Increasing Gene knock-in efficiency and specificity of CRISPR/Cas9 for future applications

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Abstract
The CRISPR-Cas9 gene editing tool has seen widespread use since its development a decade ago. The accurate and simple targeting of the Cas9 nuclease to nearly anywhere in the genome allows for a wide range of gene editing purposes. It currently is reliant on host cell DNA repair pathways to repair the generated double stranded break via either NHEJ or HDR. A reliance that is hindering the potential for further experimental and therapeutic purposes. This review aims to identify and discuss recent improvements that may improve efficiency of CRISPR-Cas9 mediated knock-ins. Several groups have attempted and achieved more efficient ways to deliver the CRISPR-Cas9 system, increased the rate of HDR DNA repair, inhibit NHEJ repair and even develop mostly host cell independent gene editing tools based on Cas9. Most of the discussed improvements require further development but do have the potential to widen the range of applications even further.

Introduction
CRISPR-Cas9, short for Clustered regularly interspaced short palindromic repeats, is a gene editing tool currently in use for a diverse range of applications. CRISPR-Cas9 has shown therapeutic potential as well, with multiple therapeutic trials currently running for both *in*- and *ex vivo* applications (Hirakawa et al., 2020).
The system is adapted from bacteria, where the CRISPR system serves as an adaptive immune response to bacteriophages. The CRISPR genes were discovered by chance in 1987, although their purpose remained unclear for 20 more years (Ishino et al., 1987). In 2007 sequencing of the region revealed that small integrated DNA sequences spaced between the CRISPR repeats were homologous to bacteriophage and plasmid sequences (Barrangou et al., 2007). The sequences were derived from the first exposure to a bacteriophage, and uptake of these sequences provided resistance to a secondary exposure (Gasiunas et al., 2012). The system from *Staphylococcus pyogenes* (spCas9) was adapted for gene editing by Doudna and Charpentier in 2012, who were awarded a Nobel prize for this effort (Jinek et al., 2012).

The CRISPR system relies on the CRISPR repeats and the integrated viral sequences in the CRISPR locus, as well as a multitude of CRISPR associated proteins (Cas). The Cas proteins serve various purposes ranging from viral DNA integration to DNA or RNA Cleaving (Keravala et al., 2006). Transcription of the CRISPR array results in the formation of CRISPR RNA (crRNA), which, together with Cas9 protein and trans-activating crRNA (tracrRNA) can locate and cleave specific DNA sequences. crRNA consists of a 20-nucleotide sequence complementary to the target DNA, while tracrRNA allows interaction between the RNA and protein components resulting in a ribonucleoprotein (RNP) complex (Mali et al., 2013). The RNA components have since been linked together, forming a single guide RNA (sgRNA) (Briner et al., 2014). In addition to this, the sgRNA-Cas9 complex requires a specific protospacer adjacent motif (PAM) downstream in the target DNA, which is *NGG* in the case of the aforementioned spCas9.

Structure of the Cas9 enzyme
The Cas9 enzyme is able to generate a double stranded break (DSB) three nucleotides upstream of the PAM site, by cleaving both target and non-target strands with HNH and RuvC like domains respectively. A study into the molecular structure of Cas9 by Jinek et al. has described that in the inactive state (without sgRNA), Cas9 consists of two lobe structures (figure 1). The REC lobe is responsible for PAM recognition via two loops that interact with the PAM site via a series of tryptophan residues. The NUC lobe contains the RuvC and HNH nuclease domains, responsible for the cleaving of the DNA three base pairs upstream from the PAM site (Figure 1b and c). Both RuvC and HNH domains rely on a metal ion mechanism to catalyze DNA cleavage, where a conserved histidine and aspartate residue, D10 and H840 respectively, play an important role (Jinek et al., 2014; W. Yang, 2008). Mutation of one or both of these residues (D10A and/or H840A) converts Cas9 into a nickase or catalytically dead Cas9 (dCas9) (Jinek et al., 2012). Both of these have since been applied in a range of applications. Furthermore, binding of the crRNA and tracrRNA to the Cas9 enzyme plays a large role in Cas9 activation.

It was shown that interaction of the sgRNA with the Cas9 protein results in substantial conformational changes leading to activation, while the sgRNA-Cas9 complex binding to its target does not result in any major further changes. The conformation change upon sgRNA loading results in the formation of a channel, facilitating the binding of target DNA with the sgRNA (Jinek et al., 2014). After cleaving the targeted strand, the success of the knock-in relies on the DNA repair pathways of the host cell.

