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Genome Editing: Breakthroughs in Double-Strand Break (DSB) Repair and What's Next

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ABSTRACT: Genome editing has revolutionized the field of genetics, offering remarkable possibilities for advancing medicine, agriculture, and microbiology. Optimizing double-stranded break (DSB) repair mechanisms is central to enhancing genome editing efficiency, which is crucial in ensuring precise gene integration. This review focuses on classical non-homologous end joining (cNHEJ), microhomology-mediated end joining (MMEJ), single-strand annealing (SSA), and homologous recombination (HR). It evaluates the current understanding of various DSB repair pathways, highlighting their strengths, limitations, and recent advancements to improve efficiency. Overall, there has been a significant enhancement in the efficiency of all four DSB repair pathways. However, we are not at a point where genome editing can be used for routine, safe medical therapy. Of the repair pathways reviewed, MMEJ emerges as the most promising due to its balanced propensity, precision, and relatively broad applicability. Lastly, while progressions on DSB-based genome editing are made, the potential benefits of switching to an alternative genome editing strategy, such as prime editor, should also be considered, which has the potential for high precision genome editing due to the avoidance of DSB creation.

KEYWORDS: Cellular and Molecular Biology, Genetics, Genome Editing, DSB Repair.

Introduction

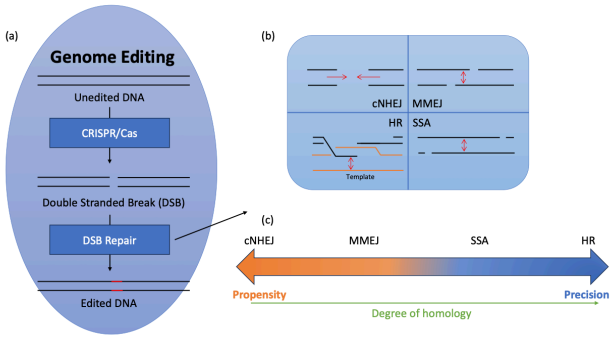

Figure 1: Graphical Abstract: (a) Overview of the process of CRISPR/Cas-based genome editing (arrows represent the proge; (b) Overview of 4 DSB repair pathways (cNHEJ, MMEJ, SSA, and HR); (c) Simplified comparison of 4 DSB repair pathways' propensity, precision, and degree of homology.

Genome editing aims to change organisms' characteristics by precisely manipulating a gene's DNA nucleotides.[1] This involves gene knock-out, gene knock-in, and single-nucleotide changes. Gene knock-out is a "loss of function mutation" in which the original gene is inserted with random DNA sequences or deleted, disrupting the gene function.[2,3] This is particularly beneficial to decipher the functions of a gene.[3,4] Gene knock-in, on the other hand, is a "gain of function mutation" that adds a new gene to an organism or replaces the original gene sequence with a correct or better-functioning allele.[5] This can be used to treat genetic disorders by correcting the mutated gene sequence.[6] Both gene knock-in and gene knock-out require the breakage of both strands of DNA. However, a single nucleotide change, also known as a point mutation or base editing, modifies a single nucleotide base by cutting only one DNA strand.[7] Single-nucleotide changes are highly beneficial in treating specific genetic mutations, offering precise and targeted therapeutic interventions.[8]

Genome editing is the cutting and gluing of DNA to create targeted changes in the genome, as illustrated in Figure 2. For DNA cleavage, sequence-specific nucleases—which include zinc-finger nucleases, transcription activator-like effector nucleases, and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system—are used to cut the DNA and produce double-stranded breaks (DSB) at predetermined genomic sites.[9,10] These DSBs can then be glued back together through DSB repair pathways.

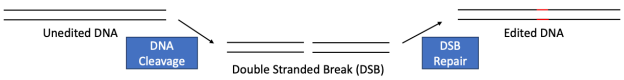

Figure 2: Simplified illustration of genome editing (arrows represent the progression of genome editing's stages): firstly, unedited DNA is cut into DSB through the process of DNA cleavage; subsequently, DSB is glued back together with the targeted edits via DSB repair.

The revolutionary power of genome editing traverses diverse biological domains, encompassing potential applications in bacteria, animals, and humans. In bacteria, genome editing has been used for industrial or medical purposes. For example, inserting the gene for human insulin into a plasmid of *Escherichia coli* bacteria cells can generate human insulin for therapeutic uses.[11] In animal trials, genome editing can serve agricultural and medical purposes. For medical purposes, genome editing techniques for treating human diseases are used on animals before human cells are used to ensure safety and efficacy.[12] For

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agricultural purposes, genome editing can be applied to expedite livestock breeding programs.[13] For example, improved meat production in Bama pigs has been achieved with genome editing.[14] Since genetic disorders are based on the fault of specific genes, there is hope that genome editing can treat genetic disorders in humans.

