to infect the cells with the CRISPR/Cas9 components will incorporate into the host genome and cause adverse effects, including the activation of oncogenes. This is a reasonable concern and viral vectors, such as AAV can have these kind of side effects but it has also been found that adenovirus vectors

do not merge into the host genome (Lino *et al*., 2018) which is one way around this problem. Another major ethical concern is that of progression to producing germline modifications that become inheritable and the view that this is interfering with the process of evolution. For this, it should be noted that there are strict regulations in place in many countries, including the UK, against the use of genetically modified embryos or gametes for any reproductive purposes (Reyes & Lanner, 2017).

Conclusion

In conclusion, CRISPR/Cas9 is a revolutionary gene editing technique with the potential to change the course of treatment for many genetic diseases. Before CRISPR/Cas9 can be introduced to human trials on a wider scale there are improvements that must be made to increase the safety and efficiency of the process. Off-target effects are still too frequent and unpredictable to make this a feasible *in-vivo* technique to treat human genetic disease. Here, some of the methods scientists have developed in an effort to make CRISPR/Cas9 a more effective and precise method of gene editing have been outlined. While already being used in laboratories all over the world for *in-vitro* experiments, once improved, the process of CRISPR/Cas9 is likely to become much more common as part of *in-vivo* gene editing used for therapeutics of genetic disease. If this happens, scientists will have to stick to strict regulations in order to make sure the applications remain controlled without any disastrous consequences.

References

Benati, D., Miselli, F., Cocchiarella, F., *et al.* 2018. CRISPR/Cas9-Mediated *In Situ* Correction of LAMB3 Gene in Keratinocytes Derived from a Junctional Epidermolysis Bullosa Patient. *Molecular Therapy*, 26(11), 2592-2603.

Chu, V. T., Weber, T., Wefers, B., *et al.* 2015. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nature Biotechnology*, 33, 543-548.

Doudna, J. & Charpentier, E. 2014. The new frontier of genome engineering with CRISPR-Cas9. *Science,* 346(6213), 1077.

Hsu, P., Lander, E. S., & Zhang, F. 2014. Development and applications of CRISPR-Cas9 for genome engineering. *Cell,* 157(6), 1262-1278.

Lino, C., Harper, J. C., Carney, J. P. & Timlin, J. A. 2018. Delivering CRISPR: a review of the challenges and approaches. *Drug Delivery*, 25 (1), 1234-1257.

Liu, C., Zhang, L., Liu, H. & Cheng, K. 2017. Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. *Journal of Controlled Release*, 266, 17-26.

Martinez-Lage, M., Torres_Ruiz, R., Puig-Serra, P., *et al.* 2020. *In vivo* CRISPR/Cas9 targeting of fusion oncogenes for selective elimination of cancer cells. *Nature Communications*, 11.

Plaza Reyes, A. & Lanner, F. 2017. Towards a CRISPR view of early human development: applications, limitations and ethical concerns of genome editing in human embryos. *Development*, 144(1), 3-7.

Szczelkun, M. D., Tikhomirova, M. S., Sinkunas, T. *et al.* 2014. Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. *PNAS*, 111(27), 9798-9803.

Yang, H., Want, H., Shivalila, C. S., *et al.* 2013. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell*, 154(6), 1370-1379.

Zhang, X.-H., Tee, L. Y., Wang, X.-G., Huang, Q.-S. & Yang, S.-H. 2015. Off -target Effects in CRISPR/Cas9-mediated Genome Engineering. *Molecular Therapy Nucleic Acids*, 4(11), e254.