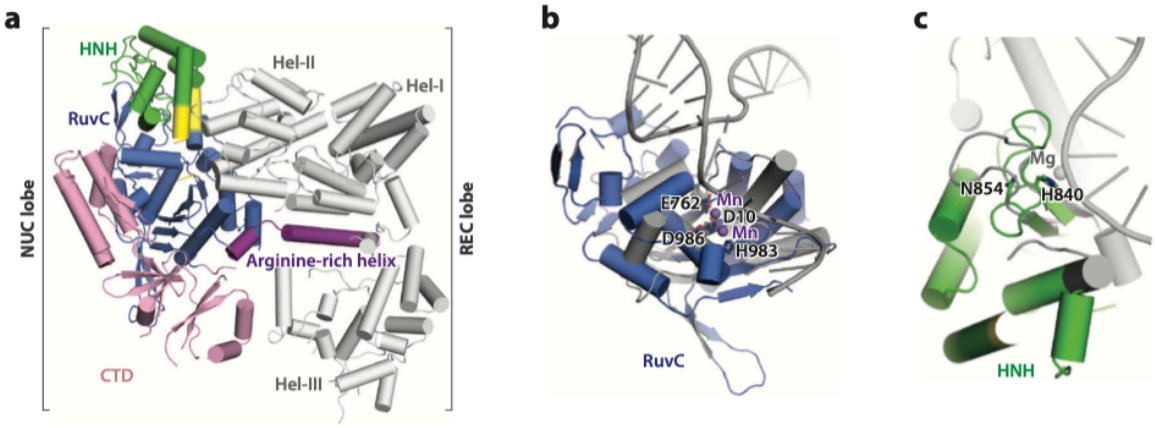

Figure 1: Schematic molecular structures of the Cas9 enzyme. A: general molecular structure of Staphylococcus pyogenes Cas9, with domains noted and colored. The NUC and REC lobes are oriented left to right. B: closer view of the Cas9 RuvC domain (in blue), overlaid with Holliday junction resolvase RuvC in gray. C: closer view of Cas9 HNH domain in green. Figure adapted from Jiang & Doudna, 2017.

DNA Repair mechanisms

Currently, CRISPR-Cas9 relies on the Host cell DNA repair pathways to repair the DSB generated by Cas9. Non homologous end joining (NHEJ) and homology dependent repair (HDR) are most commonly associated with DSB repair, although Microhomology-mediated end joining (MMEJ) is also involved (Symington & Gautier, 2011).

NHEJ is the most prevalent, efficient as well as the most inaccurate of the repair pathways, since no template is utilized to restore the original sequence (Moore & Haber, 1996). In the mammalian NHEJ pathway 53bp1, a NHEJ stimulating damage response factor, initially prevents end resection of the DSB (Escribano-Díaz et al., 2013, p. 1). The repair pathway is determined by whether this initial end resection occurs or not (figure 2). In mammalian cells, the DSB is recognized and bound by a ku70/ku80 heterodimer which prevents further end resection. DNA-PKc and Artemis are then recruited to the Ku bound DNA ends, which in turn allows for the binding of the Ligase IV complex including XRCC4, XLF and PAXX subunits. DNA-PKc and Artemis possess endo- and exonuclease activity to trim various damaged DNA overhangs while Pol X family (Pol μ/λ together with TdT) polymerases are responsible for insertion of nucleotides during this step of the repair process, leading to an increase in genetic diversity via creation of indels or insertions of nucleotides (Benedict et al., 2000; Loc'h et al., 2019). The Ligase IV complex then ligates the DNA ends, after which the repair complex dissociates from the DNA (Chang et al., 2017; Pannunzio et al., 2018).

In contrast, other DNA repair pathways rely on end resection upon the recognition of the DSB, as well as a varying degree of sequence homology in order to repair the DNA. In the HDR pathway, a 3' overhang is generated by the CtIP regulated MRN complex, which binds to the DSB site using BRCA1 and degrades the 5' strand (Roy et al., 2011). Once an initial overhang is generated, exonuclease I (EXO1) is able to degrade the 5' strand further where it can reach hundreds of base pairs in length (Mirman & de Lange, 2020). Since the 3' overhang is single stranded, it is coated by RPA proteins to prevent degradation. BRCA2 mediates replacement of the RPA coat with Rad51. Rad51 coated ssDNA is able to actively search and anneal to homologous DNA strands via ATP hydrolysis, forming a D-loop once invading a strand (Hilario et al., 2009). Usually, the sister chromatid serves as the homology partner, therefore the HDR pathway is restricted to the S-G2 phases of the cell cycle.

