CRISPR vs Prime Editing

Is it the end of an era for CRISPR-mediated genetic manipulation?

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In the era of genomics, it is almost certain we will be editing our genomes in the near future, but the question is how? And what are the consequences? Knowledge of human genetics has expanded rapidly since the complete human genome was published in 2003 (1), leading to increased interest and investment in further research. One of the most astonishing achievements in genetic research was the understanding and use of the CRISPR/Cas9 system to edit DNA sequences. However, experiments using this technique encountered many downfalls including unpredicted changes to random, untargeted DNA sequence, including the introduction of stop codons (2). Several improvements have been made to CRISPR/Cas9 over time but a new candidate technique, prime editing, published in October 2019 (3) could steal the spotlight. Prime editing claims to be a more precise gene editing technique, improving on some of CRISPR/Cas9's limitations and having a safer mechanism of action. This review deciphers the mechanisms of both CRISPR/Cas9 and prime editing. The review aims to broaden the understanding of how these gene editing techniques work and why they can be subjected to off target effects. Upon review, prime editing is less error prone, most notably with the elimination of double-strand breaks (DSBs). Though, CRISPR/Cas9 has its limitations, it is still more reliable than prime editing, primarily due to the strong evidence-based research collected over time. With the same attention and experimentation, prime editing can certainly surpass the expectations of CRISPR/Cas9 in performing small, precise edits. In conclusion, CRISPR/Cas9 could be useful for larger, less precise edits to the genome. Therefore, although there are major implications on scientific advancement, the system could be adapted and utilised more in an experimental setting using model organisms. Whereas prime editing appears to be the most suitable genome editing method for use in the human population, surpassing the accuracy capabilities of CRISPR/Cas9.

Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) along with the enzyme Cas9 were originally found to be a natural defence mechanism in bacteria. The use of CRISPR and its associated Cas9 were found to be a powerful tool for editing genomes in 2012 (4). Since, CRISPR/Cas9 has been subject to a major upheaval in scientific research, particularly biomedical, where it has been shown to cure mice of genetic disorders (5). This included correcting common cystic fibrosis (CF) causing mutations leading to restored function of the CF transmembrane conductor receptor (5). Although more recently, it has been found that despite CRISPR/Cas9 being more precise than past gene editing techniques, so called 'off-target effects' are becoming an increasingly acknowledged issue (2). Other current limitations that have slowed clinical development of CRISPR/Cas9 in its tracks include; activation of the immune system, lack of targeting specific tissues or cells and the inability to enter cells in the first place (6). While the development of CRISPR/Cas9 has potential applications for eradicating genetic disease, it is questionable whether it can be approved for human use unless these impediments are addressed. These problems have led to research into alternative, more precise, gene editing techniques with more potential for human application. In October 2019, an article was published detailing a new kind of genome editing named 'prime editing' (3). The creators of prime editing claim that it could correct up to 89% of known genetic faults associated with human diseases, and can work similarly to CRISPR/Cas9, but with fewer detrimental effects (7). This review aims to evaluate both CRISPR/Cas9 and prime editing to determine which provides a safer and more accurate approach to gene editing. The discussion aims to reflect on which method has the most potential for continued development and research when considering biomedical applications.

The history of CRISPR/Cas9

Unexpected repetitive sequences later termed CRISPR identified in many bacteria and archaea were found to be strikingly similar to those in bacteriophages. First discovered in the 90's, the sequences were named CRISPR by the founder Francisco Mojica and colleagues in 2002, when CRISPR was first mentioned in scientific literature (8). The similarity to bacteriophage sequences led to the understanding that CRISPR were part of an immune response to past viral attacks and served to identify and destroy the DNA of similar invaders (9). After a viral attack, spacers (sections of DNA interspersed along repeat sequences) were found to be incorporated into the CRISPR region. When these spacers were manipulated to include specific viral DNA it was found that there was resistance upon infection with the same virus. These findings confirmed that CRISPR sequences were important for regulating bacterial immunity (4). The specific DNA sequences stored are transcribed into RNA, and when the CRISPR RNA (crRNA) binds to a complementary target