Despite all this potential, genome editing still faces many challenges in delivering efficient DNA editing. Efficiency depends on a precise and frequent editing of the targeted DNA sequence. For genome editing to initiate, all the biomacromolecules (such as sequence-specific nucleases and inserted template DNA sequences) must enter the cell.[1] This is particularly challenging in multicellular organisms because of the difficulty in reaching or editing every single cell necessary for changing the targeted phenotype. While nanoparticle-based delivery systems can potentially improve intracellular delivery of mRNA to the target organ and tissues, improving endosomal escape, transfect efficiency, and achieving targeted delivery remains challenging.[15,16] Another source of inefficiency is that off-target cleavage can result when sequence-specific nucleases bind to unintended genomic sites that share sequence similarity with the target site.[17] These unintended DSBs may undergo DSB repair, leading to unintended mutations or gross chromosome rearrangements. Thence, the on-target/off-target rate of DNA cleavage strongly correlates with the efficiency and safety of genome editing. Inefficiency also arises from the inclusion of unintended nucleotides during DSB repair, which can lead to the formation of indels that affect the functionality of the modified sequence.[18]

To achieve the greatest possible benefits that genome editing's potential applications can create, specific strategies and methods to improve the efficiency of the intermediate steps are necessary. Since genome editing is the cutting and gluing of DNA nucleotide sequences, DNA cleavage and DSB repair are the two most fundamentally important intermediate processes to enhance overall genome editing efficiency. Many reviews have addressed the challenges and advancements in DNA cleavage, especially in the CRISPR/Cas system.[19-21] Therefore, I will focus on the DSB repair challenges in this review.

Overview of recent genome editing:

In 1987, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) were unintentionally discovered in *E. coli*.[22] It wasn't until 20 years later that it was understood that bacterial cells with CRISPRs can resist virus infection and form the adaptive immune system of prokaryotes.[23,24] Meanwhile, the endonuclease Cas protein was identified, which, when working with particular single-stranded guide RNA (sgRNA), can target a specific genomic site and create a double-stranded break (DSB) by breaking the phosphodiester bonds between nucleotides.[25,26] Compared to previous DNA cleavage mechanisms, the CRISPR/Cas system was more precise and efficient at introducing DSBs.[25,27]

DSB occurs frequently in living organisms' cells due to various causes other than the cutting by an endonuclease, including mistakes in replication, ionizing radiation, chemotherapeutic agents, etc.[28] As DSB disrupts both strands of DNA if left unrepaired, it may lead to cell cycle arrest, genomic instability, cell death, and chromosomal abnormalities.[29,30] Given the deleterious nature of DSB, living organisms have a range of mechanisms to repair DSB and restore molecular function.[31]

Scientists applied and combined the natural adaptive immune system of prokaryotes, the CRISPR/Cas system, with the natural mechanisms of repairing the DSB of living organisms to develop genome editing. The four most commonly used DSB repair pathways are classical non-homologous end joining (cNHEJ), microhomology-mediated end joining (MMEJ), single-strand annealing (SSA), and homologous recombination (HR).[29] These can be seen in Figure 3.

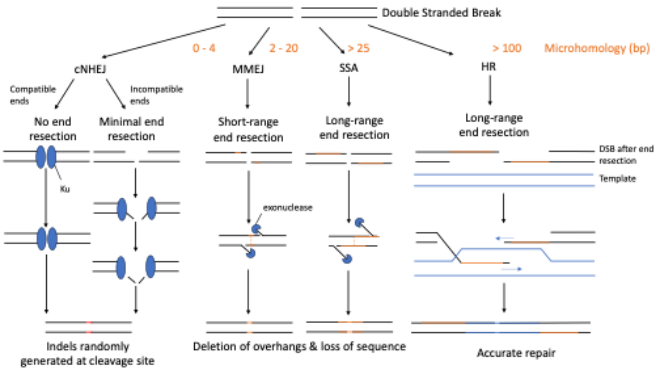

Figure 3: An overview of the four main DSB repair pathways: cNHEJ, MMEJ, SSA, and HR. cNHEJ repairs DSB (represented by black lines) regardless of microhomology (represented by orange lines), using Ku proteins (represented by blue ellipses); MMEJ and SSA repair DSB based on the annealing of microhomology (represented by orange dotted lines), using exonucleases (represented by blue ¾ circles); and HR repair DSB according to the homologous template (represented by blue lines). Black arrows indicate the progression of each DSB repair pathway. The outcomes of each DSB repair are shown at the bottom: cNHEJ – indels randomly generated at the cleavage site; MMEJ and SSA – deletion of overhangs and loss of sequence; HR – accurate repair.