Once a homologous sequence is detected the D-loop, with its exposed opposite strand, can bind the other resected end of the DSB since it also shares homology with this sequence. DNA polymerase then synthesizes new DNA using the invading strand as a primer, and the homologous strand as template. The Holliday structure migrates, via helicases and topoisomerases, until the opposing side is reached. The structure is then resolved using endonucleases to cleave both strands, which are then ligated back together (Kowalczykowski, 2015).

MMEJ (also named alternative end joining) instead relies on microhomology present in the resected ends of the DSB generated by the MRN complex and EXO1. The 10 to 25bp long homologous sequences anneal together, which in HDR is blocked by binding of Replication protein A (RPA) and Rad51 (Wang & Xu, 2017). The annealing generates flaps of non-homologous DNA, which are cleaved by XPF/ERCC1. The annealed strands then serve as primers for DNA synthesis by polymerase θ. The restored DNA sequence is ligated by Ligase I or III, in conjunction with PARP1 and XRCC1 proteins (Seol et al., 2018). Other eukaryotes as well as bacterial DNA repair mechanisms function much in the same manner, having homologues for many of the components involved.

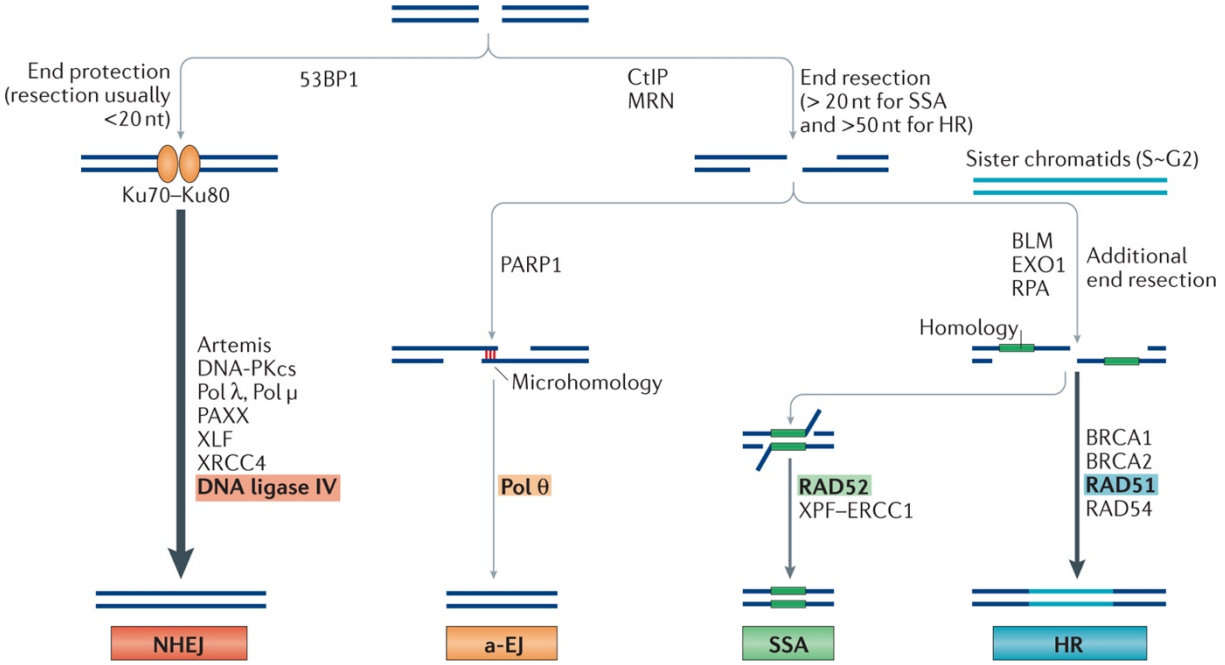

Figure 2: Mammalian DNA repair pathways with the factors involved. A DSB end is either protected from resection by 53BP1 or resected via the MRN/CtIP complex. NHEJ occurs via binding of Ku proteins, which in turn recruits DNA PKcs, Artemis and eventually the DNA ligase IV complex. Resected ends can either be restored using HR, where the sister chromatid serves as template, or via a-EJ (MMEJ) where microhomology is used to restore the DNA.

