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Chapter

Maximizing the Efficacy of CRISPR/Cas Homology-Directed Repair Gene Targeting

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Abstract

Clustered regularly interspaced short palindromic repeats/CRISPR-associated system (CRISPR/Cas) is a powerful gene editing tool that can introduce double-strand breaks (DSBs) at precise target sites in genomic DNA. In mammalian cells, the CRISPR/Cas-generated DSBs can be repaired by either template-free error-prone end joining (e.g., non-homologous end joining/microhomology-mediated end joining [NHEJ]/[MMEJ]) or templated error-free homology-directed repair (HDR) pathways. CRISPR/Cas with NHEJ/MMEJ DNA repair results in various length insertions/deletion mutations (indels), which can cause frameshift mutations leading to a stop codon and subsequent gene-specific knockout (i.e., loss of function). In contrast, CRISPR/Cas with HDR DNA repair, utilizing an exogenous repair template harboring specific nucleotide (nt) changes, can be employed to intentionally edit out or introduce mutations or insertions at specific genomic sites (i.e., targeted gene knock-in). This review provides an overview of HDR-based gene-targeting strategies to facilitate the knock-in process, including improving gRNA cleavage efficiency, optimizing HDR efficacy, decreasing off-target effects, suppressing NHEJ/MMEJ activity, and thus expediting the screening of CRISPR/Cas-edited clonal cells.

Keywords: CRISPR/Cas, homology-directed repair, gene editing, Cas9, Cas12, non-homologous end joining, microhomology-mediated end joining, knock-in

1. Introduction

Clustered regularly interspaced short palindromic repeats/CRISPR-associated system (CRISPR/Cas) technology has revolutionized biological research and holds great therapeutic potential, since it is remarkably flexible and reliable [1–3]. CRISPR/Cas genome editing (i.e., genetic engineering) is a programmable technology to introduce double-strand breaks (DSBs) at specific target sites in the genome of a living organism [1–3]. There are two major mechanisms by which Cas enzyme-mediated DSBs are subsequently repaired [4–6]. The first is by template-free end joining (e.g., non-homologous end joining/microhomology-mediated end joining [NHEJ]/[MMEJ]), which introduces insertions/deletion mutations (indels) and can lead to targeted gene

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knock outs. The second mechanism is *via* the homology-directed repair (HDR) pathway, which produces a targeted gene knock-in or other specific mutations utilizing an exogenous donor template [4–6]. Given that DSBs generated in mammalian cells are predominantly repaired by NHEJ or MMEJ, the rate of precise editing through CRISPR/Cas/HDR with an exogenous repair template is significantly compromised/reduced [4–6]. This review summarizes multiple strategies to enhance the efficacy of CRISPR/Cas/HDR as well as decrease off-target effects.

2. CRISPR/Cas history

CRISPR history began in 1987 when Ishino et al. [7–9] first observed five repetitive palindromic sequences of 29 nucleotides separated by random 32 nucleotides toward the end of the *E. coli* genome. Although Ishino et al. [7] did not decipher the biological significance of the puzzling repeat sequences, this report led to the discovery of similar patterns in other bacterial and archaea genomes [10–12]. Mojica et al. [13] then established that the unusual repetitive DNA sequences were functionally related. These curious sequences were later designated "CRISPR" by Jansen et al. [14] given that these loci harbor: 1) palindromic repeats with little sequence variation; 2) non-repetitive spacer sequences between the repeats; and 3) a several hundred base pair (bp) common leader sequence on one side of the repeat cluster. The CRISPR locus is present in approximately 40% of the sequenced bacteria and 90% of the genomes of the different domains of archaea [15]. Finally, it was demonstrated that CRISPR-associated (Cas) genes (i.e., over 40), of which only a subset is found in any given prokaryote that harbor CRISPRs, are frequently located in close proximity to CRISPR loci [14, 16, 17]. The Cas genes were predicted to encode endo- and exonucleases, helicases, polymerases, and RNA-binding proteins [14, 15, 17].

Initially, CRISPR/Cas systems were expected to have a role in DNA repair or gene regulation due to their location near the DNA repair system in the bacterial genome [18]. However, in 2005, three seminal studies revealed that the CRISPR spacer sequences were homologous to bacteriophage, prophages, and conjugative plasmid sequences and suggested that they were the remnants of past invasions by extrachromosomal elements [19–21]. These investigators further speculated that there was a relationship between CRISPR and immunity against foreign DNAs by coding an anti-sense RNA [19–21]. In the following year, Makrova et al. [17] analyzed the link between the CRISPR and the Cas proteins and how this system is similar to the prokaryotic RNAi-mediated adaptive immune system, which led them to propose that the CRISPR/Cas system, with its "memory component," may function as inheritable adaptive immunity for bacteria.

Subsequently, Barrangou et al. [22] demonstrated that after a viral challenge, phage sequence was integrated into a CRISPR locus of *Streptococcus thermophilus* and provided immunity against the corresponding phage. When the protospacer sequence was deleted from the bacterial genome, they became sensitive to phage infection [22]. These investigators hypothesized that the nucleic acid based "immunity" system in prokaryotes was dictated by the CRISPR spacer sequence and that the Cas protein machinery mediated resistance against foreign DNAs [22].

In 2008, a pivotal study by Brouns et al. [23] established that the *E. coli* spacer sequences were transcribed into a precursor CRISPR RNA (pre-crRNA) that was matured to small crRNAs by a complex of Cas proteins. Additionally, it was demonstrated that mature crRNAs serves as a "guide" to a direct a protein to target viral

nucleic acids, which results in an antiviral response in prokaryotes [23]. Subsequently, Mojica et al. [24] identified CRISPR-type-specific proto-spacer adjacent motifs (PAMs), which are important for discrimination between self and nonself sequences. Further, Garneau et al. [25] showed that CRISPR/Cas immunity resulted from the generation of DSBs at specific sites in bacteriophage and plasmid DNA. Finally, Sapranauskas et al. [26] demonstrated that the *S. thermophilus* CRISPR/Cas system could be transferred to *E. coli* and provide a Cas9-mediated immunity that required a PAM site. Their initial characterization of the Cas9 protein revealed that two domains are involved in the formation of DBSs [26]. The Cas9 McrA/HNH-like nuclease domain cleaves the DNA strand complementary to the guide RNA sequence (target strand), and the RuvC/RNaseH-like domain cleaves the noncomplementary strand (nontarget strand) (**Figure 1**) [26]. They also demonstrated that a 20-nucleotide crRNA, the trimmed version of the full-length crRNA, is sufficient for DNA target identification with efficient cleavage and that the target site of the Cas can be changed by changing the crRNA sequence (**Figure 1**) [26].

Next, Deltcheva et al. [27] discovered an additional small RNA designated the trans-activating CRISPR RNA (tracrRNA). This small RNA is transcribed from