cNHEJ is the dominant form of DSB repair, as it occurs with high frequency and repairs most of the DSBs formed in living organisms. This is because it is template-independent, and it can occur at any phase of the cell cycle.[18] As the name suggests, cNHEJ generally repairs DSB without needing a homologous sequence between the two exposed DNA ends of DSB; hence, it has the fastest processing of all repair pathways. However, when the broken DNA ends are incompatible, minimal end resection (cutting broken DNA ends by exonucleases) creates a low degree of microhomology (1 to 4 complementary base pairs).[32] Moreover, cNHEJ often results in the random generation of indels (unintended insertion or deletion of nucleotides) at the cleavage site. Due to its inability to maintain the original DNA sequences, cNHEJ repair is considered error-prone.[18,19,29]

DSB repairs through MMEJ and SSA depend on the annealing of homologous sequences between the two broken DNA ends of the DSB.[33,34] However, MMEJ requires a lower degree of homology (around 2 to 20 complementary base pairs), whereas SSA requires a higher degree of homology (>25 complementary base pairs).[35-37] Both MMEJ and SSA undergo DNA end resection to create sticky ends that expose

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homologous sequences as single-stranded overhangs. As a result of the DNA end resection, small sections of the original DNA sequences at the cleavage site can be deleted. Thus, these processes also fail to maintain the original gene sequences.[33,34]

HR is referred to as the error-free DSB repair pathway because, with the help of a homologous DNA template and a high degree of homology (>100 complementary base pairs), HR can precisely repair a DSB.[38] Despite the high fidelity, the HR pathways are limited to dividing cells because the required molecular components are only expressed in the S and G2 phases of the cell cycle.[39] Thus, it has low editing efficiency, and it often experiences extensive competitive pressure from cNHEJ, reducing the probability of cells undergoing HR.[40-42]

DSB repairs under molecular lenses:

Because inefficiency is present in every DSB repair pathway, I will now zoom in to the molecular level to understand where improvements can be made.

cNHEJ, as illustrated in Figure 4, is initiated as the ring-shaped protein heterodimer (Ku) detects and binds to a DSB. This protein prevents the DSB ends from extensive DNA-end resections and recruits other cNHEJ proteins to promote end ligations.[32] If the DSB has compatible end configurations, XRCC4-DNA ligase IV will bind to the Ku protein, forming a Ku-XRCC4-DNA ligase IV complex, ligating the two DNA ends. If the DSB has incompatible end configurations, cNHEJ proteins such as DNA-PKcs, Artemis, Pol μ, and Pol λ will bind to the Ku protein to create blunt ends via minimal end resections.[32] Once the DNA ends can be ligated, an XRCC4-DNA ligase IV complex will form, and the DSB will be repaired. These minimal end resections cause random deletion of DNA near the cleavage site, and free-floating nucleotides randomly attach to the exposed DNA ends, creating indels. Therefore, cNHEJ leads to mutations and error rates of up to 50%, reducing efficiency.[43]

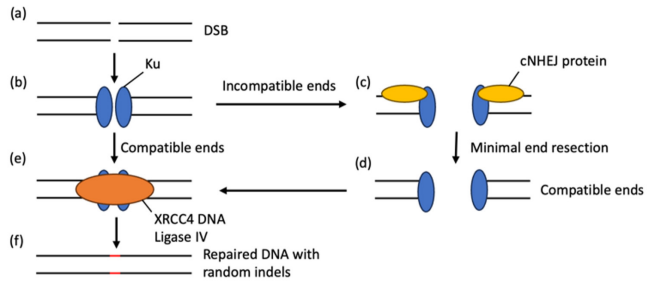

Figure 4: A simple schematic illustration of cNHEJ's molecular processes. Black arrows indicate the progression of cNHEJ. (a) A DSB, represented by black lines, is created. (b) Ku proteins, represented by blue ellipses, bind to DSB at the cleavage site. If the DNA end configurations are compatible, DSB processes to (e) in which XRCC4 and DNA Ligase IV, represented as orange ellipses, bind to Ku proteins. If the DNA end configurations are incompatible, DSB processes to (c), in which cNHEJ proteins, represented by yellow ellipses, bind to Ku proteins. (d) cNHEJ proteins undergo minimal end resection and change DNA end configurations to be compatible. Hence, DSB with compatible DNA ends undergoes (e). Finally, (f) cNHEJ completely repaired DNA, but indels, represented as red lines, are randomly generated at the cleavage site.

