

REVIEW ARTICLE

CRISPR/Cas9 Gene Editing: From Basic Mechanisms to Improved Strategies for Enhanced Genome Engineering *In Vivo*

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Abstract: Introduction: Targeted genome editing using the CRISPR/Cas9 technology is becoming a major area of research due to its high potential for the treatment of genetic diseases. Our understanding of this approach has expanded in recent years yet several new challenges have presented themselves as we explore the boundaries of this exciting new technology. Chief among these is improving the efficiency but also the preciseness of genome editing. The efficacy of CRISPR/Cas9 technology relies in part on the use of one of the major DNA repair pathways, Homologous recombination (HR), which is primarily active in S and G2 phases of the cell cycle. Problematically, the HR potential is highly variable from cell type to cell type and most of the cells of interest to be targeted *in vivo* for precise genome editing are in a quiescent state.

Conclusion: In this review, we discuss the recent advancements in improving targeted CRISPR/Cas9 based genome editing and the promising ways of delivering this technology *in vivo* to the cells of interest.

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1. INTRODUCTION

Since their discovery in bacteria and archaea, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their associated nuclease Cas9 have generated considerable excitement, akin to the unraveling of the RNA interference pathway in the late 90's. The CRISPR/Cas9 revolution offers an efficient, reliable, affordable and more amenable way to manipulate gene function and has sparked renewed enthusiasm (and ethical debate) on the use of gene editing as therapy for hereditary and acquired diseases including viral infections and cancer.

The most common CRISPR/Cas9 approach is to inactivate or knock-out a gene of interest in cells by relying on the Non-Homologous End-Joining (NHEJ) pathway and the introduction of insertions and/or deletions (indels), which ultimately disrupt the targeted locus (detailed in the section below). This way of generating knock-out cells has proven its efficacy and its affordability. On the other hand, the use of the CRISPR/Cas9 technology for precise mutagenesis in

cell and animal models, and gene correction in the case of hereditary disease, has been much more challenging. One of the core reasons is that precision gene editing requires Homologous Recombination (HR), a pathway that is less efficient than NHEJ in the repair of CRISPR-Cas9-mediated DNA breaks in part due to being restricted to proliferating cells. Engineered variants of the Cas9 nuclease, use of small molecules as well as optimization in the delivery timing of the CRISPR/Cas9 have provided alternative ways to favor precision gene editing by HR. Nevertheless, the precise editing of genes, particularly *in vivo* and in quiescent cells, remains a clear challenge, and currently limits the realization of the full potential of the CRISPR/Cas9 technology for applications in gene therapy.

This review focuses on the basic mechanisms underlying CRISPR/Cas9 genome engineering and the different techniques and approaches used to enhance genome editing and their implications for targeting non-dividing cells for gene therapy.

2. DNA REPAIR PATHWAY CHOICE AFFECTS THE OUTCOME OF Cas9-MEDIATED DNA BREAKS

Using a guide RNA, the Cas9 enzyme creates DNA double-strand breaks (DSBs) that are channeled in DNA repair mechanisms. In mammalian cells, DSBs are primarily re-

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paired by either NHEJ or by HR. During HR, a DNA repair template with homology to DNA flanking the DSB, such as a sister chromatid or exogenous DNA, can be used for precise gene editing (Fig. 1).

However, multiple factors influence the choice between NHEJ or HR, also called DNA repair pathway choice, following the detection of the DSB lesion [1]; this topic has been a very active area of research in the past decade and is highly relevant for those researchers working with CRISPR/Cas9. Below, we discuss the machinery and mechanisms underlying NHEJ and HR, which provide context for understanding how to enhance and best employ CRISPR/Cas9 for precise gene editing.

2.1. Canonical Non-homologous End Joining (c-NHEJ)

In mammalian cells, DSBs are predominantly repaired by NHEJ [2], in contrast to yeast that show a preference for HR and tend to have a poor capacity for ligating blunt DNA ends [3]. The NHEJ pathway is subdivided into two different repair mechanisms: classical/canonical NHEJ (c-NHEJ), and alternative end joining (alt-NHEJ).

During c-NHEJ, repair of the DSB is initiated by binding of the heterodimer Ku70/Ku80 to DNA ends and activation of the DNA protein kinase catalytic subunit (DNA-PKcs), which together protect the broken DNA ends as well as maintain them in close proximity [4, 5] (Fig. 1). Once activated, DNA-PKcs phosphorylates the histone variant H2AX within chromatin flanking the DSB at Serine 139. Nevertheless, the phosphorylation of H2AX relies largely on the ATM and ATR kinases, with DNA-PKcs being dispensable for DSB-induced chromatin signaling [6, 7]. Once phosphorylated, this form of H2AX is referred to as γ -H2AX, and its role is to facilitate DNA lesion detection and recruitment of DNA repair factors is elaborated in more detail below.

DNA-PKcs autophosphorylation and its phosphorylation by ATM kinase are also critical in c-NHEJ as they facilitate the recruitment of DNA polymerases lambda and mu, as well as the nuclease Artemis to the break site. For the latter, phosphorylation of DNA-PKcs regulates the 5' exonuclease activity of Artemis and its ability to cleave 3' ssDNA overhangs to generate blunt dsDNA ends that can be ligated [8, 9]. As a result of these activities at the DNA break, DNA bases are added and removed during c-NHEJ to generate small insertions and deletions (indels) (Fig. 1). DNA ligase 4 (LIG4), X-ray repair cross-complementing protein 4 (XRCC4) and XRCC4-like factor (XLF) are consequently recruited to the DNA break site. At the break, XLF then aids LIG4 in the ligation of the DNA ends to complete the repair process [2]. Thus despite being efficient and active throughout the cell cycle, DNA repair by c-NHEJ has the potential to be error prone [10].