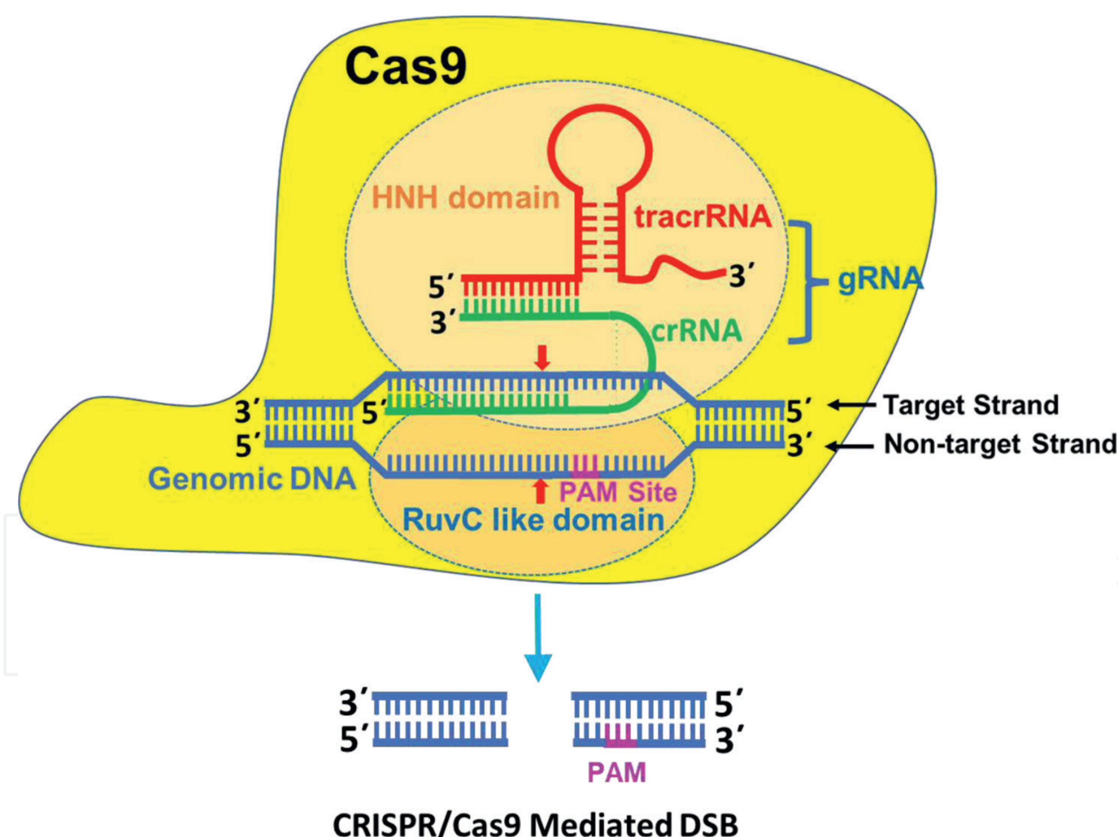

Figure 1.
Schematic representation of the CRISPR/Cas9-mediated DSB with a two-piece gRNA. The Cas9 gRNA is a two-piece RNA complex comprised of a crRNA required for DNA targeting (denoted in green) and the tracrRNA, which is necessary for nuclease activity (denoted in red) [27, 28]. The Cas9 protein (denoted in yellow) binds to the gRNA to form a RNP complex. The gRNA directs the Cas9 to a specific location in the genomic DNA (denoted in blue) through a user-defined 20 nt sequence at the 5′ end of the crRNA, which is complementary to the target DNA (denoted by green and blue hash marks). If there is a PAM site (NGG) (denoted in pink) adjacent to the 3′ end of the 20 nt sequence, then the Cas9 McrA/HNH-like nuclease domain (denoted in peach) cleaves the DNA strand complementary to the guide RNA sequence (target strand) and the RuvC/RNaseH-like domain (denoted in orange) cleaves the noncomplementary strand (nontarget strand) to introduce site-specific DSBs in the target DNA [26].

sequence upstream of the CRISPR-Cas locus of *Streptococcus pyogenes* [27]. These investigators demonstrated that, upon maturation of both the tracrRNA and the crRNA, they form a duplex that has both single- and double-stranded regions (**Figure 1**) [27]. Furthermore, Jinek et al. [28] verified that the two-RNA complex, dual RNA (i.e., the crRNA [required for DNA targeting] and the tracrRNA [necessary for nuclease activity]; now designated as a guide-RNA [gRNA]) directs the Cas9 to introduce site-specific DSBs in the target DNA (**Figure 1**). They also demonstrated that Cas9 target recognition required complementary seed sequences between the crRNA and target DNA as well as a PAM sequence containing a GG dinucleotide adjacent to the crRNA-binding region in the DNA target (**Figure 1**) [28]. Moreover, Jinek et al. [28] established that the *S. pyogenes* Cas9 endonuclease could be programmed to target and cleave any dsDNA sequence, which harbors a NGG (N denotes any nt) PAM site, with an engineered gRNA which contains a 20-nucleotide crRNA sequence that is complementary to the target DNA (**Figure 1**). CRISPR-Cas technology is now widely adopted in the scientific community due to its simplicity and precision for gene editing, which has opened the possibility of numerous applications in the field of genetic engineering.

3. DNA double-strand break repair

Pathological DNA DSBs can arise from normal endogenous metabolic cellular processes (e.g., DNA replication and transcription) or from cellular exposure to exogenous sources (e.g., reactive oxygen species, ionizing radiation, radiomimetic chemicals, and anticancer chemotherapeutic drugs) [29–32]. However, physiologically important DNA DSBs are also required for several developmental and physiological cellular activities including chromosomal disjunction, meiosis, V(D)J, and immunoglobulin heavy chain (IgH) class switch recombination [29, 33]. Notably, both pathological and physiological DNA DSBs require efficient repair processes since these lesions can result in insertions, deletions, chromosomal translocations, and genomic instability, which can lead to numerous hereditary human diseases, including cancer, developmental disorders, and premature aging [29–31, 33]. Mammalian cells employ multiple DNA repair pathways to protect the integrity of their genomes. However, the two predominate DNA DSB repair pathways that are template-free NHEJ/MMEJ and templated HDR [32, 34, 35]. It is important to note that NHEJ and HDR are two competing pathways [4–6]. In mammalian cells, template-free NHEJ is favored over templated HDR since NHEJ is a rapid high-capacity pathway, which is active throughout the cell cycle and directly represses HDR [4–6]. In contrast, HDR is largely restricted to the S and G2 phases [4–6].

At the most basic level, the CRISPR/Cas genome editing technology is utilized to introduce a DSB at a specific target site in the genome and then relies upon the cellular machinery to repair this lesion by either the NHEJ/MMEJ or HDR repair pathways to yield the desired repair outcomes [32, 34, 35]. If the experimental goal is to knockout the function of a given gene of interest, then the error-prone NHEJ or MMEJ pathways would be utilized to repair DNA DSBs created by the Cas endonuclease at a programmed target site to introduce indels, which can shift the open reading frame (ORF) and result in targeted gene loss of function [32, 34, 35]. In contrast, if the experimental goal is to edit out or to introduce mutations at specific genomic sites (i.e., targeted gene knock-in), then the HDR pathway would be utilized to repair the Cas endonuclease-created DNA DSBs with an exogenous repair template harboring

specific nucleotide (nt) changes [32, 34, 35]. The cellular NHEJ/MMEJ and HDR repair pathways of endogenous and CRISPR/Cas-generated DSBs will be discussed in more detail below.

3.1 Template-free error-prone end joining NHEJ/MMEJ pathways

Non-homologous end joining (NHEJ) rejoins DNA DSBs as quickly as 30 minutes after break induction with minimal processing [4–6]. Briefly, after a DSB (i.e., DNA ends can be either blunt or possess a short 5′ overhang) has formed, the ring-shaped XRCC6 (X-ray repair cross complementing 6, also known as Ku70)/XRCC5 (X-ray repair cross complementing 5, also known as the Ku80) protein heterodimer quickly binds to the broken DNA ends [4–6]. This binding protects the DNA ends from further resection, preventing MMEJ and HDR pathway initiation [36, 37]. The XRCC6/XRCC5 heterodimer (Ku) then recruits and activates the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) [38, 39]. The XRCC6/XRCC5 heterodimer subsequently recruits additional NHEJ factors including XRCC4 (X-ray repair cross complementing 4), NHEJ1 (non-homologous end joining factor 1, also known as XLF), and (DNA ligase IV) to the complex to ligate the DNA DSB ends [40]. Therefore, in the absence of DNA end processing, NHEJ-mediated repair is error-free [40]. In contrast, if the DSB ends are not ligatable due to nucleotide overhangs, DCLRE1C (DNA cross-link repair 1C, also known as Artemis), a single-strand-specific 5′ → 3′ exonuclease, and specialized DNA polymerases POLL (DNA polymerase) and POLM (DNA polymerase μ) generate compatible DNA blunt ends, which can then be ligated by LIG4 [41]. Importantly, this process limits DNA end processing and minimizes mutagenesis (i.e., indels) [41].