A CRISPR-Cas9 knock-in is currently achieved by providing the Cas9 enzyme, the specific sgRNA together with a gene of interest, flanked by sequences homologous to the target site. The knock in relies on the homology directed repair pathway to repair the generated DSB and using the provided gene of interest as a repair template, the gene is integrated at the selected location.

The reliance of the current CRISPR system on the host cell presents limitations, such as the need for dividing cells to achieve a knock-in, the low efficiency, and the optimization necessary to function properly in different cell types. To expand the range of applications further, some of these drawbacks need to be amended. Many solutions are currently being developed and tested, and this review aims to summarize and discuss current efforts to increase CRISPR Cas9 efficiency and versatility, that may enable the use of CRISPR for even more purposes in the future.

The ongoing efforts to improve the CRISPR-Cas9 system are focused on different aspects of the procedure. The main focusses are the improvements in delivery, increasing the likelihood of HDR, and finally creating host cell independent gene editing tools

Advancements in Delivery methods

Successfully delivering the CRISPR system to the target plays are large role in increasing the likelihood of a successful knock-in. By delivering all the components to the best location, using the most ideal delivery method, the knock in efficiency can be increased. The best delivery method may differ depending on the targeted cell type. A Cas9 plasmid for example may not be suited for cells with a very low rate of protein expression while it might be the best option when a longer-term presence of Cas9 is necessary.

Plasmid based CRISPR-Cas9 delivery

Currently plasmid-based delivery methods are the most common. These are integrated into the target cell by using viral vectors, lipid particles or electroporation (Liang et al., 2015; Liu et al., 2019). The expression plasmids contain both the sgRNA and Cas9 genes in one plasmid, and usually also have the advantage of a selectable marker (Ran et al., 2013). Viral delivery vectors such as Adeno- associated (AAV) and lentiviruses, often impose a size limitation to the plasmid, sometimes requiring a separate plasmid for the sgRNA and Cas9 DNA. This has been shown to reduce efficiency, but is a cheaper and simpler alternative to mRNA and protein delivery (Yip, 2020). In addition, using viral delivery vectors has been used to effectively deliver genetic material *in-vivo* for decades (C. S. Lee et al., 2017). The knowledge of long-term effects and wide range of data allows gives a head start in the development of gene therapies. Non-viral plasmid delivery is also common, although mostly limited to in vitro use. Transfecting a plasmid into the cell is a well-documented and relatively simple, and the DNA components are quite stable and easy to assemble. Longer term expression of the Cas9 enzyme also increases gene editing efficiency, although with a considerably higher risk of off target indels (J.-H. Zhang et al., 2016). Recently a polyethylenimine (PEI) carrier molecule was adapted for delivering Cas9 using host cell mediated nuclear transport of the plasmid (Abdallah et al., 1996; Zuckermann et al., 2015). This method reduces stress to the cell and is transported to the nucleus without the requirement for any additional nuclear localization signals.

Protein based CRISPR-Cas9 delivery

In light of increasing efficiency, ribonucleoprotein (RNP) delivery has shown potential. Here, the Cas9-sgRNA protein is expressed and assembled beforehand. RNP delivery is interesting, since it is only active for a short term and does not rely on the host cell to express the protein. This decreases the chances of off target indels that can result from longer term expression and presence of Cas9 (Kim et al., 2014). In addition, the independence from the host cell widens the range of cell types suitable for gene editing. The RNP does not have any inherent ability to enter the target cell on its own, and therefore relies on co-carrier molecules, lipid vesicles or electroporation. Recent studies have developed several nano-molecule co-carriers, including gold and diamond particles (K. Lee et al., 2017; T.-C. Yang et al., 2020). Such non-viral delivery systems are also desirable for in-vivo gene editing, as viral particles may generate an immunogenic response (Barnes et al., 2019). In cells with a low rate of gene expression RNP delivered Cas9 offers the benefit of being immediately active, for a relatively short period. Compared to DNA delivered Cas9 in human embryonic kidney cells (HEK293FT), which accumulates over time, RNP was most active immediately after transfection and was only detectable for 48 hours resulting in a decrease in off target indels (Liang et al., 2015).