HR, as shown in Figure 5, is initiated by the long-range end resection of the 5' ends of the DSB site to produce 3' single-stranded DNA overhangs. This resection is carried out by a series of nucleases, including the MRN complex (MRE11-RAD50-NBS1), CtIP, EXO1, and Bloom helicase (BLM)-DNA2.[44,45] The activation of some of these key DNA end resection factors is restricted mainly to the S/G2 phases of the cell cycle, hence limiting HR's efficiency.[29] Following the extensive end resection, ssDNA overhangs are then coated by replication protein A (RPA), and eventually, the ATP-dependent DNA recombinase RAD51 replaces RPA bound onto the ssDNA overhangs, forming long helical filaments that search for a homologous sequence.[46] Once a homologous sequence is found, RAD51 facilitates strand invasion, where the ssDNA overhangs invade the template and pair with the complementary strand. This creates a displacement loop (D-loop) that starts generating new DNA nucleotides, with the help of DNA polymerases, along the 3' overhangs using the intact homologous sequence as a template.[47] This synthesis continues until enough DNA has been generated to copy the DNA nucleotide sequence of the template.

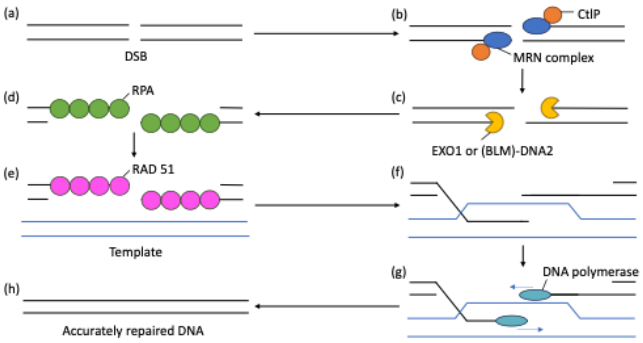

Figure 5: A simple schematic illustration of HR's molecular processes. Black arrows indicate the progression of HR. (a) A DSB, represented by the black lines, is created. (b) MRN complex and CtIP, represented by dark blue ellipses and orange circles, respectively, bind to DSB, removing DNA at the cleavage site. (c) EXO1 or (BLM)-DNA2, represented by yellow ¾ circles, replaces the MRN complex and CtIP, further removing DNA at the cleavage site. (d) After DNA end resection, RPA, represented by green circles, binds to the overhangs. (e) RAD 51, represented by pink circles, replaces RPA and searches for the homologous template, represented by blue lines. (f) DSB binds with the template according to homology. (g) DNA polymerases, represented as light blue ellipses, bind the DNA ends and repair DSB according to the homologous template. (h) Finally, HR completes with an accurately repaired DNA.

MMEJ and SSA share similar processes: end resections, annealing of microhomology, and ligation.[36,48] In particular, MMEJ resembles cNHEJ, as it involves short-range end resection, whereas SSA is more similar to HR due to its long-range end resection. According to Figure 6, both MMEJ and SSA initiate with the short-range end resection when MRN-complex (MRE11-RAD50-NBS1) and CtIP (C-terminal binding protein interacting protein) bind onto the DSB to prevent cNHEJ by removing Ku protein from the DNA ends and activate 5'-exonuclease activity, removing DNA sequences from 5' to 3' direction and generating 3' overhangs.[29,49] At this stage, the exposed microhomology suffices for MMEJ. SSA, however, requires additional steps to expose the microhomology further. Similar to the HR, exonucleases such as EXO1 or Bloom helicase (BLM)-DNA2 take over the job of MRN-complex and CtIP, continuing to remove DNA from the 5' to 3' direction

and elongating the exposed microhomology.[50,51] After end resections, MMEJ anneals the microhomology by DNA polymerase θ and fills any gaps via template-directed DNA synthesis.[29,49] Eventually, the DNA nucleotides are ligated together by DNA Ligase I and DNA Ligase III.[36] SSA, however, anneals the microhomology via RAD52: following the end resections, the resulting overhangs are bound by RPA, like HR; but unlike HR, RAD52 replaces the RPA instead of RAD51 and promotes the annealing of the microhomology.[37,52] Eventually, the DNA nucleotides are ligated together by an unidentified DNA Ligase, which some scientists have hypothesized to be DNA Ligase I.[37]