3.2 Microhomology-mediated end joining (MMEJ)

Although it was originally thought that most CRISPR/Cas-generated DNA DSBs were repaired by the NHEJ pathway [4–6], it is now apparent that a significant number of these DSBs are also fixed by the MMEJ pathway (>50%) [42, 43]. MMEJ, like NHEJ, does not require a template for repairing DNA DSBs [4–6]. However, in contrast to NHEJ, MMEJ begins with a short-range resection of the DNA DSBs and functions independently of XRCC6/XRCC5 and LIG4 [44]. MMEJ resection is initiated by the MRN (i.e., MRE11 [MRE11 homolog double-strand break repair nuclease]-RAD50 [RAD50 double-strand break repair protein]-NBN [Nibrin, also known as NBS]) DNA DSB repair damage sensing complex with its stimulatory factor RBBP8 (RB-binding protein 8 endonuclease, also known as CTIP) [4–6, 44]. RBBP8 phosphorylation stimulates MRE11 endonuclease activity to create a nick at the 5′ strand near to the DSB, which promotes the removal of XRCC6/XRCC5 and DNA-PKcs, thus preventing NHEJ [45–47]. The resulting nick allows the MRE11 3′-to-5′ exonuclease to resect back toward the DNA DSB, which generates short 3′ overhangs, thus exposing potential single-strand DNA microhomologies (5–25 bps) on opposite strands, which allows the broken ends to realign and anneal [4–6, 44]. Any resulting heterologous 3′ single-stranded DNA (ssDNA) flaps must be removed by the ERCC1 (ERCC excision repair 1 endonuclease non-catalytic subunit)/ERCC4 (ERCC excision repair 4 endonuclease catalytic subunit, also known as XPF) endonuclease [48]. POLQ (DNA polymerase theta) is recruited to stabilize the annealed ssDNA and fills any gaps *via* template-directed DNA synthesis. LigI (DNA ligase 1) or LigIII (DNA ligase 3) subsequently seals the break [49]. Importantly, due to the resection

step, MMEJ is "error prone," and therefore, this repair mechanism can lead to indels, chromosomal translocations, and end-to-end chromosomal fusions [42, 43].

3.3 CRISPR/Cas9-induced error-prone end joining DNA repair outcomes

Then, the Cas9/gRNA complex binds to its target site, and the Cas9 HNH nuclease domain cleaves the target strand 3 bp upstream of the PAM site [50]. In contrast, the Cas9 RuvC-like nuclease domain cleaves the non-target strand 3, 4, or 5 bp upstream [50]. Therefore, Cas9-induced DSB ends are either blunt or have 1–2 bp 5′ overhangs [50]. As described above, the blunt ends can be directly ligated with the XRCC4/NHEJ1/LIG4 complex through NHEJ, without any further processing (i.e., "error-free" NHEJ) (**Figure 2**) [42]. Importantly however, even when DNA DSBs are repaired by nontemplated NHEJ, it has been established that the Cas9 cleavage cycle is repeated over and over until NHEJ mutagenic events prevent gRNA target recognition [51]. Thus, this repeated cleavage process enhances the number of non-templated indels (**Figure 2**) [51–55]. Likewise, Cas9-induced DNA DSB ends that have 1–2 bp 5′ overhangs are not ligatable and must be processed further by DCLRE1C, POLL, and POLM, which subsequently generates blunt ends followed by ligation through NHEJ [43]. Importantly, this process results in 1–2 bp indels (**Figure 2**) [55].

Similarly, Cas-induced DSBs repaired by the MMEJ pathway are also innately mutagenic due to the loss of sequence information when the extraneous heterologous 3′ ssDNA flaps are cleaved off [56, 57]. The frequency of deletions mediated by MMEJ is positively correlated with GC base content, and microhomology length, with deletions of two or more nucleotides occurring most often [50–55]. Interestingly, recent studies have established that MMEJ repair outcomes of Cas9-induced DSBs are not random and can be predicted [50–55].

Given the mutagenic nature of Cas9-induced DSBs repaired by NHEJ and MMEJ (i.e., the generation of non-templated indels), this type of end joining is leveraged frequently to silence gene expression (i.e., gene-specific knockout or loss of function)

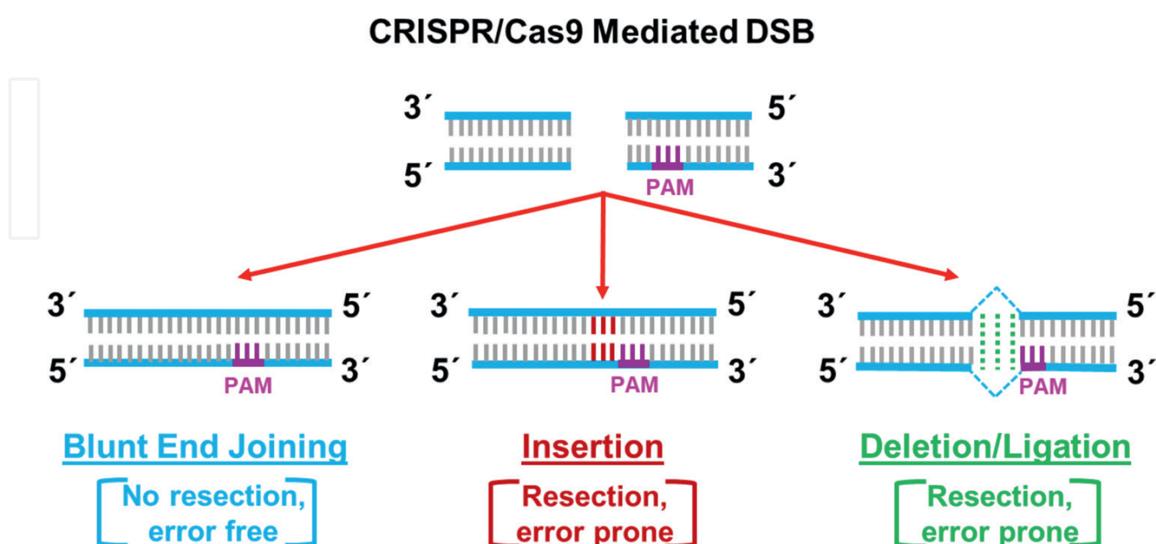

Figure 2.
Schematic representation of potential outcomes of error-prone NHJE/MMEJ repair of CRISPR/Cas9-mediated DSBs. The PAM site (denoted in pink) is shown relative to the DSB generated by CRISPR/Cas9 cleavage. Nontemplated DNA repair is mediated by the NHEJ/MMEJ pathway as described in the text. Three potential outcomes are shown. Nontemplated error-prone repair of CRISPR/Cas9-mediated DSBs can cause frameshift mutations leading to a stop codon and subsequent gene-specific knockout (i.e., loss of function).

(**Figure 2**) [50–54]. Cas9-mediated error-prone NHEJ and MMEJ repair has been utilized to study the function of a wide variety of genes and noncoding elements in cellular and animal models [35, 37, 58]. Additionally, precise template-free end-joining-mediated genome editing through MMEJ has also been achieved [35, 37, 58].

4. Templated homology-directed repair

HDR of endogenously generated DNA DSBs requires extensive DSB end resection and necessitates the physical base pairing interactions between the broken DNA strands and an identical sister chromatid, a homologous chromosome, or an ectopic site (i.e., a double-strand DNA [dsDNA] repair template) [4–6]. Therefore, HDR is most prominent during S and G2 cell cycle phases when an identical sister chromatid is available for recombination [59, 60]. Although HDR is typically an error-free process, indels, point mutations, genomic rearrangements, and subsequent genomic instabilities can result in a DNA donor-dependent or donor-independent manner [61].