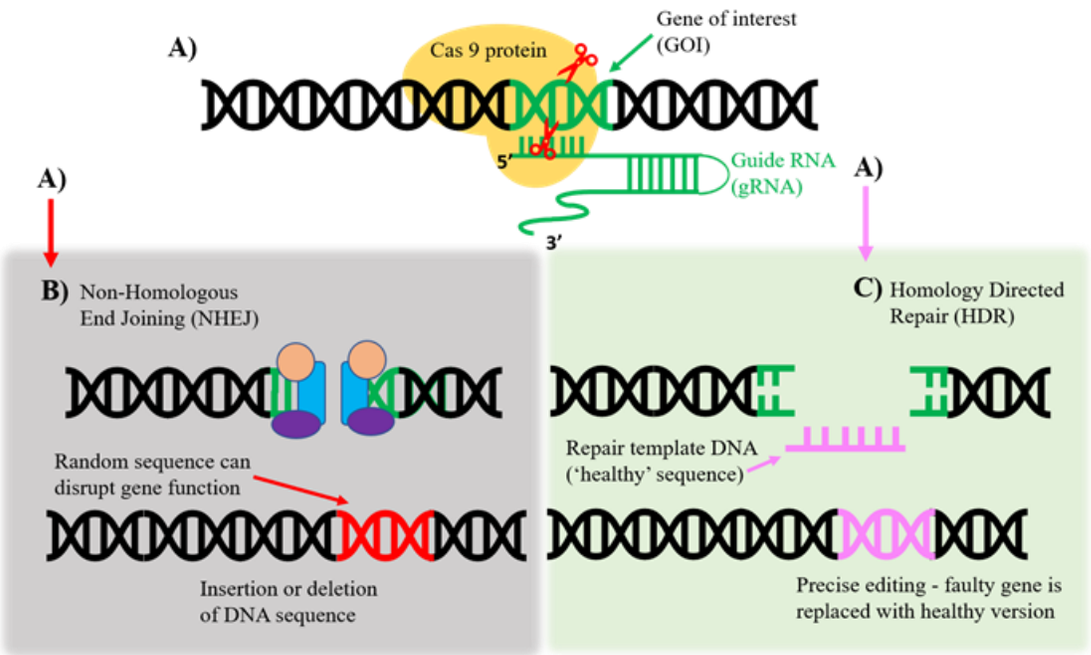

Figure 1. CRISPR/Cas9 mechanism of action. A) The Cas9 protein is guided to the gene of interest (GOI) with the gRNA which is complementary to the target gene. The Cas9 enzyme cuts both DNA strands in the GOI region; B) To disrupt a gene, the DNA is left to repair itself using NHEJ which is an error prone repair mechanism. This can lead to random DNA sequence being inserted or deletion of the DNA sequence, making the gene non-functional; C) If a repair template is added with a desired sequence to insert, HDR can take place. If the repair template DNA is ligated, the other strand can be filled in using this template, leading to a precise insertion of desired sequence.

sequence, the CRISPR system is activated. The system most commonly uses the endonuclease protein Cas9 to cleave the 'recognised invader' sequence (10) as shown in Fig. 1A. Studies in 2012 concluded that Cas9 could be guided to specific regions of DNA if the crRNA was designed to be complementary to the target of choice. The development of a guide RNA (gRNA) through fusing both crRNA and trans-activating crRNA (tracrRNA) could be used along with Cas9 as a two-component gene editing system (Fig. 1) (4).

When a target site is cut by Cas9, two different DNA repair pathways can be initiated, non-homologous end joining (NHEJ) or homology directed repair (HDR). Depending on the desired outcome of the gene edit, both can be utilised (Fig. 1B, 1C) (11). To correct a mutation, a DNA repair template which contains the corrected code can be added along with CRISPR/Cas9 and subsequently utilised in HDR.

Whilst this technique appears infallible, the organisation and function of the mammalian genome is still largely undetermined. Published research using CRISPR/Cas9 has highlighted this gap of knowledge in more ways than one. Schaufer *et al.* (2) compared the genomes of identical mice treated with CRISPR/Cas9 and those untreated (controls). A concerning number of single nucleotide variants (SNVs) across the whole genome were found upon analysis. In particular, 60 SNVs in coding regions of genes induced by CRISPR/Cas9, including one deleterious SNV in the form of a premature stop codon (2). This means that targeting one gene can lead to the knock-out of another gene outside of the targeted region. To reduce this effect the use of bioinformatics tools has proven to be useful. These computational tools use homology of sequences to predict the number off-target sites CRISPR/Cas9 gRNA could potentially interact with (6), essentially acting as a quality control procedure before confirmation and use of the designed gRNA sequence in an experiment. Although this reduces the harmful effects of CRISPR, it does not eliminate them, bringing into question whether there are other off-target effects of CRISPR/Cas9. Later in 2018, Simhadri *et al.* (12) conducted a different study using CRISPR coupled with Cas9 and found that humans could have an immune response to the Cas9 protein. If Cas9 proteins are displayed on the cell's surface and recognised as 'foreign' by CD8+ killer T cells, any Cas9 containing cell would be destroyed by the body as part of a natural defence mechanism (12). Additionally, CRISPR/Cas9 containing cells are traditionally engineered *in vitro* and reintroduced into model organisms due to the complex mechanism of action. Many components need to be intertwined into the DNA sequence of target cells before CRISPR/Cas9 can carry out its function (6). The development of these concerning problems over the years since the introduction of CRISPR has instigated a search for a CRISPR like tool, the most recent of which recycles CRISPR technology.