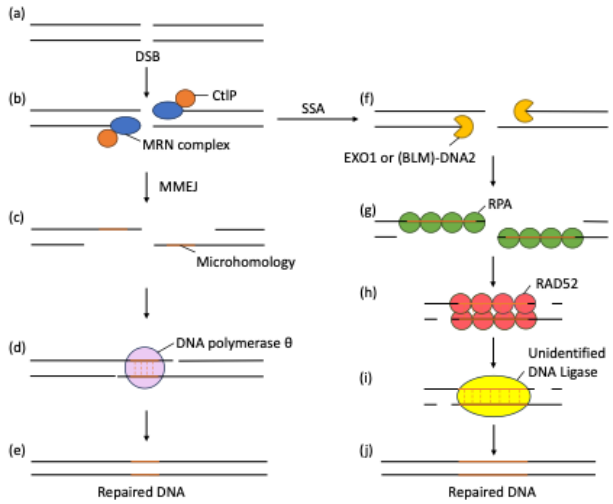

Figure 6: A simple schematic illustration of MMEJ and SSA's molecular processes. Black arrows indicate the progression of MMEJ and SSA. (a) A DSB, represented by black lines, is created. (b) MMEJ and SSA involve the attachment of the MRN complex and CtIP to DSB, as represented by blue ellipses and orange circles, which remove DNA from DSB at cleavage sites. Subsequently, MMEJ and SSA's processes separate into (c), (d), (e) and (f), (g), (h), (i), (j), respectively. (c) For MMEJ, microhomology, represented by orange lines, is exposed on overhangs after short-range end resection. (d) DNA polymerase θ, represented by pink circles, anneals the microhomology. (e) Finally, MMEJ is completed with repaired DNA. (f) For SSA, EXO1 or (BLM)-DNA2, represented by yellow ¾ circles, replace the MRN complex and CtIP, further removing DNA at the cleavage sites. (g) RPA, represented by green circles, binds to the overhangs after DNA end resection. (h) RAD 52, represented by red circles, replaces RPA and anneals microhomology. (i) Unidentified DNA Ligase, represented by a yellow circle, binds and ligases to the DSB. (k) Finally, SSA is completed with repaired DNA.

Comparison of the four DSB repair pathways:

Among the four main DSB repair pathways discussed, cNHEJ and HR were identified as the earliest and most extensively studied. cNHEJ has the least dependence on homology and the least restriction on cell phases; therefore, it occurs relatively fast and is the most frequently occurring DSB repair pathway in a cell (i.e., highest propensity). Despite having a high propensity, cNHEJ is less useful in genome editing due to its low precision. HR, on the other hand, has the greatest precision and would be preferable for genome editing. However, due to its complex mechanisms and restriction on cell phase, HR is generally slow and outcompeted by other pathways, occurring with low frequency. Therefore, among the current pathways used for genome editing, there is a trade-off between propensity and precision: the DSB repair pathway that involves more homologies receives higher precision, but its process also becomes more complex, reducing the likelihood of occurrence. Compared to cNHEJ and HR, MMEJ and SSA are more balanced in this propensity and precision trade-off, granting a more efficient approach overall. However, since SSA is also restricted to cell cycles like HR, MMEJ emerges as the more promising repair pathway for genome editing. Regarding their inefficiencies in nature, all DSB repair pathways require new strategies to improve. (Table 1)

Table 1: Detailed and comprehensive comparison of the four DSB repair pathways – cNHEJ, MMEJ, SSA, HR – over precision, propensity, degree of homology, phase dependency, and application scenarios.

	cNHEJ	MMEJ	SSA	HR
Precision	Low	Medium	Medium	High
Propensity	High	Medium	Low	Low
Degree of Homology	None	Low	Medium	High
Phase Dependency	None	Low	High	High
Application scenarios	Gene knock-out only	Both gene knock-out and gene knock-in	Both gene knock-out and gene knock-in	Both gene knock-out and gene knock-in

Possible improvements:

cNHEJ: dual-cutting for PAM-in configurations:

The CRISPR/Cas system is currently the most popular DNA editing mechanism. It often integrates with cNHEJ, known as CRISPR/Cas-based cNHEJ, to perform genome editing. According to Figure 7, the process starts with the Cas endonuclease, guided by its single-stranded RNA (sgRNA), detecting and binding to a region called Protospacer Adjacent Motif (PAM), which is a specific short sequence adjacent to the target sequence; then, it cuts the DNA at the target sequence, resulting in a DSB. According to the location of the PAM region, the two DNA ends of the DSB can be differentiated into the PAM-proximal ends and the PAM-distal ends.[19] After cleavage, the Cas endonuclease initially releases the PAM-proximal end but remains bound to the PAM-distal end for a prolonged period.[53,54] This prolonged attachment of Cas endonuclease prevents the Ku protein from binding onto the PAM-distal end, preventing cNHEJ from occurring, but it also limits the generation of indels.[54] Therefore, the precision of cNHEJ is relatively higher at the PAM-distal end than that of the PAM-proximal end, which contains the free generation of indels.[18,55]