4.1 Rad51-dependent homology-directed repair

The repair of DNA DSBs using an endogenous dsDNA repair template can occur through a RAD51 (RAD51 recombinase)-dependent mechanism [32, 34, 35, 62]. Initially, HDR, like MMEJ, begins with a short-range 3′-to-5′ resection (5–25 bps) of DNA ends mediated by the MRN/RBBP8 complex [32, 34, 35, 62]. The short-range resection is then followed by long-range 5′-to-3′ resection (>1000 bps) catalyzed by EXO1 (exonuclease 1) or DNA2 (DNA replication helicase/nuclease 2) with the assistance of BLM (BLM RecQ-like helicase) or WRN (WRN RecQ-like helicase) [32, 34, 35, 62]. The resected 3' ssDNA overhangs are subsequently stabilized by the binding of multiple RPA (heterotrimeric Replication Protein A) complexes [63]. RPA complexes are then replaced by the ATP-dependent nucleoprotein Rad51 (RAD51 recombinase) that forms long helical filaments on the resected 3′ ssDNA overhangs [64, 65]. RAD51 promotes the invasion of the overhangs (i.e., strand exchange), aligns, and pairs the ssDNA with a homologous sister chromatid sequence to form a displacement loop (D-loop) [32, 34, 35, 62]. The invading 3′ ssDNA overhang within the D-loop can then be extended by POLD1 (DNA polymerase delta 1) to synthesize sequences lost at the break site and by end resection using the homologous sister chromatid sequence as a template [66]. Finally, the resulting HDR intermediates can be resolved by multiple mechanisms, which include SDSA (synthesis-dependent strand annealing), crossover and non-crossover dHJ (double Holliday junction), and BIR (break-induced replication) [32, 34, 35, 62].

4.2 Rad51-independent homology-directed repair

Alternatively, endogenously generated DSBs cans also be repaired by a RAD51-independent HDR pathway designated single-strand annealing (SSA). Like Rad51-Dependent Homology-Directed Repair, SSA also requires long-range 5′-to-3′ resection (>1000 bps) catalyzed by EXO1 or DNA2/BLM [32, 34, 35, 62]. The resected 3' ssDNA overhangs are subsequently bound with RPA complexes; however, they are replaced by RAD52 (RAD52 homolog, DNA repair protein), which promotes the annealing of homologous sequences within the two DSB ends [67, 68]. The heterologous DNA flaps generated by SSA annealing are removed by the ERCC1 endonuclease complex, thus producing genomic deletions [48].

4.3 CRISPR/Cas-induced homology-directed repair (HDR) DNA repair outcomes

For HDR, subsequent to CRISPR/Cas-generated DSBs, an exogenous DNA template that shares homology to ends of the DSB and contains the desired gene-specific nucleotide changes, mutations, or additions is required to incorporate these alterations intentionally and precisely *via* the HDR pathway (**Figure 3**) [32, 34, 35]. If a donor DNA template is not provided, then error-prone NHEJ/MMEJ will be the predominant mechanism utilized to repair the DSB and unwanted indels will occur [32, 34, 35].

If exogenous plasmids, PCR products, or chromatinized templates are utilized as dsDNA donor templates, then the Rad51-dependent HDR pathway described above is employed [32, 34, 35, 69]. In contrast, if single-strand oligodeoxynucleotides (ssODNs) are used as homologous donor templates to repair CRISPR/Cas-generated DSBs, then a RAD51-independent mechanism designated, single-stranded DNA donor-templated repair (SSTR) occurs through SSA and synthesis-dependent strand annealing (SDSA) [67, 68, 70, 71]. Like RAD51-dependent HDR, SSTR is initiated by resection of the DSB [67, 72–74] and like SSA, SSTR requires RAD52 to promote annealing of 3′ resected ssDNA tails with ssODN donor templates followed by DNA-templated synthesis [68, 70, 74].

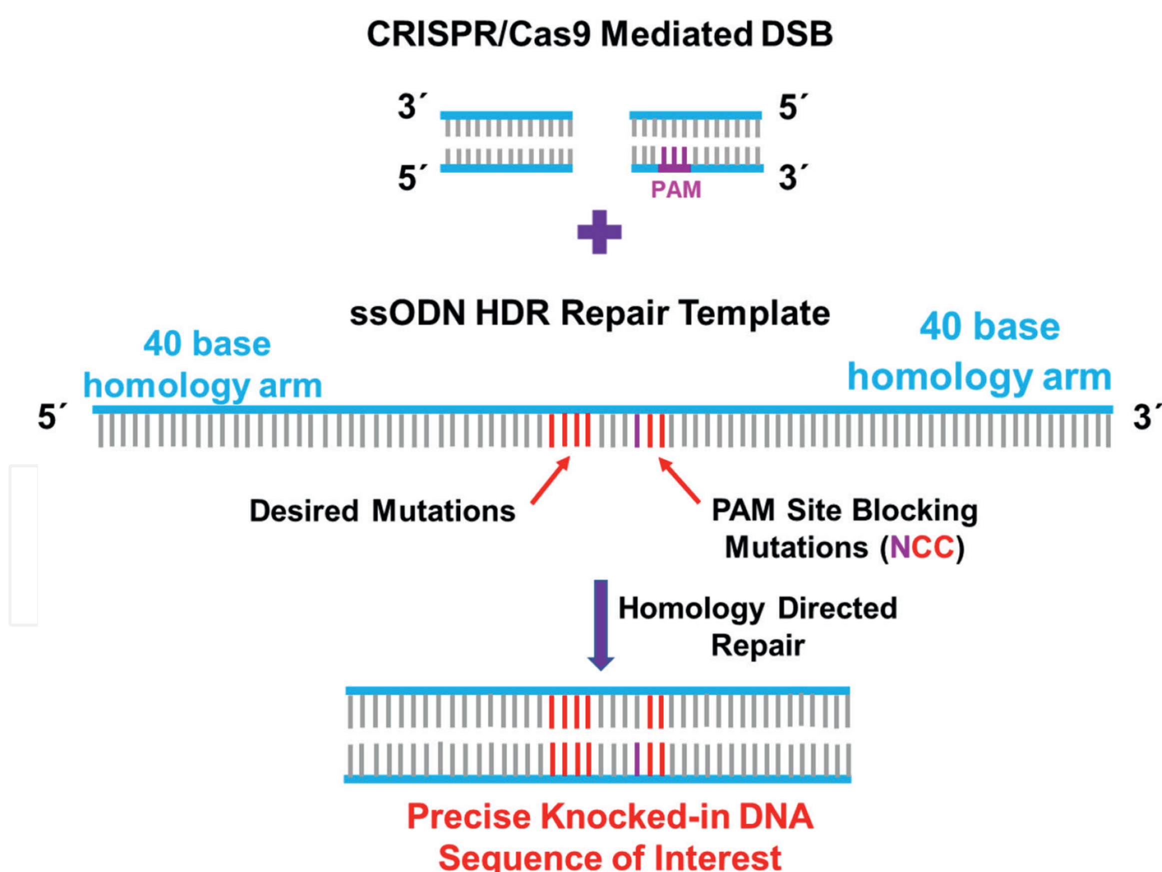

Figure 3.
Schematic representation of precise gene modification mediated by HDR of CRISPR/Cas9-mediated DSBs. The PAM site (denoted in pink) is shown relative to the DSB generated by CRISPR/Cas9 cleavage. The ssODN donor template with symmetric 40-nt homology arms with the desired modifications placed in the middle of the template (denoted four red hash marks). Blocking PAM mutations (i.e., NGG → NCC) are also shown denoted with the pink and two red hash marks). After co-transfection of the Cas/RNP complex with the ssODN donor template, this template is utilized to repair the generated DSB by the HDR pathway. This allows for the precise knock-in of the sequence of interest.