2.2. Alternative Non-homologous End Joining (alt-NHEJ)

The alt-NHEJ pathway, also known as microhomology-mediated end joining (MMEJ), is Ku70/80-independent and requires microhomology in the range of 4-14 nucleotides [11] (Fig. 1). Alt-NHEJ employs different effector proteins than c-NHEJ and can result in deletions and gene rearrangements, including chromosome translocations on mice [12,

13]. However, it should be noted that c-NHEJ is the predominant mechanism underlying chromosomal translocations in human cells [14]. One of the major effector proteins in alt-NHEJ is Poly(ADP-ribose) polymerase 1 (PARP1), which modifies several proteins by ADP-ribosylation during DNA repair including the SUPT16H (a nuclear exchange factor) [15]. In addition, the poly(ADP-ribose) polymer chains formed by PARP1 also facilitate protein-protein interactions [16]. PARP1 can directly compete with DNA-PKcs and the Ku heterodimer for DNA end-binding [15, 17]. The importance of this competition for DNA end-binding is illustrated by the fact that Ku70/80 can repress alt-NHEJ and HR [18, 19], and thus competition between PARP1 and the Ku complex is a likely contributing factor in DNA repair pathway choice. PARP1 can also enhance the spread (and retention) of H2AX on chromatin by modifying SUPT16H, thereby inhibiting the exchange of γ -H2AX in nucleosomes [3]. This inhibition facilitates the recruitment of alternative DNA damage sensors such as the MRE11/RAD50/NBS1 (MRN) complex and thus shifts DNA repair pathway choice away from c-NHEJ [3]. In addition, it has been demonstrated that phosphorylation of XRCC1 by casein kinase 2 (CK2) following ionizing radiation-induced DNA breaks can promote the interaction of XRCC1 with MRE11 to favor alt-NHEJ [20]. Alt-NHEJ is active throughout the cell cycle although evidence suggest that it may primarily occur in the S/G2 cell cycle phases [21-23]. Like HR, alt-NHEJ involves DNA end resection by the MRN complex and the nuclease RBBP8/CtIP [12, 19] but the extent of the DNA end processing is much more limited than in HR. End resection facilitates the exposure of DNA microhomologies that contribute to the larger deletions, rearrangements and chromosomal translocations that occur during alt-NHEJ [12, 19]. In contrast to c-NHEJ, DNA ligase activity during alt-NHEJ relies on DNA ligase 1 (LIG1) (microhomology-independent alt-NHEJ) and DNA ligase 3 (LIG3)/XRCC1 (microhomology-dependent alt-NHEJ) rather than LIG4 [13]. In addition, alt-NHEJ relies on the activity of DNA polymerase theta (POLQ), which provides an opportunity for selective modulation of alt-NHEJ *via* genetic or chemical inhibition of this polymerase [1, 24].

2.3. Homologous Recombination

Due to the requirement for a sister chromatid as template, HR-mediated DSB repair is primarily restricted to the S/G2 phases of the cell cycle [22, 23]. Similar to NHEJ, the phosphorylation of the histone variant H2AX at DSB by the kinases ATM, ATM and Rad3-related (ATR) and DNA-PK is a pre-requisite for the initiation of HR [25]. γ -H2AX is then detected by both MDC1 [26] and the MRN complex (likely through direct binding of NBS1 to γ -H2AX and/or *via* interaction with RAD17)[27, 28]. There is also evidence that the MRN complex can bind to DNA ends even in the absence of γ -H2AX [29], which may facilitate both initial lesion detection and subsequent "trapping" of broken DNA ends in close proximity [4]. If the DNA ends are not easily ligated due to base damage, the 5' ends of the DNA are resected by nucleases such as MRE11 and CtIP (short tract resection), or EXO1 and DNA2-BLM (long tract resection) to yield 3' ssDNA [23, 30] (Fig. 1). Extensive DNA resection is counteracted by the helicase HELB in order to protect