mRNA based CRISPR-Cas9 delivery

mRNA is another delivery approach that has been utilized to effectively deliver Cas9. The in vitro-transcribed mRNA shares benefits with the RNP delivery approach. Namely the short presence of the nuclease, resulting in lower off target effects (Pruett-Miller et al., 2009). Compared to plasmid delivery, mRNA does not need to enter the nucleus in order to be transcribed which leads to more efficient translation (Leonhardt et al., 2014). The major drawback of mRNA is its inherent instability compared to proteins and DNA. This can be remedied by packing the RNA in lipid vesicles or using nanoparticles to prevent the RNA from degrading (Finn et al., 2018). Another consideration is that all components require separate delivery, unlike a plasmid which can encode all components within a single DNA molecule. For in vitro purposes the mRNA can be effectively integrated into the cell using electroporation, but for in vivo applications viral delivery is required (H.-X. Zhang et al., 2019).

Efficient delivery can help in increasing the Cas9 presence across the targeted cells, but the inherent inefficiency of a CRISPR-Cas9 mediated knock in is not remedied. To achieve this, modifications are required to the Cas9 enzyme itself. Such modifications can serve a variety of purposes to increase gene editing efficiency.

Promoting homology directed DSB repair

Since the HDR pathway is to the late S to G2 phases of the cell cycle while NHEJ can operate in all phases, one approach to increase the likelihood of HDR occurrence is to restrict Cas9 activity to the S and G2 phase. Multiple groups have achieved such a system by linking the Cas9 enzyme to cell cycle specific proteins.

Cas9-Gem, simultaneously developed by both Gutschner et al. and Howden et al. combines Cas9 with geminin, a substrate of the APC/Cdh1 complex. This complex is active in the late M and G1 phases and promotes ubiquitination of multiple proteins which leads to degradation. N-terminal linking of the first 110 amino acids of geminin to Cas9 successfully restricted Cas9 activity to only the S and G2 phases via post-translational regulation. Comparing to wild type Cas9, Gutschner found the relative rate of HDR was increased by 28 to 87% in HEK298T cells (Gutschner et al., 2016), while Howden describes a 2-fold reduction of NHEJ in Cas9-Gem transfected human pluripotent stem cells (Howden et al., 2016). This approach was since then adapted to edit genes in human hemopoietic stem cells (hHSC), where a four-fold increase in the HDR/NHEJ ratio was achieved in vitro (Lomova et al., 2019).

In another study by Vicente and colleagues, Cas9-gem was compared to Cas9 linked to CyclinB2. Like geminin, cyclinB2 is a substrate for the APC/Cdh1 complex and is degraded in the M and G1 phases. Cas9-Gem is compared to this Cas9-Cyclin fusion. In HEK293 cells, Cas9-Gem showed 0.31 HDR/NHEJ ratio versus wild-type Cas9 0.11 while Cas9-Cyclin achieved a 0.48 HDR/NHEJ ratio (Vicente et al., 2019).

Besides the Cell-cycle based approaches, another group has used HDR promoting protein CtIP to locally promote the initial DSB end resection once the DNA has been cleaved (Charpentier et al., 2018). Cas9 was fused to an N-terminal fragment of CtIP, a key co-factor for DSB end resection by the MRN complex in the early stage of HDR. Due to the proximity of the CtIP to the generated DSB, the end resection occurs before NHEJ components can assemble. Cas9-CtIP was tested in HEK293 cells targeting the AAVS1 locus with GFP integration. Measurement by FACS shows a 1.8-fold increase in HDR rate relative to WT Cas9, while nearly halving indels. The CtIP fragment was also coupled with the 110bp Geminin fragment to restrict Cas9 to S and G2 phases The combination did not show a further increase in HDR rates, as the pathway is already restricted to the S and G2 phases following CtIP mediated end resection.

Inhibition of NHEJ DSB repair

Opposite to the promotion of HDR is the inhibition of the NHEJ pathway. Something that has to be done carefully as deficiencies in the function of NHEJ have severe consequences for VDJ recombination, stem cell ageing, and cancer susceptibility (Woodbine et al., 2014). Therefore global inhibition of the NHEJ pathway should ideally be avoided, although a 53BP1 inhibiting protein (i53) was identified that resulted in a 5.6 fold increase in knock-in efficiency (Canny et al., 2018). The aforementioned risks of global NHEJ inhibition limit the application potential of such an inhibitor.