5. Optimizing HDR efficiency

5.1 Allelic considerations

Before initiating any CRISPR/Cas genome editing projects, regardless of whether knockout (i.e., NHEJ/MMEJ) or knock-in (i.e., HDR) experiments are planned, one must explore how many target gene alleles of interest are present in the cell line to be edited. This is a crucial consideration given that many cancer cell lines utilized for gene editing experiments often exhibit extensive somatic gene copy number variation (CNV) [75, 76]. Therefore, a chosen gene of interest could vary from a single copy (e.g., heterozygous deletion), two copies (e.g., normal), several copies (e.g., aneuploidy), or many copies (e.g., gene amplification) depending on which cell line is utilized for the CRISPR/Cas/NHEJ/MMEJ or CRISPR/Cas/HDR experiments.

For example, many CRISPR/Cas studies (i.e., from 2020 to 2022, greater than 100 published as per PubMed) have utilized K562 cells (an immortalized chronic myelogenous leukemia cell line) that are known to contain widespread aneuploidy and numerous obvious structural abnormalities [77–80]. Recently, Zhou et al. [81] published a comprehensive characterization of the K562 genome. This publication proved to be invaluable as our laboratory initiated CRISPR/Cas/HDR studies utilizing an anticancer drug (etoposide)-resistant K562 clonal subline, K/VP.5, previously generated by our laboratory [82, 83].

Briefly, our laboratory studies human DNA topoisomerase IIα (170 kDa, TOP2α/170), which generates transient double-strand DNA breaks to resolve nucleic acid topological entanglements [84, 85]. TOP2α/170 is an important target of anticancer drugs (such as etoposide), whose efficacy is often compromised due to decreased TOP2α/170 levels [86, 87] and resultant attenuation of cytotoxic drug-induced TOP2α-DNA covalent complexes [84, 85]. Compared to parental K562 cells, etoposide-resistant K/VP.5 cells contain reduced TOP2α/170 levels and express high levels of a novel C-terminal truncated TOP2a isoform (90 kDa, TOP2α/90) [88, 89]. TOP2α/90 is the translation product of a short TOP2α mRNA that is generated from a cryptic poly(A) site harbored in intron 19 (i.e., I19 intronic polyadenylation; I19 IPA) [90, 91]. TOP2α/90 lacks the active site tyrosine 805 harbored in exon 20 of full-length TOP2α/170 necessary for TOP2α-mediated DNA strand breaks [88–91]. We hypothesized that, by utilizing CRISPR/Cas/HDR to enhance the TOP2α gene's suboptimal exon 19/intron 19 5′ SS (E19/I19 5′ SS), removal of intron 19 would be enhanced, which in turn would result in decreased TOP2α/90 mRNA/protein, increased TOP2α/170 mRNA/protein, and circumvention of etoposide resistance [92].

Since the human TOP2α gene is harbored on chromosome 17 (i.e., mapped to chromosome 17q21–22) [93], Zhou et al.'s [81] study was utilized to determine the number of TOP2α alleles (i.e., copy number) present in the K562/K/VP.5 cells before initiation of CRISPR/Cas/HDR experiments [92]. It was found that K562 and the isogenic-acquired resistant cell line, K/VP.5, contained three TOP2α alleles [81]. Therefore, our CRISPR/Cas9/HDR strategy was focused on editing all three TOP2α alleles in K/VP.5 cells at the E19/I19 5′ SS to maximize the desired phenotypic change (i.e., decreased TOP2α/90 mRNA/protein, and increased TOP2α/170 mRNA/protein levels) and to circumvent etoposide resistance [92]. qPCR and Sanger sequencing demonstrated that the ratio of wild-type to edited genomic sequence decreased by 1/3 with each allele edited [92]. TOP2α/90 progressively decreased and TOP2α/170 increased with each allele edited by CRISPR/Cas9/HDR. Etoposide resistance was completely reversed when all three TOP2α alleles were edited to enhance the E19/I19

5′ SS [92]. RNA seq confirmed that intron 19 was effectively spliced out in the three allele-edited clone [92].

5.2 PAM site considerations

Multiple studies have demonstrated that CRISPR/Cas-generated DSBs should be in close proximity to the edit site to achieve high HDR efficiencies [93–100]. These investigators established that if a Cas9 PAM site (i.e., NGG; N denotes any nt) was located more than 14 bp (on either DNA strand) from the desired gene-specific nt changes, mutations, or additions, then the efficiency of CRISPR/Cas9/HDR was dramatically reduced. However, Renaud et al. [96] observed that the 14-bp limitation may be pushed to 20 bp utilizing chemically modified ssODN donor templates (see "HDR Considerations" below). Paquet et al. [94] also demonstrated that it was easier to create homozygous gene edits when the PAM site was closer to the intended nucleotide changes and heterozygous gene editing by distance-dependent suboptimal mutation incorporation.

Importantly, Schubert et al. [100] indicated that although guide selection in close proximity with the required HDR changes is important, it was more significant that the gRNA utilized not only targeted Cas9 to the appropriate sequence but also activated Cas9 endonuclease activity. Therefore, since all gRNAs are not equally efficient in activating Cas9, it is essential that the cleavage efficiency for each gRNA utilized is calculated using a T7 endonuclease mismatch cleavage assay (i.e., measuring the extent of indel formation) before initiating HDR experiments [97, 100]. If several Cas9 PAM sites are identified within the 15 base HDR parameter, then the gRNA eliciting the highest cleavage efficiency should be utilized for CRISPR/Cas9/HDR experiments (see "gRNA Considerations" below).

Since the lack of gRNAs with appropriate cleavage efficiency and proximity to the desired HDR-mediated changes is a significant limitation for many CRISPR/Cas9/HDR studies, Schubert et al. [100] also demonstrated that Cas9 D10A nickases (i.e., induce single DNA nicks) can be utilized for HDR mutation experiments if gRNAs target PAM sites on opposite strands of the genomic DNA to generate a staggered DSB provided that the desired mutation is placed between the two nick sites. Alternatively, the number of possible CRISPR/Cas/HDR editing sites can be expanded with the utilization of Cas12a (also known as Cpf1), which recognizes a unique PAM site (TTTV; V denotes an A, C, or G nt) [101].

Since Cas9/Cas12a PAM site recognition restricts targeting and affects CRISPR/Cas/HDR editing efficiency and flexibility, there are efforts to genetically re-engineer CRISPR enzymes to target heretofore inaccessible PAMs [102–105]. For example, Kleinstiver et al. [106] have successfully altered *S. pyogenes* Cas9 (SpCas9) PAM specificity by utilizing bacterial selection-based directed evolution. Walton et al. [104] utilized structure-guided engineering to develop several "near-PAMless" SpCas9 variants capable of targeting NGN and NRN (R denotes an A or G), respectively. Finally, Kleinstiver et al. [105] have also utilized structure-guided protein engineering to improve the targeting range of *Acidaminococcus sp.* Cas12a. Together these studies suggest that the PAM site constraints that currently limit CRISPR/Cas/HDR editing will be circumvented in the future.

5.3 gRNA considerations

Most CRISPR/Cas genome editing experiments are now performed by delivering purified Cas9/Cas12 proteins and chemically synthesized gRNAs as a ribonucleoprotein (RNP) (i.e., Cas/RNP) complex to restrict their temporal activity, improve

precision, decrease the immune response, and reduce off-target effects [106, 107]. Specifically, engineered gRNAs have been chemically modified to increase their stability and decrease off-target editing resulting in enhanced cleavage efficiency and improved HDR efficacy [108, 109].

gRNAs can be synthesized in two formats. First, like the endogenous Cas9 gRNA, the crRNA/tracrRNA is a two-piece gRNA where the crRNA (~36–42 nt) and tracrRNA (~67–89 nt) are synthesized as two independent oligonucleotides and are subsequently annealed together through a complimentary linker region to form a functional gRNA [28]. Second, a single guide (sgRNA, 100 nt) can be synthesized, which comprises both the crRNA and tracrRNA in a single oligonucleotide (no annealing is required) (**Figure 4**). It is important to note that the PAM sequence is not included in either gRNA format [28]. One advantage of the two-piece gRNAs is that the tracrRNA sequence is the same for all CRISPR/Cas9 experiments and only the crRNA sequence varies, based on the DNA site to be targeted [28]. Therefore, one chemically synthesized tracrRNA can be annealed to any chemically synthesized crRNA. Chemically synthesized chimeric sgRNAs have the advantage that they exhibit equivalent or greater efficiency compared to the native dual RNA system [109, 110]. We advocate for the use of sgRNAs since our laboratory tested several two-piece gRNAs that exhibited no activity [92] that when resynthesized as sgRNAs displayed high cleavage efficiency [111].