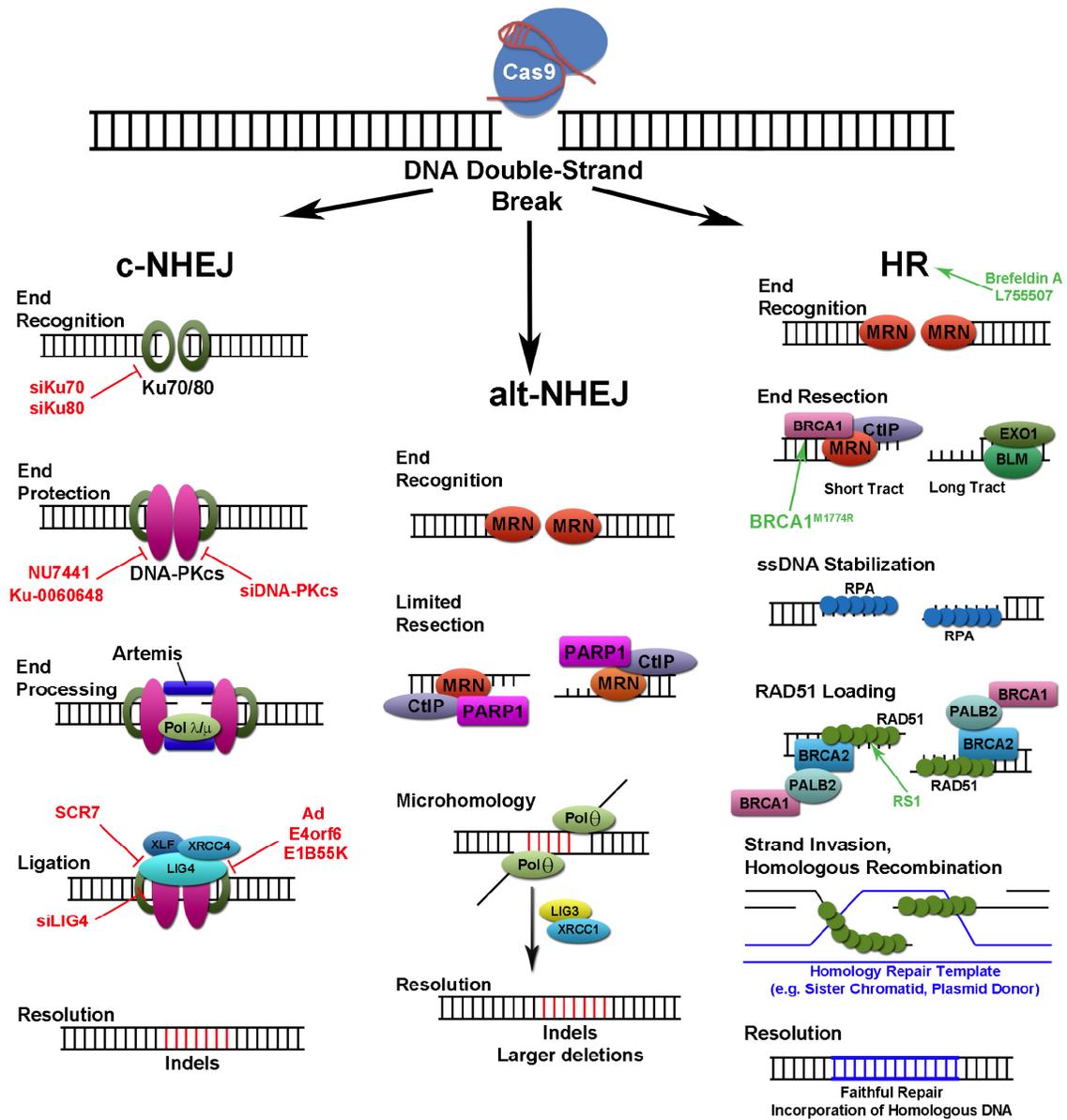


Fig. (1). Overview of DNA repair pathways involved in repair of Cas9-mediated DNA DSBs. The Cas9/small guide RNA (sgRNA) complex can generate a site-specific DNA double-strand break (DSB) resulting in exposed DNA ends that are recognized by either Ku70/80 or MRN (MRE11/RAD50/NBS1) end-recognition complexes. When bound by KU70/80, repair proceeds through classical non-homologous end joining (c-NHEJ), which is the major DNA repair pathway, particularly in G1 cells. The recruitment of DNA-PKcs, Artemis and DNA polymerase (Pol λ/μ) coordinates end processing, resulting in small insertions and deletions of less than 20 nucleotides. The ends are then ligated with the DNA ligase 4 (LIG4), XRCC4 and XLF1 complex. Suppression of NHEJ using siRNA, small molecules (SCR7, NU7441, Ku-0060648) or adenoviral proteins E4orf6 and E1B55K (shown in red) can result in increased DNA repair through alternative NHEJ (alt-NHEJ) or homologous recombination (HR). DNA repair using the alt-NHEJ pathway begins with the recognition of DNA ends by the MRN complex. The recruitment of PARP1 and CtIP results in limited 5' end resection and exposure of 3' single stranded DNA (ssDNA) overhangs that can locally interact with regions of microhomology (4-14 nucleotides). Single-stranded DNA overhangs are created by annealing of complementary DNA strands, which are removed and the intervening gap in DNA sequence is filled in by the activity of DNA polymerase theta (Pol θ , also known as POLQ). XRCC1 and DNA ligase 3 (LIG3) then contribute to the resolution of DNA break, resulting in end ligation with larger genomic deletions compared to c-NHEJ. During the S and G2 phases of the cell cycle, when sister chromatids are present, DNA repair can proceed through HR. The recognition of DSBs by the MRN complex initiates HR where the recruitment of BRCA1 and CtIP facilitates limited 5' end resection (short tract). This is followed by longer end resection by the BLM/EXO1 protein complex (long tract). The exposed ssDNA is initially bound and stabilized by the RPA protein, which is then exchanged for RAD51 with the assistance of the BRCA1-BRCA2-PALB2 complex. RAD51-bound ssDNA can then search and invade homologous DNA such as sister chromatid or foreign homology repair template, including plasmid DNA. Resolution of the HR process results in faithful incorporation of the homologous sequence with the broken DNA ends. This process can be exploited to introduce novel DNA sequences into the genomic DNA at the site of the DSB. HR can be enhanced through the use of small molecules such as brefeldin A, L755507 and the Rad51 activator RS1 or over expression of BRCA1 (shown in green).

the genome from over-resection [31]. The 3' ssDNA ends are then bound and protected by RPA, which is later exchanged for RAD51, a process that requires the breast cancer genes 1 and 2 (BRCA1 and BRCA2) and the Partner and Localizer of BRCA2 (PALB2) proteins (reviewed in [32-34]). RAD51-DNA filaments formed on the 3' ssDNA in turn enable homology search and DNA strand invasion to initiate the recombination process. Strand invasion leads to the formation of displacement loops (D-loops) in the homologous DNA template which allows DNA synthesis by DNA polymerase ϵ or δ [35, 36]. In mitotic cells, synthesis-dependent strand annealing (SDSA) is the major pathway used to complete HR, by displacement of the extended D-loop with annealing with the second extremity of the break, leading to noncrossover products (reviewed in [37]) (Fig. 1).