The New Prime Editing

Introduced by Anzalone *et al.* (3) in October 2019, 'prime editing' serves to edit genomes without the creation of DSBs or use of donor templates. The system uses the Cas9 protein which is altered to 'nick' only a single strand of the helix and is coupled with a new prime editing guide (pegRNA). PegRNA contains a specific RNA template and has a reverse transcriptase (RT) enzyme attached (Fig. 2A) (7). PegRNA is multi-functional, acting as a guide with

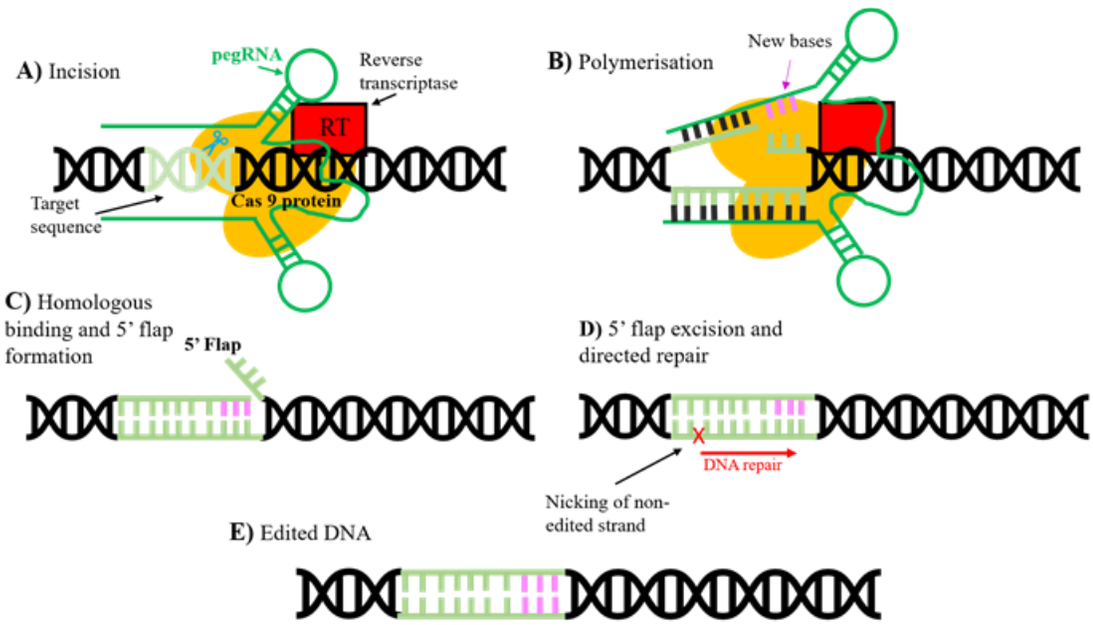

Figure 2. Prime editing mechanism of action. A) The complementary pegRNA with attached reverse transcriptase (RT) guides the Cas9 protein to the target sequence. The Cas9 protein has been adapted to only cut one strand of the DNA; B) Once cut, the broken strand hybridises to the complementary pegRNA and primes reverse transcriptase into action, inserting new desired bases; C) The newly transcribed strand re-anneals to the uncut strand, most frequently leaving a 5' flap as it is preferred during DNA repair using structure specific endonucleases and exonucleases; D) A second nick is introduced into the unedited strand to cause mismatch repair which incorporates the new edit; E) This process results in precise DNA edits being incorporated into both strands of the DNA sequence.