Since RNAs are inherently unstable and susceptible to endo- and exonucleases, considerable effort has been devoted to chemically modifying RNAs to improve their stability. Importantly, Hendel et al. [112] established that chemical modification of

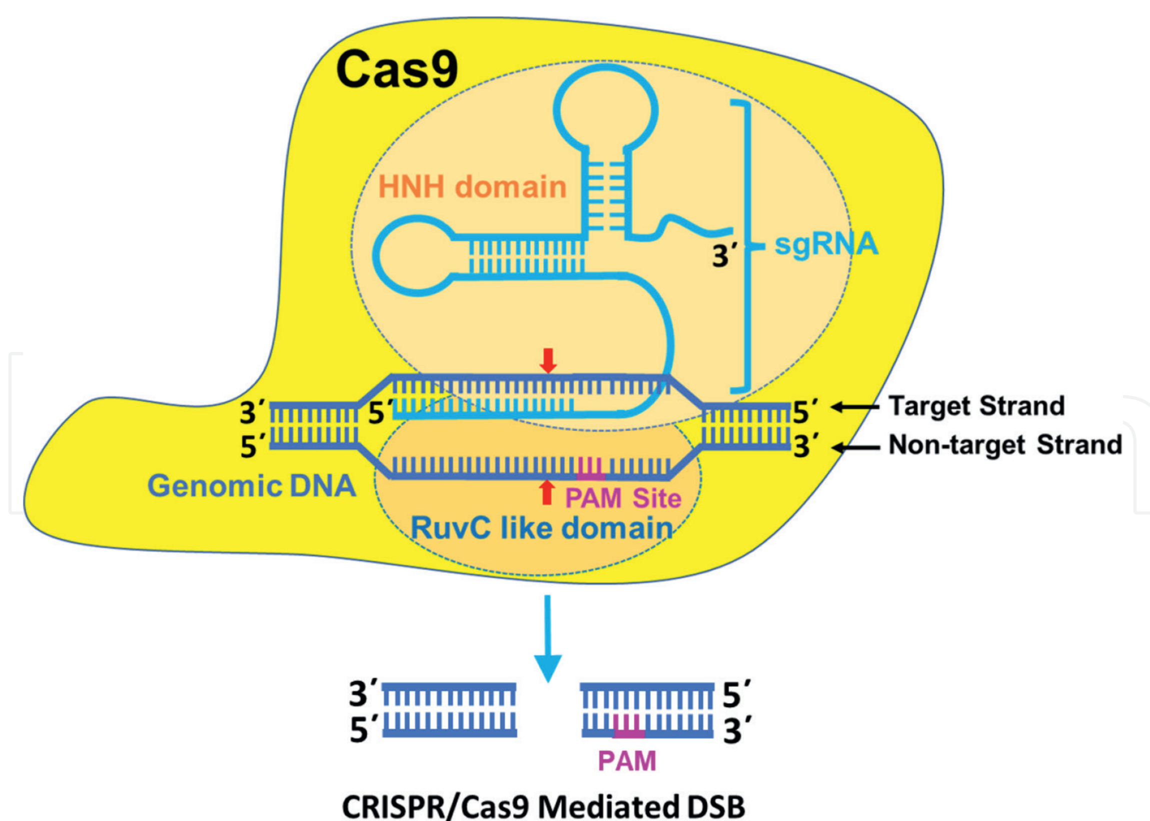

Figure 4.
Schematic representation of the CRISPR/Cas9-mediated DSB with a one-piece sgRNA. The CRISPR/Cas9 schematic is denoted as described in ***Figure 1****, except Cas9 sgRNA is synthesized as a single molecule, which harbors both the crRNA and the tracrRNA (denoted in light blue). The DSB is created as described in* ***Figure 1****. sgRNAs have the advantage that they can exhibit greater efficiency compared to the native dual RNA system with no crRNA/tracrRNA annealing step required [109, 110].*

gRNAs protected them from degradation and enhanced genome editing efficiency. Specifically, these investigators demonstrated that when 2′-O-methyl 3′phosphorothioate (MS), or 2′-O-methyl 3′thioPACE (MSP) chemical modifications were incorporated at both the 5′ and 3′ three terminal nucleotides, and indel formation and HDR were significantly increased [112]. They concluded that chemically synthesized/modified sgRNAs offer significant advantages over sgRNAs expressed by plasmids or by *in vitro* transcription, including 1) scalable and robust production for many applications; 2) greater sgRNA design flexibility; 3) lower toxicity; and 4) increased efficacy [112]. In conclusion, the studies reviewed in this section clearly suggest that the continued optimization of synthetic gRNAs will increase cleavage and on-target efficiency, which will help leading to future efficacious CRISPR-based therapies.

5.4 HDR template considerations

Regardless of whether dsDNA or ssODN donor templates are utilized by distinct HDR pathways to mend Cas RNP complex generated DSBs, the same precise, intentional repair outcomes can be achieved. However, ssODNs donor templates are most frequently used to introduce specific changes (e.g., introduce or correct mutations, and to create short insertions) into specific DNA sequences through HDR due to their superior efficiency, fidelity, and ease of synthesis (**Figure 3**) [71, 74, 95–97, 113]. Importantly, recent studies investigating 1) chemical modifications at the 5′ and 3′ ends of ssODNs donor templates; 2) optimal complementary length; 3) homology arm polarity and asymmetry; and 4) donor template design to prevent the re-cleavage of edited alleles have resulted in empirical rules to rationally design ssODN donor templates to maximize HDR efficiency and flexibility [94–100].

Renaud et al. [96] established that phosphorothioate (PS) chemical modifications at the two terminal nucleotides at both the 5′ and 3′ ends of ssODNs repair templates strongly enhanced genome editing efficiency in cultured cells. These investigators also demonstrated that PS-modified ssODN donor templates also permitted efficient insertion of over 100 nucleotides, while only limited integration was observed with non-chemically ssODNs [96]. Likewise, a higher frequency of insertions was attained in mice and rats using modified ssODNs [96]. The importance of utilizing PS-modified ssODN repair templates to enhance HDR editing efficiency was validated by Liang et al. [97].