2.4. Repair Pathway Choice

Although the activation or mobilization of several DNA repair factors is dependent on cell cycle-regulated events, the molecular mechanisms that determine repair pathway choice between HR and NHEJ are complex and have only been partially elucidated (reviewed in [1]). The occupancy of DNA ends by either the Ku70/80 or the MRN complex is a critical step in the choice between HR and NHEJ, as it controls the initiation of DNA end resection by MRN/CtIP [1, 2]. It is well documented that CtIP activity is directly controlled by CDK-dependent phosphorylation in vertebrates, restricting resection activity needed for HR to the S/G2 phases [38]. But CDK activity promotes DNA end resection by targeting several additional DNA repair factors, including EXO1 and NBS1 [39-42]. Finally, CDK2 may promote DNA end resection by controlling the nuclear concentration of the ssDNA translocase HELB [31].

One of the major molecular events controlling DNA repair pathway choice relies on the antagonizing roles of 53BP1 and BRCA1 at DSBs [43]. 53BP1 accumulates at DSBs by recognizing both methylation of histone H4 at Lys20 and RNF168-dependent ubiquitylation of H2A Lys15 [44, 45]. This event is primarily favored during G0/G1 and promotes NHEJ while opposing HR, in part by preventing the accumulation of BRCA1 and CtIP to DSBs and by blocking DNA end resection [46-49]. It relies on the recruitment of several effectors including RIF1, PTIP and MAD2L2/REV7 [46-53]. In contrast, during S/G2, BRCA1 association with DSBs peaks, excluding 53BP1 and RIF1 from sites of damage and promoting HR [48]. DNA-PKcs also regulates the choice between NHEJ and HR by acting as an antagonist of DNA end processing [54]. However, various autophosphorylation states of DNA-PKcs can alter its association with DNA ends and can promote either NHEJ or HR [54-56].

Homology search is also tightly regulated by the cell cycle. Early evidence has shown that RAD51 association to BRCA2 is restricted to S/G2 phases of the cell cycle through the CDK-dependent phosphorylation of BRCA2 C-terminus [57]. More recently, the identification of cell cycle-regulated ubiquitylation events dictating the association of the BRCA1-PALB2-BRCA2 complex have further confirmed that homology search is primarily restricted to S/G2 [58].

A mechanistic understanding of the various DSB repair pathways and their regulation is critical in the choice of gene-editing strategy. On one hand, since the NHEJ pathway is available throughout the cell cycle, it can be exploited for the introduction of indels in target genes to disrupt or “knock-out” their function in both cell and animal models. HR, on the other hand, is better suited to “knock-in” strategies and site-specific gene correction or mutation. However, the restriction of HR to S/G2 phases of the cell cycle and its low efficiency may limit the use of this pathway for gene therapy strategies in non-dividing tissues. One means of overcoming this limitation is to edit the germline cells of individuals to “repair” their genome and that of their progeny in future. However, ethical considerations notwithstanding, it is not clear whether germline gene editing is actually necessary, due to well-established alternative methods, including prenatal genetic diagnostics or *in vitro* fertilization (IVF) coupled to pre-implantation genetic profiling [59, 60]. Nevertheless, patients currently experiencing genetically linked diseases have great hopes that genome editing in somatic cells may resolve some of their issues, in absence of any alternative therapy.

3. MANIPULATION OF THE CRISPR/Cas9 SYSTEM

To efficiently edit the genome of cells by HR *in vivo*, optimizing each step of the CRISPR/Cas9 system is critical. It starts by properly designing specific sgRNAs to generate DSBs that will enable correction/editing of the desired DNA sequence. But it may also require several additional elements including the use of mutants of the Cas9 enzyme and the generation of the best template for repair. The different strategies for improving the CRISPR/Cas9 system are discussed in the section below, discussing non-exhaustively the parameters that can be altered to favor HR.

3.1. sgRNA Design Considerations

The minimum requirement for Cas9 small guide RNA (sgRNA) design is that the sgRNA be homologous to a 20nt sequence upstream (5') of a NGG protospacer adjacent motif (PAM), where N = any nucleotide [61, 62]. Although there is some tolerance for mismatches, which can contribute to off target effects, the Cas9 enzymatic activity is directed to cut the DNA backbone between the 3rd and 4th nucleotide upstream of the PAM motif [62]. Many academic and commercial online resources are now available for designing and choosing appropriate sgRNAs with minimal predicted off-target cutting for HR-based genome editing [63]. For HR-based editing using Cas9 nickases (Cas9n), the location of potential sgRNAs can be restricted to small regions (50-100nt) around a desired cut site. However, for efficient HR, it is desirable to cut as close to the location of the site of change as possible [64], which may limit the use of Cas9n variants. Therefore, compromises must sometimes be made while selecting sgRNAs between lowest predicted off-target activity and efficient on-target editing to facilitate HR. Mutations to the PAM and sgRNA target site in the homology repair template can also be introduced to prevent re-cutting of edited gene loci and potential introduction of indel mutations. If the sgRNA targets coding regions, it is possible to modify the homology donor using conservative mutations

that prevent changes to the coding sequence yet prevent re-cutting of the edited gene.