Recently Jayavaradhan and colleagues presented an approach that aims to locally inhibit the function of NHEJ initiator 53BP1 only at the Cas9 generated DSB site. The group identified domains of 53BP1 essential for DSB end binding, and removed domains required for function, thereby creating a shortened dominant negative 53BP1 variant (DN1s). By tethering the DN1s to Cas9 NHEJ protein recruitment is suppressed at the DSB generated by the Cas9, but not at otherwise occurring DSB sites. Testing in a HEK293T reporter line at two different loci showed a decrease in local NHEJ repair, resulting in HDR efficiencies of on average from 21% to 33.3% at the AAVS1 locus, and from 27% to 54.6% at the LMO2 locus respectively. Further testing in hematopoietic lines confirmed the findings, with HDR frequency reaching up to 86% in K562 cells (Jayavaradhan et al., 2019). Thus far no other groups have attempted similar NHEJ inhibition approaches, even though the Cas9-53DN1s system has proven to be effective.

Cas9 donor DNA tethering

Another approach to increasing the efficiency of a HDR mediated knock-in is to optimize conditions required to initiate homology directed repair. By loading the DNA repair template onto the Cas9 enzyme, all components of the system are localized at the target site which was shown to increase the percentage of HDR by up to 90% in HEK293 cells (Roche et al., 2018). This was achieved by linking Cas9 to a monoavidin domain, which strongly interacts with biotin. A biotinylated single stranded oligodeoxynucleotide (ssODN) is used as donor DNA (dDNA), resulting in a firm connection between the Cas9 enzyme and the dDNA. The Cas9-Mav-sgRNA-dDNA was complexed with the PEI delivery method mentioned earlier, delivering all components in a single package. Co-localization of the dDNA is believed to be a solution to a significant rate limiting step during the DNA repair process, the localization of a homologous DNA template. The in vivo applications of a Cas9-Avidin system were demonstrated by successfully generating mice embryos carrying homozygous double-floxp alleles with roughly 20% efficient editing in the modified embryos. Furthermore, a two-fold HDR frequency increase was observed with an in vitro reporter assay in HEK293 cells (Ma et al., 2017).

Similarly, Aird and colleagues used Porcine Circovirus Rep protein (PCV) to covalently link an unmodified ssODN to Cas9. PCV was linked to the n-terminal end of the Cas9 RNP and HDR activity was tested by restoring mCherry in a dual fluorescent HEK293 reporter cell line. Tethering by PCV requires for a 13bp recognition sequence to be attached at the 5' end of the dDNA without needing further chemical modification, as is the case for the Cas9-avidin based approaches. Up to a 30-fold increase in HDR rates were demonstrated by providing PCV-Cas9 with a repair template to restore a base mutation in the mCherry gene (Aird et al., 2018).
In studies both in vitro and in vivo, co-delivery of the Cas9 RNP with the intended repair template has shown to be a simple solution to increasing knock-in efficiency without requiring significant adjustments.

Host cell independent DNA editing

All previously described advancements are still reliant on the host cell DNA repair pathways. In the further future, complete independence from the host cell is preferable to create a more standardized gene editing procedure across cell types and organisms. There are currently two types of Cas9 based mostly host cell independent DNA editing systems: Prime editing and Cas9 transposases (figure 3). Both operate by combining the targeting ability of Cas9 with DNA modifying enzymes.