Richardson et al. [95] demonstrated that although Cas9 dissociates slowly from dsDNA substrates, Cas9 releases the 3′ end of the cleaved nontarget strand (NT strand, the DNA strand that is not complementary to the gRNA and harbors the "NGG" PAM sequence) before complete Cas9 dissociation. They subsequently showed that ssODNs donor templates complementary to the NT strand increased HDR frequencies compared to donor templates complementary to the target strand (T strand; the DNA strand that is complementary to the gRNA and does not contain the "NGG" PAM sequence) [95]. Finally, these investigators established that ssODN donor templates asymmetrically oriented relative to the 5′- and 3′-side of the generated DSB and complementary to the NT strand also increased HDR rates [95]. In support of these results, Liang et al. [97] also showed that asymmetric ssODNs with 30-bp homology arms 3′ to the insertion and greater than 40 bp of homology at the 5′ end were preferred. This report indicated that the optimal amount of asymmetric ssODN was 10 pmol. However, in contrast to Richardson et al. [95], these investigators only observed a slight increase in HDR efficiency with NT strand compared with T strand ssODN repair templates [97]. Okamoto et al. [99] demonstrated that the optimal ssODN donor template should have a total length of ~75–85 nt with 30 to

35 nt perfectly matched homology arms on the 5′ and 3′ ends and complementary to the gRNA strand (i.e., T strand). Recently, Schubert et al. [100] established further design parameters to improve HDR efficiencies by testing hundreds of genomic loci and multiple cell lines. First, they demonstrated that the ssODN donor template (i.e., NT or T strand) that leads to the highest HDR efficiencies varies greatly depending on the genomic locus and cell type utilized [100]. Second, they observed that the preferred strand (NT or T), relative to the gRNA, is dependent on where the desired HDR modification is located. For example, there is no repair strand preference when the HDR modification is placed precisely at the Cas9 cleavage site [100]. However, if the HDR modifications occur further from the Cas9 cleavage site, a NT strand ssODN donor template is preferred for PAM-distal mutations and a T strand ssODN donor template is ideal for PAM-proximal mutations [100]. Additionally, they showed that asymmetric homology arms did not improve HDR beyond symmetrical homology arms when arm length was ≥30-nt from both the mutation location and the Cas9 cleavage site [100]. These investigators advocated for ssODN donor templates with 40-nt homology arms with modifications placed in the middle (**Figure 3**) [100].

The CAS RNP complex can regenerate DSBs in alleles already appropriately edited, thereby lowering HDR efficiency [94, 99]. In another design innovation using ssODN donor templates, Paquet et al. [94] strategically prevented re-cutting of HDR-edited sites by introducing CRISPR/Cas-blocking PAM site mutations in their repair templates and observed increased HDR accuracy and effectiveness. Okamoto et al. [99] subsequently established that ssODN repair templates with a single mutation in the PAM site (i.e., NGG → NGC) showed the highest HDR efficiency. Their results clearly indicated that the re-cutting of edited alleles resulted in very low HDR efficiencies, and that introducing PAM site mutations within ssODN repair templates to prevent re-cutting is essential for efficient HDR knock-in [99]. Schubert et al. [100] also demonstrated that adding a blocking PAM mutation to the second or third base of the PAM (i.e., NGG → NCG or NGC) in ssODN repair templates resulted in greater HDR efficiency. Donor templates containing two blocking PAM mutations (i.e., NGG → NCC) resulted in the highest HDR efficiency (**Figure 3**). Finally, another important indication for blocking PAM mutations in HDR repair templates is to ensure that when multiple rounds CRISPR/Cas/HDR transfections are required to edit all gene-specific alleles in cell lines that exhibit aneuploidy; the previously edited alleles will not be re-cut in the subsequent rounds of transfection [91, 92].

5.5 Pharmacological strategies to enhance HDR efficiency

Most CRISPR/Cas/HDR genome editing experiments are now performed by transfecting Cas/RNP complexes and ssODN repair templates to restrict temporal activity, thereby reducing off target effects, decreasing immune responses, and increasing HDR efficiency [94–100, 106–110, 112, 113]. Discussion below of pharmacological and genetic strategies for gene editing by HDR will be limited to this experimental paradigm.

Since NHEJ/MMEJ are rapid high-capacity pathways which are active throughout the cell cycle (i.e., G1, S and G2 phases), while HDR is active only after DNA replication is completed and sister chromatids are available to serve as repair templates (i.e., late S and G2 phases) [60], one of the first attempts to pharmacologically enhance CRISPR/Cas/HDR efficiency was to time the delivery of Cas RNA complexes after synchronization of cells using aphidicolin or nocodazole [114]. Lin et al. [114] demonstrated that synchronization, with either aphidicolin or nocodzole, resulted in increased HDR rates (up to 38%) compared with unsynchronized cells.

Since DNA repair is also influenced by the accessibility of DNA binding factors, like Cas RNP complexes [115, 116]. Li et al. [117] hypothesized that CRISPR/Cas/HDR efficiency would be enhanced with histone deacetylase (HDAC) inhibitors, trichostatin A (TSA) and PCI-24781, by promoting a more open chromatin structure [118]. These investigators established that HDR, single strand annealing and ssODN mediated HDR were all increased with HDAC inhibitor treatment [118]. Moreover, this study demonstrated that TSA and PCI-24781 usage also favored HDR by arresting the cell cycle in the G2/M phase [118].

Another pharmacological strategy to improve HDR efficiency is to target the competing NHEJ/MMEJ pathways. Riesenberg et al. [119], explored the efficacy of a wide range of small molecules reported to inhibit the NHEJ/MMEJ pathways or to activate/increase the HDR protein components. These investigators determined that NU7026 (DNA-dependent protein kinase, DNA-PK inhibitor), TSA, MLN4924 (NEDD8 E1 Activating Enzyme Inhibitor), and NSC 15520 (replication protein A1, RPA1 inhibitor) increased HDR efficiencies in various genes and in specified cell lines when DSBs were generated by nickase Cas9n and Cas12/RNP complexes [119]. When Cas9/RNP complexes were utilized to generate DSBs, only NU7026 significantly increased HDR efficacy [119]. NSC 19630 (WRN RecQ like helicase, WRN inhibitor), AICAR (protein kinase AMP-activated catalytic subunit alpha 1, PRKAA1 activator), RS-1 (RAD51 recombinase, RAD51 stimulator), Resveratrol (selective inhibitor of COX–1), SCR7 (DNA ligase IV inhibitor), and L755507 (potent β3-adrenergic receptor partial agonist), showed no clear effect on any Cas/HDR efficiency [119]. Finally, it was demonstrated that the combination of NU7026, TSA, MLN4924, and NSC 15520 resulted in the highest HDR levels observed with Cas9n and Cas12/RNP complexes [119].

In contrast to the results presented above, other investigators have successfully utilized SCR7 (DNA ligase IV inhibitor) to specifically impede the NHEJ pathway to increase HDR activity [120–124]. However, there are also conflicting reports on the ability of this compound to increase HDR [119, 125, 126]. The lack of consistency with this compound regarding HDR efficacy may have resulted from the use of different chemical derivatives of SCR7 [127]. Additionally, Greco et al. [127] demonstrated that SCR7 exhibited greater inhibitory activity against DNA ligases I and III than DNA ligase IV and therefore should target the MMEJ pathway (i.e., also involved in error-prone repair).

6. Conclusion

CRISPR/Cas/HDR is a robust gene editing methodology to purposefully edit out or introduce mutations or insertions at specific genomic sites (i.e., targeted gene knock-in) by creating a DSB along with the introduction of an exogenous template harboring the desired nt changes for DSB repair *via* the HDR pathway [32, 34, 35]. Since DSBs generated in mammalian cells are predominantly repaired by NHEJ/MMEJ pathway [4–6], success of CRISPR/Cas/HDR gene editing will depend on maximizing overall HDR efficacy. We propose the following "workflow" strategies to facilitate the knock-in process (**Figure 5**). First, after developing an experimental CRISPR/Cas/HDR hypothesis, serious consideration must be given to the appropriate cellular system to utilize and the number of gene alleles of interest that may need be edited to obtain a resulting altered phenotype. If the cell line of choice is not well characterized, sequence analysis of CRISPR-edited cells may help determine the number of edited and non-edited alleles [91, 92, 111].