3.2. Chemically Modified sgRNAs

Another critical factor to consider when manipulating the CRISPR/Cas9 system for HR is the synthesis step of the RNA components for genome editing. SgRNAs are typically generated enzymatically using *in vitro* transcription assays, which is a very efficient way of obtaining unmodified sgRNAs. However, it requires several purification steps to remove the different components following the completion of the *in vitro* reaction and can result in errors in the sgRNA sequence.

Alternatively, sgRNAs can be chemically generated using a solid-phase synthesis approach. This method provides several advantages: (i) it rapidly and accurately produces sgRNAs of different length and sequences; (ii) it can be used to generate sgRNAs for both wild-type and alternative Cas9 versions; (iii) it is the only method that permits the incorporation of targeted chemical modifications in the sgRNA. Several chemical modifications of the sgRNA have proven to increase significantly genome editing efficiency, specificity and delivery. They range from base modification (2-thiouridine, N6-methyladenosine, pseudouridine), to backbone modification (alteration of the phosphate bond between the two sugars), sugar modification (2'OMe, 2'F-, LNA, 2'NH₂) or covalent conjugations on the 5' or the 3'-end of the sgRNA sequence. For instance, the chemical modification of both the phosphate backbone and the sugar of the sgRNA has been used to improve resistance to nucleases [65-67] and resulted in up to ten times higher editing efficiency than the unmodified sgRNA in human primary cells.

These chemically modified sgRNAs have great potential for therapeutic applications *in vivo*. By improving *in vivo* stability, intracellular availability and potentially escaping the immune system, chemically modified sgRNAs could improve the therapeutic properties of the CRISPR/Cas9 system and favor its use for genome editing in somatic cells.

3.3. Nuclease Choice

Many Cas9 and related nucleases have been described and made available to the research community. Beyond wild type humanized *S. pyogenes* Cas9 (hSpCas9), "nickase" versions of the Cas9 enzyme harboring either the D10A and H840A mutations have been developed, termed Cas9n variants, which are capable of cutting only one strand of DNA and when paired with two specific sgRNAs can deliver site-specific DNA breaks with a 50-1500-fold reduced off-target activity [62, 68, 69]. Enzymatically-dead Cas9 (dCas9- *i.e.* harboring both D10A and H840A mutations) fused to half of the homodimeric FokI endonuclease has also been developed as an approach to reduce off-target DNA cleavage [70, 71]. Again, two sgRNAs are used to direct the dCas9-FokI fusion protein (fCas9) to target DNA loci, and upon dimerization of the FokI domains the nuclease can generate a specifically programmed DNA DSB, with the potential benefit of eliminating unwanted ssDNA breaks generated by Cas9n.

Through site-directed mutagenesis, two high fidelity variants of SpCas9 have also been developed eSpCas9(1.1) and SpCas9-HF1 [72, 73]. These high fidelity variants retain on-target activity while demonstrating greatly reducing off-target activity. Given their enhanced fidelity, it is likely the use of eSpCas9(1.1) and SpCas9-HF1 will be widely adopted as the gold-standard approach for gene editing. Nonetheless, it is prudent to always measure off-target activity during gene editing and several methods have been developed to facilitate these measurements (reviewed in [74]), including the genomic PCR and Surveyor nuclease assay [75], as well as high-throughput sequencing approaches such as Genome-wide Unbiased Identification of DSBs Enabled by sequencing (GUIDE-seq) [76], Breaks Labelling, Enrichment on Streptavidin and next-generation Sequencing (BLESS) [77], and most recently Circularization for *In vitro* Reporting of Cleavage Effects by sequencing (CIRCLE-seq) [78].

Another consideration is the GC-content of the gene (or genome) to be edited. The Cpf1 enzyme is isolated from *Francisella novicida* favours a PAM motif that is T-rich (5'-TTTV-3', where V= A, C, or G) and thus may be more useful in targeting AT-rich gene regions. In addition, Cpf1 is smaller and cuts the DNA in a staggered fashion creating a 5 nucleotide 5' overhang starting 18 nucleotides 3' of the PAM. Although this property of Cpf1 has yet to be exploited, it could be used potentially in the design of gene editing strategies that promote NHEJ-based directional insertion of DNA at specific genetic loci.

3.4. Cas9 Chimeras: The Future of Precise Genome Editing?

A recent and novel approach to introduce point mutations emerged from the use of the catalytically inactive version of the nuclease Cas9 (dCas9 D10A H840A) fused a cytidine deaminase (APOBEC1 or AID) [79, 80]. This chimera can be directed to a specific genomic region through the help of a sgRNA, but unlike the wild-type version of Cas9, it does not induce DSBs but rather promotes the conversion of cytosine into uracil, which results in C-T or G-A substitutions. One potential drawback of this strategy is that the deaminase is processive and can convert all C's to U's within a ~5 base pair window near the distal end of the protospacer from the PAM. Thus, editing may occur on undesired cytosine bases nearby. But this type of "tweaking" of the CRISPR/Cas9 system may open new avenues for the precise introduction of point mutations in a desired locus. For example, recently the spectrum of sequences that are targetable in the genome and the precision of base-editing using Cas9-deaminase fusions has been greatly enhanced by employing various species of Cas9 (with different PAM motifs), as well as site-directed mutagenesis of the deaminase itself to allow editing at near base pair resolution [81].