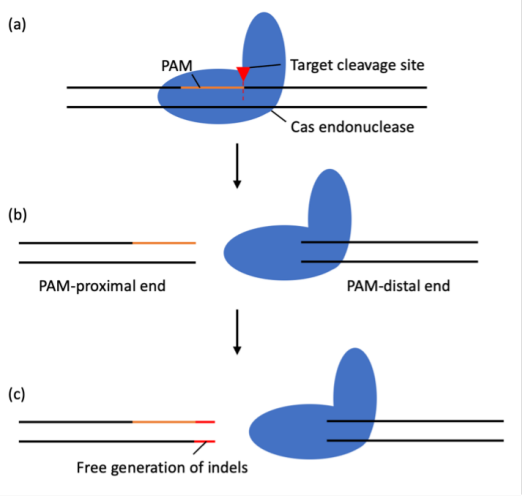

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Figure 7: A simple schematic illustration of the CRISPR/Cas system. (a) Cas endonuclease, represented by blue shapes, binds to the PAM region of DNA, represented by orange lines, and cleaves the DNA at the target cleavage site, represented by a red dotted line. (b) After cleavage, a DSB is created with a PAM-proximal and PAM-distal end, and the Cas endonuclease remains bound to the PAM-distal end. (c) As Cas endonuclease remains bound, indels are randomly generated at the PAM-proximal end, as red lines represent.

Since PAM-distal ends of the DSBs are more precise than PAM-proximal ends during CRISPR/Cas-based cNHEJ, a strategy of performing cNHEJ between two PAM-distal ends of the DSB can lead to higher precision. This is done by cleaving the DNA twice along a DNA sequence, generating two DSBs, and isolating a short sequence of DNA, which is released. Specifically, the two PAM regions from the dual cutting should be included in the target sequence through design (PAM-in), which is illustrated in Figure 8.[18,55] The two remaining ends of DSBs are both PAM-distal, which can perform cNHEJ more precisely. This strategy has produced a more precise gene knock-out in HEK 293T cells (human embryonic kidney cells) with ~79% accuracy.[18] However, this dual-cutting strategy is limited to gene knock-out, as the Cas endonuclease's attachment is too short for the inserted DNA sequence to stay attached. While restricted to gene knock-out, this dual-cutting strategy improves the precision (so as efficiency) of cNHEJ-based genome editing.

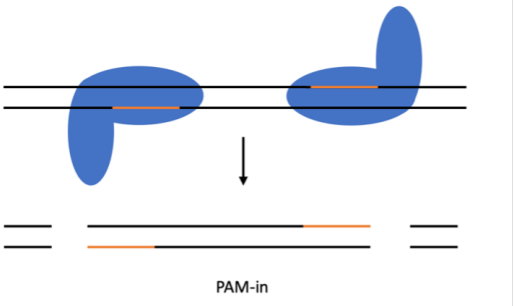

Figure 8: A simple schematic illustration of PAM-in configurations. Cas endonucleases, represented by blue shapes, bind to two PAM regions of DNA, represented by orange lines, cleaving the DNA at a certain target site. After dual cleavage, a short fragment of the DNA is isolated, which includes both PAM regions, as represented by orange lines.

HR: eliminating all its competitors:

The efficiency of HR is limited due to its low propensity, which is caused by multiple reasons. Firstly, as HR is significantly dependent on a homologous DNA template, the availability of a DNA template near the genomic cleavage site influences its efficiency. In yeast, the efficiency of HR drops by >50% when the homologous template is located more than 1 kb away from the break site.[56] Seconds, HR is limited to S and G2 phases of the cell cycle, as RPA, which is essential for recruiting RAD51; hence, HR depends on Cdk2, which has a low concentration in cells other than S and G2 phases.[57] Thirdly, HR experiences strong competition from other DSB repair pathways, especially cNHEJ, which occurs faster within the cell environment. Although the specific balance between HR and other alternative DSB repair pathways varies between species, cNHEJ always outcompetes HR. Lastly, the delivery of the large molecule of DNA template into cells also limits HDR application. Despite high precision in achieving target gene changes, HR is still far from prevalent in medical applications.

To improve the efficiency of HR, several strategies that assist HR to outcompete cNHEJ have been proposed, such as inhibiting the key components of cNHEJ, including DNA-PK and DNA Pol θ.[58,59] In addition, AZD7648 was identified as a selective DNA-PK inhibitor, which, combined with DNA Pol θ inhibitors, led to the development of the 2iHDR approach and significantly boosted the propensity of templated insertions in Jurkat cells (immobilized human T lymphocytes). Moreover, 2iHDR also reduces off-target effects of Cas9.[60] Overall, the discovery of this new treatment marked an innovative practice of combining multiple inhibitors to improve the overall efficiency of HR-based CRISPR/Cas genome editing.