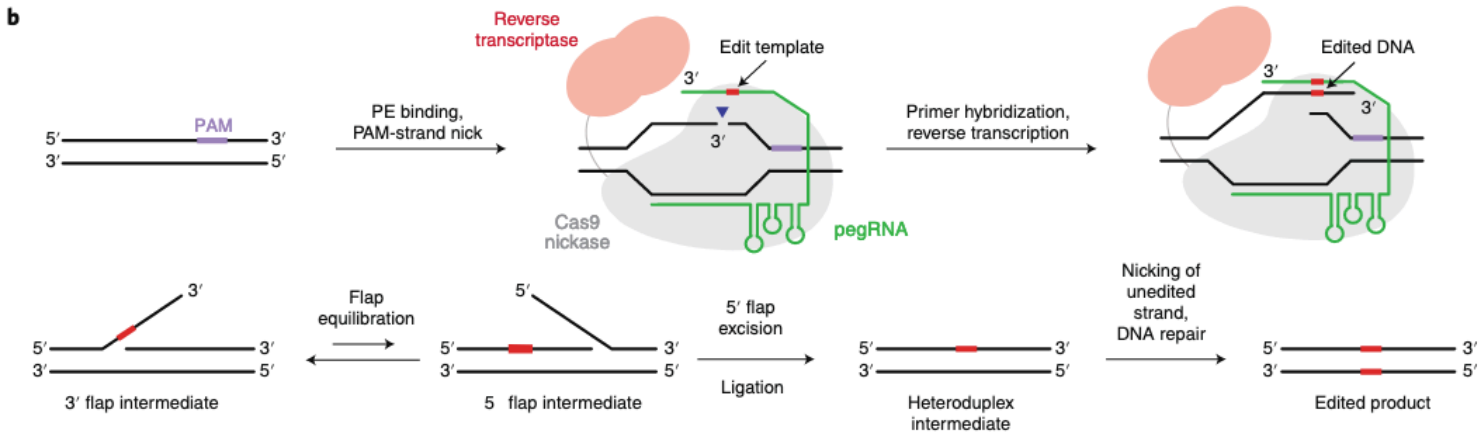

Figure 3: Schematic representation of the Prime Editing tool. The PE binds the target site and nicks the PAM strand. The 3' end serves as a primer for reverse transcriptase, using the pegRNA as a template. The 5' flap is excised by the host cell, and the edited 3' flap is ligated to the unedited strand. By nicking the opposite strand using a separate sgRNA, the opposite strand is repaired following the edited strand, integrating the edit into the genome. Adapted from Anzalone et al., 2020.

Prime editing was recently developed by the Anzalone lab as a versatile tool combining base editing, short insertion and deletion abilities. It relies on Cas9 nickase, an engineered reverse transcriptase and modified guide RNA named prime editing gRNA (pegRNA). The pegRNA serves as both the gRNA for the Cas9 and encodes the desired edit at the 3' end., the HNH domain of Cas9 is disabled, resulting in only the PAM site containing strand being nicked upon targeting. The now freed 3' end serves as a primer sequence for the reverse transcriptase, while using the pegRNA as a template. The pegRNA contains a homologous sequence to the nicked strand, allowing for the primer sequence binding to occur as well as the desired edit, deletion up to 80bp, or insertion of up to 44bp in length. This process results in two overlapping flaps of DNA after reverse transcription, the newly edited strand being a 3' overhanging flap. Anzalone and colleagues utilized the cells tendency to cleave the 5' flap to resolve such situations, leaving the edited 3' strand to bind the unmodified strand. Ligation of the edited strand leaves a non complementary intermediate, which is resolved by using a separate sgRNA with the prime editor to target and nick the unedited strand, resulting in the final product. The prime editor was thoroughly tested in four human cell lines, with successful deletions up to 80bp, insertions up to 44bp, and all 12 possible base editing point mutations. The prime editing system was also compared to an HDR optimized CRISPR knock-in and shown to be as effective in editing efficiency but with a significant reduction in off target indels, since the prime editing system does not rely on DSBs to edit the sequence (figure 3) (Anzalone et al., 2019, 2020). Although the prime editing system has shown to be an impressively versatile and effective editing system, it is limited by the size of the edited sequence. Only small tags and repairs can be integrated into the target.

Another recent development is the use of the efficient DNA transposases to integrate a desired sequence. Transposases operate without requiring DSBs, reducing the off target indels plaguing current CRISPR methods but are currently not able to target a specific location. Several groups have therefore tried to combine the targeting abilities of Cas9 with the easier DNA transposition abilities of transposases. One of the most promising efforts was a dCas9-Himar fusion by Chen et al (Chen & Wang, 2019). The Himar transposase in dimer form is a highly active transposase in a wide range of organisms without requiring any further factors to function. The transposase recognizes TA motifs, and can excise and insert sequences up to 7kb in length when such a motif is recognized (Lampe et al., 1998). This group sought to target the transposase by fusing it to a catalytically dead Cas9. The system successfully managed to facilitate a low rate of transposition to a targeted plasmid in E.coli cells, but since no modifications were made to the independent transposition abilities of the Himar transposase there was a high rate of off-target integration. In addition, all similar efforts thus far have observed that CRISPR guided transposases are restricted to bacteria only, no one has yet achieved successful

transposition in mammalian cells (Bhatt & Chalmers, 2019; Hew et al., 2019). These two factors have limited the utility as a gene editing tool until further advancements are made (Anzalone et al., 2020).