3.5. ssODNs for Point Mutations and Short-length Gene Edits: (<100bp)

For point mutations or the introduction of small peptides for epitope-tagging (*e.g.* FLAG, HA, V5) it is possible to employ a single-stranded oligodeoxynucleotide (ssODN) as the homology donor for Cas9-mediated HR. Typically less

than 200 nt long, ssODNs are generally affordable, easy to synthesize and thus highly versatile gene-editing tools. The homology arms are usually 30-60 in length and flank the intended point mutation or epitope-tag sequence of anywhere from 1-100 nt in length [75, 82]. Upon Cas9 cleavage of the DNA, the ribonuclease can locally release the 3' end of the non-target DNA strand, making it accessible to ssODN donor sequences that are complementary to the non-target strand, thus facilitating gene editing by gene conversion [83]. As a rule of thumb, the intended point mutation should be at a distance of no more than 10-100 nt from the DNA break induced by Cas9, as the efficiency of gene conversion drops dramatically beyond 100 nt [64]. Design of asymmetric ssODN homology arms (*e.g.* 91 nt PAM-proximal, 36nt PAM-distal) flanking the genomic DNA break can also facilitate enhanced HR rates of up to 60% [83].

4. FURTHER OPTIMIZATION STRATEGIES FOR HR

Besides altering the CRISPR/Cas9 components directly, alternative options have been explored to improve genome editing, including the use of small molecules and drugs that will directly or indirectly inhibit the downstream DNA repair pathways. This section discusses the large spectrum of small molecules and genetic tools that can bias the DNA repair pathways toward more HR potential in mammalian cells.

4.1. Small Molecules: NHEJ Inhibitors and HR Enhancers

Pharmacological intervention has been a particularly attractive strategy for shifting DNA repair pathway choice in favor of HR (Fig. 1, red and green arrows). For instance, inhibition of the DNA ligase LIG4 by the small molecule SCR7 was used to suppress NHEJ in mammalian cells in conjunction with the Cas9 system, resulting in ~2 to ~20-fold enhancement of HR and genome editing [84-86]. However, we [87] and others [88-91] have found limited enhancement of Cas9-mediated HR with SCR7, suggesting that this drug is not universally useful for enhancing HR in all experimental systems. Alternatively, NHEJ suppression has also been accomplished through inhibition of DNA-PKcs using the small molecules NU7441 [92] and Ku-0060648 [93], resulting in ~2-4 fold enhancement of Cas9-mediated HR in HEK293T cells and mouse embryonic fibroblasts (MEFs) using donor plasmids or ssODNs as repair templates [85]. Altogether, NHEJ suppression by various pharmacological methods has shown promising results in enhancing Cas9-mediated HR.

Using small molecules to directly promote HR has been another route to increase precise genome editing. We have recently shown that enhancement of HR using the Rad51-stimulating small molecule RS1 [94] can increase Cas9-mediated HR ~3-6-fold in HEK293 and U2OS cells [87]. RS1 is also effective at enhancing Cas9-mediated HR ~2-5-fold in rabbit embryos [88]. The above studies generally report little to no cell toxicity at effective doses for these small molecules.

Finally, high-throughput screening of chemical compounds has identified alternative modulators of precise genome editing with unclear role in modulating DNA repair

pathway choice. For example, the small molecules Brefeldin A, an ER-Golgi transport inhibitor [95] and L755507, a β -adrenergic receptor agonist [96], enhance Cas9-mediated HDR ~2 to 9-fold [97]. Altogether, these small molecules expand the efficiency of CRISPR/Cas9-mediated precise genome editing in human cells and provide a foundation for further applications *in vivo*.

4.2. Genetic Tools to Promote HR-mediated Genome Editing

Akin to reprogramming of somatic cells to induced pluripotent cells (iPS), where knocking down p53 or providing a mutant/negative regulator of this factor is an essential step [127], manipulating the DNA repair pathways in a similar way may be critical to improve precise genome editing. For example, Chu *et al.* demonstrated a 2.5-5-fold enhancement of HDR by silencing the NHEJ effector molecules Ku70, Ku80, and Lig4, individually and in combination [84]. Similarly, silencing Lig4, DNA-PK, Ku70 and Ku80 suppressed NHEJ and increased HDR by approximately 2-fold [85].

The adenoviral proteins E1B55K and E4orf6 can also suppress NHEJ when ectopically expressed in target cells. These viral proteins promote the proteosomal degradation of Lig4 [98-100] and co-expression of E4orf6 and E1B55K from adenovirus serotype 5 [85] or serotype 4 [84] can enhance HDR by 3.5- to 7-fold respectively. E1B55K and E4orf6 from some adenovirus serotypes, including type 5, can lead to the degradation of other cellular proteins involved in DNA repair, such as p53 and MRE11 [99]. Thus, while this approach helps to demonstrate the ability to enhance HDR through suppression of NHEJ, the multiple cellular targets for these proteins may confound their use in more therapeutic applications of gene editing where more precision and control is desirable.