MMEJ: computational algorithms:

MMEJ was once regarded as a backup DSB repair pathway of cNHEJ (alt-NHEJ), and it was not until recently that this pathway gained more attention in genome editing applications.[61,62] MMEJ is 10-fold more frequent than HR but still not as high as cNHEJ.63 Despite areas of improvement in its propensity, MMEJ's efficiency is mainly limited by its imprecision. As only a small degree of microhomology is used, the formation of indels at the exposed DNA ends may interrupt the original microhomology, leading to deletions and insertions at the cleavage side.[33] MMEJ uses microhomology between the two ends of the DSB that become exposed. Therefore, computational algorithms (such as MENTHU, inDelphi, and Lindel) were developed to assist in identifying the microhomologies and predicting the outcome of DSB repair across the genome. These computational algorithms achieved considerable success in correct prediction rates. Further improvement was obtained by combining MENTHU and Lindel to produce a new algorithm known as MENdel, which resulted in up to 90% successful prediction.[62]

SSA: microhomology elongation:

Similar to HR, the inefficiency of SSA is mainly attributed to its relatively lower propensity. Since SSA's mechanism is more complex than cNHEJ, it is outcompeted by cNHEJ to different degrees (for other cell and animal types). Moreover, like HR, SSA also undergoes extensive DNA end resection, experiencing similar limitations in cell phases. Specifically, RPA, an essential protein in recruiting RAD51 (so as extensive DNA end resection), depends on Cdk2 and has a low concentration in cells other than in the S phase. Therefore, SSA's efficiency is also limited to specific cell phases and non-dividing cells. One recent study identified that increasing the length of microhomology to over 500-2000 base pairs significantly enhances the frequency of successful SSA.[64] Thus, a strategy to improve SSA's propensity is to increase the length of microhomology, which can provide a better substrate for the annealing process, leading to greater efficiency overall.

HR & SSA: cell cycle synchronization:

As previously discussed, HR and SSA are limited to S and G2 phases. This restriction has reduced their propensity, hindering their overall efficiency. To overcome this challenge, scientists developed a strategy to synchronize the timing of

genome editing to specific cell phases (i.e., S and G2 phases) to maximize the efficiency of genome editing.[65] This is done by fusing Cas9 with the N-terminal region of human Geminin (hGem(1/110)). As a result, the Cas9 expression is synchronized with the cell-cycle progression, allowing Cas9 to be expressed at high levels during the S and G2 phases of the cell cycle, where HR and SSA are more active. Overall, this provides a potential for overcoming the limitation of the cell cycle and boosting the propensity of HR and SSA by 87%.[65]

DSB repair: Where do we go from now?:

Because cNHEJ and HR are the most studied pathways, they are still the most used DSB repair pathways in genome editing despite their low efficiency. cNHEJ's precision has been improved through a dual-cutting PAM-in approach, and HR's propensity has increased by actively inhibiting cNHEJ through the combined treatment: 2iHDR. However, they are still not ready for medical applications. For cNHEJ, the dual-cutting strategy is only viable for knock-outs; medical uses usually require knock-ins. Also, even in the same organism, different cell types vary slightly in their cellular compositions and biological mechanisms. This improvement strategy for cNHEJ has only been tested on HEK 293T cells; hence, its applicability in other cell types remains unknown. Similarly, for HR, 2iHDR has only been tested in a limited number of cell types, and its general applicability still needs to be determined. Confirming their reliability and adaptability under different cellular conditions is important for future investigations.

Regarding MMEJ and SSA, as the scientific community overlooked them in the past, they are still in the early stages of development. Although strategies such as MENdel and microhomology elongation improve the efficiency of MMEJ and SSA, respectively, their improvements are less significant compared to cNHEJ and HR's improvements via dual-cutting and 2iHDR treatment. Moreover, a strategy for controlling the timing of Cas9 expression has been developed to address the cell cycle restriction of HR and SSA. This benefits both HR and SSA by overcoming limitations in their propensity. However, concerns remain regarding their adaptability to non-dividing cells, leaving areas for future investigations. Collectively, there have been improvements in all DSB repair pathways, especially in HR. This constitutes one more step towards the ultimate goal of genome editing. However, none of these fields are yet mature, and more investigations are required in the future. I think more attention should be given to MMEJ in particular. Given the propensity and precision trade-off, DSB repair's overall efficiency can only be maximized if propensity and precision can be balanced. Hence, MMEJ and SSA deserve more focus than cNHEJ and HR. Between MMEJ and SSA, SSA's applicability is greatly restricted to dividing cells, whereas MMEJ can be applied to both dividing and non-dividing cells. Therefore, MMEJ should gain the most focus for DSB-based genomes in the future.