Discussion

Currently, CRISPR-Cas9 is at the forefront of gene editing. Its potential for further and therapeutic use is held back by its reliance on host cell DNA repair pathways and low overall efficiency. This review aimed to summarize and discuss current efforts to increase CRISPR Cas9 efficiency and versatility.

Several Cas9 delivery strategies were discussed that each have their benefits and downsides. The delivery of the CRISPR-Cas9 system is very dependent on the requirements of the experiment, and no one current method is specifically better than the other. Further developments could increase efficiency, and especially in in-vivo situations could contribute to widen the therapeutic potential of the CRISPR-Cas9 system (Yip, 2020). Current therapeutic applications of CRISPR-Cas9 mostly focus on ex-vivo gene editing and transplanting the edited cells, because of off target effects of Cas9 and inefficient delivery. Developments in targeted delivery to specific tissues and cell types, in combination with improvements to the CRISPR-Cas9 system itself, could allow for more precise and less invasive therapies in the future (Hirakawa et al., 2020).

The improvements to the CRISPR-Cas9 system itself discussed here each focus on a single aspect, while a combination of approaches could increase efficiency even further. This has not yet been attempted for many of the mentioned approaches, except for a Cas9-CtIP combination with Geminin (Charpentier et al., 2018). Even though no major benefit was observed in this case, most likely due to the fact that the CtIP and Geminin already restrict the knock-in to the same cell cycle phases, a combination of a dDNA tethering approach with a HDR promoting variant might operate more synergistically. Even though delivery is relatively more difficult because of their size, fusions of Cas9 with various proteins can provide an effective yet simple way of increasing Cas9 efficiency on the short term.
Another important consideration is that comparison between different CRISPR-Cas9 approaches is currently difficult. There is currently no standardized assay that can used to compare the up- and downsides of each modification made to Cas9. Efforts would have to be made in order develop and select a representative assay that can quantify various properties and aspects of a CRISPR-Cas9 knock-in (Tycko et al., 2016).

Currently, two mostly host cell independent gene editing tools are in development. Prime editing has shown to be an incredibly versatile tool but has not yet achieved widespread applicability. Anzalone and colleagues, who developed the technique, state that it is difficult to reproduce effective editing, and that there are many factors to consider when applying the technique (Anzalone et al., 2020).
Cas9-transposases were shown to have great potential in theory, but as of yet no activity in mammalian cells has been reported. Although the exact limitation has not yet been discovered, it is suspected that steric hindrance may play a role. Comparisons between PiggyBac transposase fused to Zinc Finger, TALE and dCas9 nucleases revealed that only cas9 fused PiggyBac was unable to facilitate targeted transposition in mammalian cells (Luo et al., 2017). Further investigation into the cause of this phenomenon is needed in order to continue development of such fusions. The groups that have developed dCas9 have thus far not been able to prevent the autonomous DNA transposition of the fused transposase. It is paramount that this issue is solved if the technique is ever used for gene editing purposes. Hew et al. propose the creation of an engineered transposase devoid of DNA binding abilities via directed evolution strategies (Hew et al., 2019). In the future, the ideal gene editing tool would likely be a completely synthetic protein that combines the precise RNA targeting of Cas9 with the transposition abilities of a transposase in a single protein.

Conclusion

The goal of this review was to summarize efforts to increase CRISPR-Cas9 knock-in efficiency for use in future applications.

CRISPR-Cas9 is currently widely used but its further potential is held back by the dependence on DNA repair pathways and overall inefficiency. Several ongoing developments aiming to remedy these drawbacks were discussed that may aid in expanding the potential applications of the CRISPR-Cas9 system. Improvements were focused on optimizing delivery methods, increasing the HDR rate over NHEJ repair, and gaining independence from the host cell for gene integration. Some of the discussed strategies can be applied immediately, while more development is required for others.

Much additional research is needed to refine and develop even more effective gene editing tools. Hopefully in the future, some of the discoveries and improvements mentioned here will benefit research and unlock applications which were not available before now.

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