We also used a similar over-expression approach, to promote HDR by expressing BRCA1 variants that promote hyper-recombination [46, 87]. BRCA1 promotes HR and expression of wild-type BRCA1 or the K1702M or M1774R hyper-recombination variants in our system showed a moderate increase in HDR up to ~3-fold enhancement with the M1774R variant [87]. Overexpression of RAD51 can also enhance HR [101] alone, or in combination with the RAD51 stimulatory compound RS1 may further enhance HDR in the presence of Cas9-mediated DSBs. More recently, the Durocher lab developed an ubiquitin variant that has the unique property of specifically inhibiting 53BP1, thus inhibiting NHEJ [102]. This genetic inhibitor enhanced precise gene targeting by about 2-fold. Altogether, the greatest limitation on the use of HDR in genome editing is the low efficiency of HDR compared to NHEJ. This in part can be bypassed using genetic tools discussed previously. However, the impact of genetic and chemical modulation of DNA repair mechanisms to promote HDR on the genomic integrity of the cell has not been fully evaluated. Finally, an additional element to take into account regarding the factors and limitations that can restrict genome editing efforts in non-proliferating cells, is cell cycle positioning.

4.3. The Effects of the Cell Cycle on Gene Editing

Since the HR pathway of DNA DSB repair is most active in S and G2 phases of the cell cycle, one possible strategy to enhance precision gene editing by HR is to promote and/or prolong S and G2 phase in target cells. Several well-established methods have been described that can synchronize cells by preventing progression through the various stages of the cell cycle and these have been applied to CRISPR/Cas9 gene editing strategies. For example, the Doudna laboratory tested several compounds inhibiting the cell cycle for their ability to enhance HR-mediated repair of a Cas9-mediated DNA break [103]. Pre-synchronization of cells in mitosis using nocodazole (a microtubule poison) enhanced Cas9-mediated gene editing by ~2-fold without a detectable increase in off-target Cas9 activity [103]. Similarly, synchronization with the microtubule inhibitor ABT-751 increased HDR (3- to 6-fold) in human pluripotent stem cells and neural precursor cells [91]. Synchronization and release from S-phase by pretreatment with aphidicolin or thymidine was also shown to increase HR by ~2-3-fold in both human primary neonatal fibroblasts and embryonic stem cells [103]. Although cell cycle synchronization did not produce drastic improvements in HR-mediated gene editing, these approaches can be combined with other drug treatments or genetic manipulations (e.g. knock-down of NHEJ repair factors) [84] to further improve precision gene editing efficiency. Of course, cell synchronization approaches rely heavily on the ability of the target cell to proliferate and are pointless for the enhancement of gene editing by HR in quiescent or post-mitotic cells

Yet another approach is to minimize the activity of Cas9 in G1 phase of the cell cycle when NHEJ prevails and HR is inhibited. Gutschner *et al.* [89] accomplished this feat by generating a Cas9-Geminin (Cas9-Gem) fusion protein that is a substrate for the APC/Cdh1 E3-ligase complex during G1 to trigger the degradation of the Cas9 enzyme in this phase of the cell cycle by the proteasome. When the cell enters S/G2/M of the cell cycle, the APC/Cdh1 complex is inactivated and Cas9 is stabilized. Since HR is active in S/G2 of the cell cycle, the DNA break induced by the Cas9-Gem fusion protein can be repaired by HDR. Using this approach it was possible to achieve an 87% enhancement of HR at the AAVS1 locus in HEK-293T cells [89]. Similar to drug-induced cell synchronization, this approach is only effective in proliferating cells.

Until very recently, it was not possible to achieve repair of DNA breaks in G0/G1 cells using HR. However, in a recent study [58] the Durocher laboratory described a novel mechanism of HR inactivation by the KEAP1/CUL3 ubiquitin E3 ligase complex in G1 and provided a mechanism through which HR can be reactivated in non-cycling cells. In G0/G1 cells there are several barriers to the initiation of HR repair at a DNA DSB. First, recruitment of BRCA1 to DNA breaks, a pre-requisite for efficient HR, is inhibited by 53BP1 [46]. Secondly, the endonuclease CtIP, which is involved in end-resection during HR, is not fully active due to that fact that the CDKs responsible for its phosphorylation are also not active in this phase of the cell cycle [38, 104]. Finally, ubiquitination of PALB2 by KEAP1/CUL3 complex results in inhibition of BRCA1's interaction with the

PALB2/BRCA2 complex, which is a requirement for RAD51 filament formation during HR [58]. To overcome these barriers, this team expressed constitutively active CtIP (T847E mutated) in a 53BP1 null cell combined with the co-depletion of the KEAP1/CUL3 complex by siRNA, which in turn allowed HR to precede G0/G1 cells [58]. This work provides a new conceptual basis for strategies to edit the genomes of non-cycling cells, and thus may enable new approaches for gene editing in non-dividing cells of the brain, spinal cord and muscle.

These HR-enhancing strategies discussed above have yielded increases in HR in the range of 2-20-fold. This may be sufficient for some experimental systems, allowing relatively efficient selection of gene edited cells in an experimental context. However, even combining various HR strategies has generally shown only modest additional increases in HR overall [84, 85, 87]. A corollary to this observation is that there may be a practical experimental limit to the overall enhancement of HR that can be achieved in a given experimental system. The factors affecting this limit could include the overall cell cycle profile, the efficiency of cell transfection, and cell-intrinsic recombination activities.