Primer Editing: another way of genome editing:

Despite the advancements in DSB-based genome editing, none are efficient enough for medical applications. Hence, DSB-based precise genome editing is particularly challenging and may not lead to an efficient stage. However, genome editing does not necessarily have to start with a DSB. Prime editing represents a revolutionary approach that circumvents many of the limitations of the DSB-based genome editing experience. Instead of cutting both DNA strands, prime editor, a fusion protein combining a catalytically impaired Cas9 and a reverse transcriptase, only cuts one DNA strand.[66,67] This nick in the DNA then allows the reverse transcriptase to synthesize a short strand of DNA, also known as a flap, according to the prime editing guide RNA, and replace part of the original DNA strand through a process called flap equilibration. Finally, the mismatch between the nucleotides of the edited strand and those of the original strand undergoes cellular mismatch repair to restore the complementary base pairing.[68] Compared with DSB-based genome editing, which frequently exhibits indel rates exceeding 20–30% or more, prime editing shows significantly improved precision, achieving indel rates as low as <1–10% at specific loci.[69] Moreover, prime editing exhibited much lower off-target editing compared to Cas9 nucleases at known off-target sites. For example, average off-target rates for prime editing were less than 0.1% at specific loci, while Cas9 with sgRNAs showed much higher frequencies of off-target activity, averaging from 16% to 60%.[69] Therefore, compared with DSB-based genome editing, prime editing allows for the direct and precise introduction of insertions, deletions, and substitutions without generating DSBs, circumventing the need for error-prone DSB repair pathways used in traditional CRISPR/Cas9 systems. As a result, prime editing significantly reduces the risk of off-target integrations and enhances the accuracy of genetic alterations.[70] However, prime editing also faces the risk of reverting the edits back to the unedited sequence, as the outcome of flap equilibration and cellular mismatch repair is not entirely predictable. Moreover, prime editing also faces challenges in scalability due to the complexity of delivering the larger prime editor complex and the design of prime editing guide RNAs, which requires careful optimization to ensure functionality. Overall, while the overall efficiency of prime editing is also limited, its lower indel rate and off-target rate make it a more promising tool, particularly in therapeutic settings where exact genetic corrections are critical.

Conclusion and Future Perspectives

As the scientific community continues to harness the power of genome editing, understanding the strengths and limitations of DSB repair pathways is paramount. cNHEJ, while fast and frequent, often sacrifices accuracy for speed. Therefore, it can only efficiently perform gene knock-out, where precision is not required, but not gene knock-in or other precise genetic modifications. HR, on the other hand, offers high fidelity but is constrained by its phase-specific nature and competition with faster repair pathways like cNHEJ. Hence, despite its potential for efficient and precise genetic modifications, HR's low propensity makes it an unreliable option for genome editing. Comparatively, MMEJ and SSA have a relatively balanced propensity and precision. However, given that SSA is also limited by specific cell phases, MMEJ emerges as a more promising pathway for future investigations.

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Recent advancements in the field of genome editing have focused on enhancing the overall efficiency of DSB repair pathways, yielding several promising improvements. Notably, the optimization of cNHEJ has seen developments like the dual-cutting strategy, which utilizes two Cas endonucleases to target close genomic sites, reducing the likelihood of unwanted indels at the repair site and enhancing precision for gene knockouts. In the realm of HR, the introduction of small-molecule inhibitors such as DNA-PKcs and DNA Ligase IV inhibitors has significantly improved the propensity of HR over the error-prone repair pathways. These inhibitors effectively increase the fidelity and efficiency of HR by blocking competing pathways, particularly cNHEJ. Additionally, advancements in MMEJ have included the development of computational tools like MENTHU and inDelphi, which predict the outcomes of MMEJ with high accuracy and help in designing genome editing strategies that leverage existing microhomologies. These tools enhance the practicality of MMEJ by allowing researchers to anticipate and mitigate potential errors in the genome editing process. Last, it has been suggested that increasing the length of microhomology between 500-2000 base pairs can significantly enhance SSA efficiency, providing a more effective substrate for the annealing process in genome editing.

Amid these advancements, prime editing stands out as a revolutionary alternative that bypasses the need for DSBs altogether, potentially offering a solution to the error-proneness of traditional methods. By only nicking one strand of DNA, prime editing allows for precise edits with reduced risk of unintended mutations, setting the stage for its future development and integration into therapeutic contexts.

As we look to the future, the field should pursue a dual approach: continue to refine DSB-based genome editing technologies, with a particular focus on optimizing MMEJ, while also advancing research into prime editor-based methods. This balanced approach ensures that as we improve existing technologies, we also explore new modalities that could redefine what is possible in genome editing. These developments pave the way for human health improvements via gene therapies. However, ethical considerations must be carefully examined as we approach the reality of efficiently changing genomes. Concerns regarding the equity of access, the potential for genetic discrimination, and the implications of germline editing raise significant questions about the responsible use of these powerful technologies. As such, a balance between the excitement for the bright future of genome editing and a thoughtful approach to its ethical implications is crucial to ensure these advancements are used responsibly, fairly, and for the benefit of all humanity.[71,72]

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