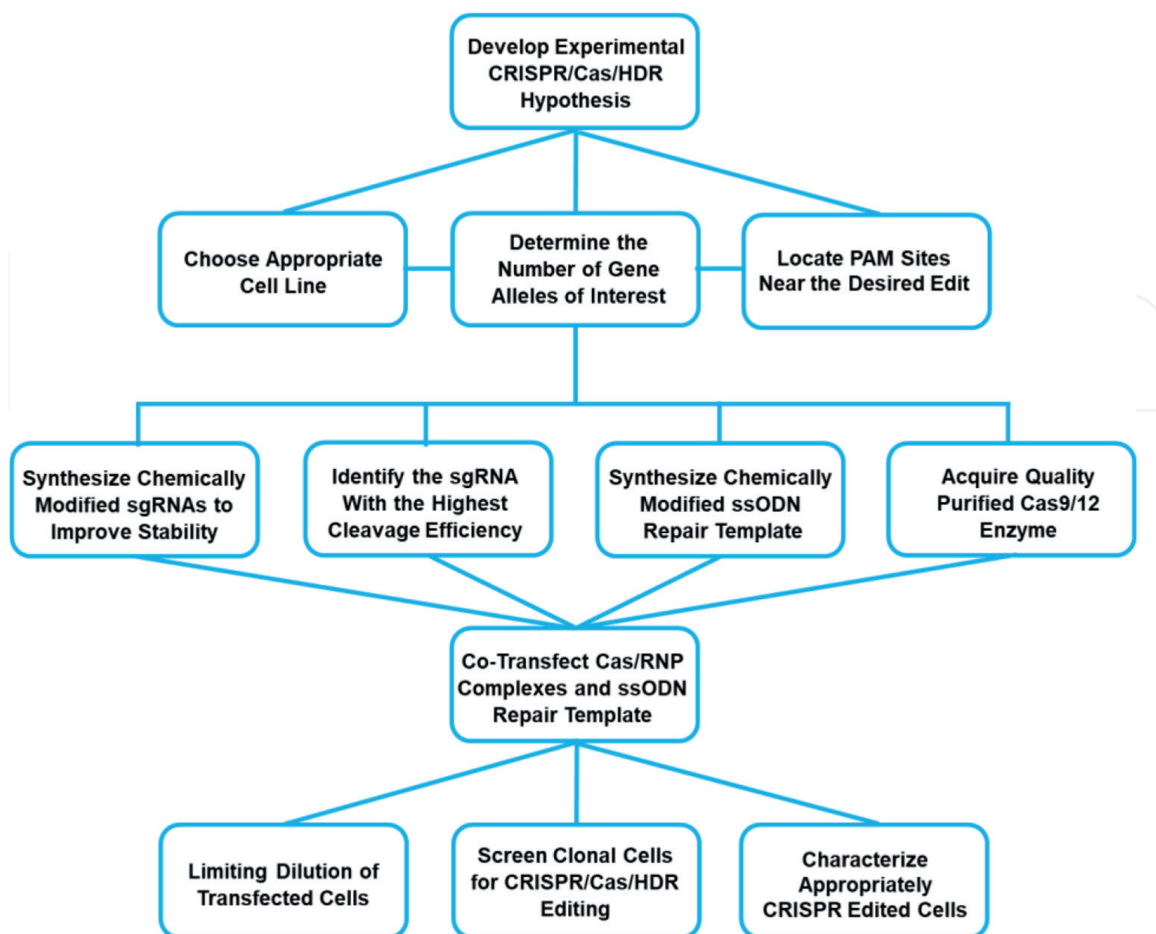

Figure 5.
Proposed experimental flow diagram to maximize CRISPR/Cas/HDR gene editing efficiency. An explanation of the flowchart is discussed in the conclusion section.

Second, the location of the targeted gene knock-in must be analyzed to determine if a Cas9/12 PAM site(s) is/are harbored within 20 nt of the DNA sequence to be edited (both DNA strands should be analyzed) [93–100]. If more than one PAM site is identified that is in close proximity to sequence to be edited, we advocate that multiple chemically modified 5′ and 3′ terminal nucleotide sgRNAs be synthesized. Although chemically modified sgRNAs exhibit increased stability and decreased off-target editing [108, 109], not all gRNAs are equally efficient in activating Cas enzymes [100]. Therefore, the cleavage efficiency for each sgRNA should be determined by employing a T7 endonuclease mismatch cleavage assay [97, 100] before proceeding with CRISPR/Cas/HDR editing experiments. The sgRNA with the highest cleavage efficiency should be utilized [100].

Third, for CRISPR/Cas/HDR, ssODN repair templates chemically modified at their 5′ and 3′ ends should be used to introduce specific changes (e.g., introduce or correct mutations, and to create short insertions) due to their superior efficiency, fidelity, and ease of synthesis [71, 74, 95–97, 113]. Although there are conflicting opinions regarding the optimal homology arm length, homology arm polarity, and asymmetry [94–100], it is now well established that HDR efficiencies varies greatly depending on the genomic locus and cell type utilized [94–100]. The most recent CRISPR/Cas/HDR editing data suggest that ssODN donor templates with symmetric 40-nt homology arms with the desired modifications placed in the middle of the template should be an appropriate standard approach (**Figure 3**) [100]. Regarding

the polarity of the ssODN donor templates with respect to the gRNA (see **Figure 1**), an NT strand ssODN donor template is preferred for PAM-distal mutations and a T strand ssODN donor template is ideal for PAM-proximal mutations [100]. Finally, blocking PAM mutations (i.e., NGG → NCC) should always be introduced in ssODN repair templates to further increase HDR efficiency [94, 99, 100] and to allow for potential repeated rounds of CRISPR/Cas/HDR transfections when editing multiple alleles in cell lines, which exhibits aneuploidy (**Figure 3**) [91, 92, 111].

Fourth, the chemically modified sgRNA with the highest cleavage efficiency should be incubated with high-quality, purified Cas9 or Cas12 enzymes that harbor a nuclear localization sequence (NLS) to form a Cas/RNP complex followed by co-transfection with an optimized ssODN donor template as above. Importantly, this procedure improves precision, restricts the temporal activity, decreases the immune response, and reduces the off-target effects of Cas proteins (**Figure 3**) [94–100, 106, 107].

Fifth, after transfection, small aliquots of cell suspension should be sampled to determine genomic cleavage efficiency (i.e., T7 endonuclease mismatch cleavage assay) [97, 100] to validate success in transfection, targeting, and DSB formation by the Cas/RNP complex at the appropriate genome location [91, 111]. Remaining cell suspensions should then be diluted (i.e., limiting dilution) and transferred to 96-well plates at a concentration less than one cell per well and allowed to grow until individual colonies are identifiable in some wells [128]. Individual clonal populations can be split into larger wells and then qPCR and/or Sanger sequencing [91, 92, 111] utilized to determine which clones contain the desired CRISPR/Cas/HDR editing. If electropherogram visualization of genomic sequence reveals only edited sequence, then this clone can be characterized for the hypothesized phenotypic changes. In contrast, if wild-type and edited genomic sequences are identified in the electropherogram, then the ratio of edited to wild-type to edited alleles can be determined. Editing all gene alleles of interest may be required to detect the hypothesized phenotypic change(s) [91, 92, 111]. If sequencing results reveal that a significant number of clonal cells have undergone NHEJ/MMEJ with no HDR editing, then pharmacological inhibitors (as described above) can be considered in an attempt to increase HDR efficiency.

The unique CRISPR/Cas/HDR gene editing experimental outline described in **Figure 5** incorporates the most comprehensive sgRNA and ssODN design considerations along with several important practical details that will help maximize the frequency of precise HDR. It is anticipated that, as strategies to enhance CRISPR/Cas/HDR efficacy continue to advance, that this tractable experimental workflow will accelerate the development of therapeutic gene editing.

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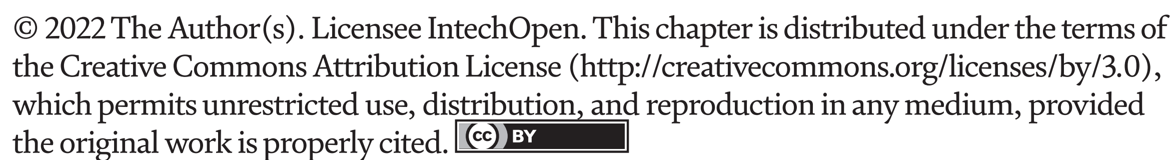
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