4.4. Co-selection Strategies

The introduction of a selectable marker to gene editing strategies has become a popular way to overcome the intrinsic limit of gene editing in a given cell model. For example, given the multiplex gene editing capabilities of the CRISPR/Cas9 system, a selective marker can be co-targeted to a region of the genome to allow selection of correctly edited cells by antibiotic resistance. Ideally, editing of these "safe harbor" loci should have minimal effect on the host cell function and several possible safe harbors have been identified including: *AAVS1*, *CCR5* and *ROSA26* loci in humans; and the *Rosa26* locus on chromosome 6 in mice (reviewed in [105]) – all of which can be used for a co-selection strategy. The AAVS1 locus has been used to insert a variety of gene expression cassettes (e.g. cDNA), reporter assays (e.g. HDR, Luciferase) or selection markers (puromycin resistance, GFP) [106-109]. Recently, the Doyon lab has developed a robust CRISPR/Cas9 co-selection strategy based on the generation of resistance to the Na⁺/K⁺ ATPase (ATP1A1) inhibitor, ouabain [110]. By combining this approach to targeted HR-mediated genome editing in the locus of interest, they observed a ~13-15- fold increase in the number of cells displaying precise genome editing. Importantly, they were able to transpose this co-selection strategy to primary human cord blood cells to revert a mutation in the beta-globin gene (*HBB*), which is associated with sickle-cell anemia. Altogether, this co-selection strategy has the great potential of further increasing HR-mediated genome editing. However, these approaches have not been extensively evaluated for their appropriateness for therapeutic applications where disruption of the safe harbor genes at or near the target loci or the introduction of mutations in the *ATP1A1* locus may have adverse effects [111].

4.5. Efficient and Safe CRISPR/Cas9 Gene Delivery

A major issue to promote the therapeutic use of the CRISPR/Cas9 system is the lack of a safe and efficient *in*

in vivo delivery method. Initially, an adeno-associated virus (AAV) assisted approach has been used to efficiently edit the mouse apolipoprotein (*ApoB*) gene, the proprotein convertase subtilisin/kexin type 9 (*Pcsk9*) gene, the neuronal methyl CpG binding protein *Mecp2* gene and a family of DNA methyltransferases (*Dnmt1*, *Dnmt3a* and *Dnmt3b*) [112, 113]. More recently, *in vivo* genome editing of the cardiac and skeletal muscle cells in the *mdx* mouse model, which recapitulates the Duchenne Muscular Dystrophy (DMD) syndrome, used an AAV approach coupled to the CRISPR/Cas9 system [114-116]. Although AAVs have a low immunogenic potential and reduced risk of oncogenic transformation, its limited loading capacity of ~4.7 kb of DNA and its persistence in the host could prevent the wide use of this approach for the delivery of the CRISPR/Cas9. Other viral vectors with larger capacities, including adenoviral and lentiviral vectors, are currently being investigated (Cheng *et al.*, FEBS Letter 2014); however, the safety profile of integrative viruses including retroviruses is less than ideal given their documented ability to induce oncogenesis in early gene therapy trials [117].

As an alternative, several groups have reported the development of cationic lipid-based and cationic polymer-based particles. These lipid-like nanoparticles (LLNs) or a hybrid system using this technology [118-120] allows the Cas9 mRNA and the sgRNA to be entrapped in these LLNs and efficiently delivered *in vivo*, in particular to the liver. Successful *in vivo* editing of the *Pcsk9* gene, the blood clotting factor IX (*FIX*) gene and the fumarylacetoacetate hydrolase (*Fah*) has been reported [118-120]. *In vivo* delivery of the Cre recombinase and the Cas9/sgRNA complexes to hair cells in a mouse inner ear has also been achieved using this approach [121]. These non-viral delivery approaches have the benefit of bypassing some of the limitations of the AAV system but may be challenging to use for specific organs of limited accessibility.

Finally, physical means have been explored to the genomes of cells *in vivo*. For example, hydrodynamic injections have been used to deliver the CRISPR/Cas9 system into mouse hepatic tissue *in vivo* and target the *Fah* and *Pten* genes [122, 123]. But it remains unclear whether electroporation and micro-injection could have a clear potential in favoring precise genome editing *in vivo*.

CONCLUSION

Since the initial discovery of the CRISPR-Cas9 and its application for editing the genome of cells, many advancements and improvements to this technology have been made, from the design of more suited sgRNAs to the generation of variants of the Cas9 nuclease. However, one major challenge of the CRISPR/Cas9 technology is the limited efficacy of precise genome editing, which in part relies on a balance between cellular DNA repair pathways. Deciphering the potential of each cell type to promote HR remains a critical element to promote precise genome editing approaches *in vitro* and *in vivo*. Alternatively, a more regulated spatiotemporal delivery of the CRISPR/Cas9 technology could provide an avenue for the use of precise genome editing in clinical applications. While this approach provides great promise for dividing cells, derived for instance from the hema-

topoietic system, quiescent cells in G0/G1 phase of the cell cycle would require alternative approaches and thus, represent a very important area of future study. One possibility is the use of alt-NHEJ-mediated targeted integration using PITCh (precise integration into target chromosome) [124] or NHEJ-based HITI (homology-independent targeted integration) [125] approaches, which are less restricted by cell cycle phase. In particular, HITI has shown efficacy in post-mitotic and quiescent cell types such as primary neurons [125].

While the development of precise genome editing carries great hope for basic researchers and clinicians for the study and treatment of genetic diseases, it also bears great concerns about its safety and the ethical implications of its use. As a community, we need to remain vigilant about the impact and purpose of using the CRISPR/Cas9 technology and its derivatives when safer and more proven technologies already exist for the prevention of genetic disease, including embryo screening by pre-implantation genetic diagnosis (PGD) [126].

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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