a target DNA sequence and containing an edit-encoding extension to be utilised RT. Cas9 nicks the DNA once at the target site exposing the 3' hydroxyl group which primes RT into action (Fig. 2A, 2B). As 5' flaps are the preferred substrate for structure specific endonucleases, they tend to be created upon hybridization of the strands after synthesis by RT (Fig. 2C) (3). Then a single nick of the non-edited strand biases DNA repair to this strand, favourably incorporating the new bases changes (Fig. 2D, 2E) (3). Using human and mouse cells, the creators of prime editing introduced mutations that cause sickle cell anaemia and Tay-Sachs disease and then corrected them. This resulted in few off-target effects and a high efficacy of successful editing (7). They utilised multiple human cell lines and additionally introduced a mutant allele conferring resistance to prion disease in human and mouse cells. They found that their most efficient variant of prime editor was successful in 53% of cells, with 1.7% indels (3). Indels are random insertions and deletions which are also referred to as 'off target effects'. Prime editing is precise and less error prone due to the accuracy of inserting small numbers of bases, although this methodology does not allow for large insertions and deletions comparable to those that CRISPR/Cas9 can achieve (13). Different pegRNAs and sgRNAs with varying 'nick' locations, along with many RT template lengths can be chosen to increase editing efficiency and specificity. This is an advantage that prime editing has over many other precision gene editing methods (3).

As prime editing is such a novel addition to the scientific community, there is a requirement for validation protocols. This novelty means a lack of research and therefore results to analyse in comparison to CRISPR/Cas9. It has been noted that similarly to CRISPR, delivery into living cells will prove to be difficult due to the complex nature of the machinery (7). Nonetheless, the vigorous analysis carried out by Liu and colleagues suggests that in theory prime editing could correct up to around 89% of the approximately 75,000 known human disease-causing variants (3).

Mechanisms	CRISPR/Cas9	Prime Editing
Targeting apparatus	Guide RNA	PegRNA
Cas9 cutting style	Double strand break	Single strand break
New template source	Homologous repair template DNA free in cell	PegRNA – Edit encoding extension
DNA repair induced	NHEJ/HDR	Mismatch repair with DNA poly-
DNA repair error class	NHEJ = Error prone	Error free
Approximate length of edits	300-1000bp	< 100bp

Table 1. Comparison of CRISPR/Cas9 and prime editing mechanisms (3, 15).

How will Prime Editing overcome CRISPR's limitations?

The most significant triumph for prime editing over CRISPR/Cas9 is the elimination of Cas9 induced DSBs. These DSBs are detrimental to cells and can lead to undesirable outcomes including insertions, deletions and more deleterious translocations (3). The resulting mixture of edits between cells determines CRISPR/Cas9 as a very non-precise editing technology. Even with a repair template it is much more likely that the DSB will incorrectly repair itself than incorporate the new sequence (13). Prime editing overcomes this issue using RT and an edit encoding extension held in place at the site of interest as it is part of the pegRNA. This significantly reduces the chance of faulty repair, along with the resection of only a single strand of DNA. Both strands of DNA are nicked during prime editing, but this is controlled to occur at different times to avoid depending on the cells genome repair system which creates incorrect edits (13).

At this moment in time, a downfall for prime editing is the complexity when it comes to delivering the large pegRNA construct and enzymes into living cells. There is no evidence yet that proves it will be functional in an animal model (7). In comparison, CRISPR/Cas9 has had time on its side with the successful generation of live animals expressing CRISPR/Cas9 in brain tissues. In this case, functioning animal models were created by injecting Cas9 and guide RNA into zygotes to modify the early embryos (14). Though the clear wider application of using CRISPR/Cas9 is shown, it is only a matter of time before prime editing could progress in the same way.

CRISPR/Cas9 edits can be of variable length, allowing large deletions or insertions of template DNA (15). The problem here is the lack of specificity, which prime editing brings, mediating all 12 possible base-to-base conversions and combinations of bases in human cells with no DSBs. Most genetic diseases are caused by insertions, deletions and duplications up to and smaller than 30bp (3). This makes increased size of edit with CRISPR/Cas9 less advantageous in these applications.

What is the future for CRISPR and prime editing?

The comparison of CRISPR/Cas9 and prime editing highlights the journey towards improving gene editing technologies for human application. It can be argued there would be more safety in human gene editing with the use of precise, low off target effect technologies like prime editing. However, it may not be the end of an era for CRISPR/Cas9, which has progressed and improved in many ways since its beginning. Instead of one or the other, these technologies could take different directions with more suited paths determined by their differences. CRISPR/Cas9 is ideal for research into gene expression and disease with the ability to create 'knock-out' organisms, whereas prime editing is an ideal candidate to take over CRISPR's proposed 'human editing' role.

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