Molecular Therapy
Nucleic Acids
Review

CRISPR-Cas9-mediated homology-directed repair for precise gene editing

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CRISPR-Cas9-mediated homology-directed repair (HDR) is a versatile platform for creating precise site-specific DNA insertions, deletions, and substitutions. These precise edits are made possible through the use of exogenous donor templates that carry the desired sequence. CRISPR-Cas9-mediated HDR can be widely used to study protein functions, disease modeling, and gene therapy. However, HDR is limited by its low efficiency, especially in postmitotic cells. Here, we review CRISPR-Cas9-mediated HDR, with a focus on methodologies for boosting HDR efficiency, and applications of precise editing via HDR. First, we describe two common mechanisms of DNA repair, non-homologous end joining (NHEJ), and HDR, and discuss their impact on CRISPR-Cas9-mediated precise genome editing. Second, we discuss approaches for improving HDR efficiency through inhibition of the NHEJ pathway, activation of the HDR pathway, modification of donor templates, and delivery of Cas9/sgRNA reagents. Third, we summarize the applications of HDR for protein labeling in functional studies, disease modeling, and *ex vivo* and *in vivo* gene therapies. Finally, we discuss alternative precise editing platforms and their limitations, and describe potential avenues to improving CRISPR-Cas9-mediated HDR efficiency and fidelity in future research.

INTRODUCTION

Since Watson and Crick first unraveled the DNA double helix in 1953, scientists have employed a variety of methods to study the structure and function of DNA. The fields of molecular biology and genomics have greatly benefited from the discovery of tools such as DNA polymerases, DNA ligases, and restriction endonucleases. Together with subsequent inventions such as polymerase chain reaction (PCR), DNA Sanger sequencing, next-generation sequencing, and other innovative technologies, researchers are now able to quickly obtain a variety of DNA fragments, modify them at specific sites *in vitro*, and monitor the result. However, there was a lack of efficient and quick approaches to DNA editing of target sites *in vivo*. Early discovery of natural DNA repair pathways in bacteria and yeast revealed that cells can use a variety of mechanisms to repair double-strand DNA breaks (DSBs) which would otherwise be lethal. More recently, scientists have discovered that nucleases can be used to create targeted DSBs *in vivo*, and the cell's DNA repair mechanisms can be manipulated to make precise DNA edits. This approach of creating targeted DSBs and harnessing the cell's DNA repair mechanisms has become an important part of an ideal gene editing strategy.[1]

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9), termed CRISPR-Cas9, originated as a part of the bacterial adaptive immune system and has been identified as a powerful genome editing tool. CRISPR sequences were first identified in the genome of *E. coli* in 1987.[2] By 2007, the function of CRISPR as a defense mechanism against phages was clarified.[3] It was discovered that foreign phage DNA could be integrated into the bacterial genome within the CRISPR repeats in *S. thermophilus*.[3] This groundbreaking discovery revealed that bacteria could "remember" and defend against specific viruses using these sequences. Then in 2012, the group of Doudna and Charpentier made a groundbreaking discovery by systematically explaining how the CRISPR-Cas system effectively cuts double-stranded DNA (dsDNA) from invading foreign entities.[4] Their work provided a crucial foundation for the future use of CRISPR-Cas9 in gene editing. This mechanism paved the way for the first CRISPR-Cas-based genome editing in cell culture, reported by Cong et al. in 2013.[5] Since then, CRISPR-Cas9 has been widely adopted, revolutionizing biological research in both animals and plants over the past decade.[5–7]

Unlike early genome editing platforms such as zinc-finger nucleases and transcription activator-like effector nucleases,[8,9] which both specifically bind to DNA through complex engineered proteins, the CRISPR-Cas9 system relies on the specific binding of an engineered single guide RNA (sgRNA) with homology to the target DNA. These easily programmable sgRNAs bind to Cas9 and recruit the nuclease to cleave the target DNA, forming DSBs.[4,10] DSBs will activate the cell's

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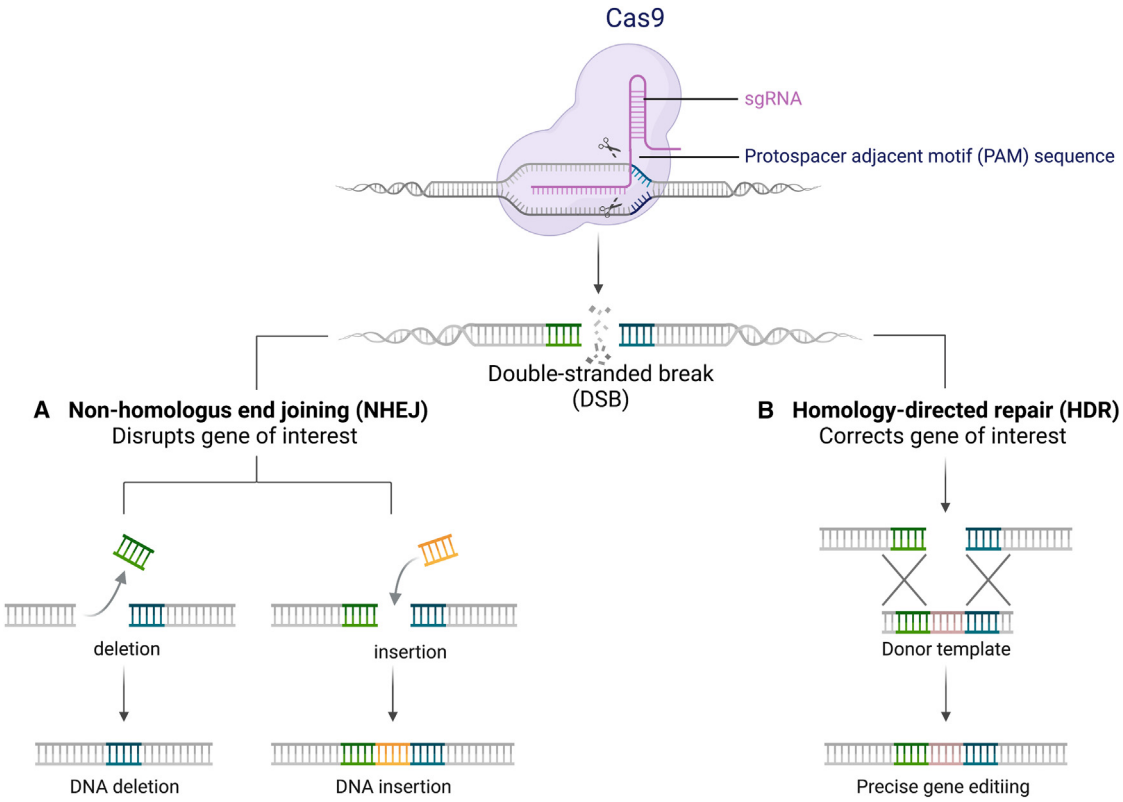

Figure 1. CRISPR-Cas9 gene editing strategies

CRISPR-Cas9-mediated gene editing can be achieved through either non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways. NHEJ introduces semi-random insertion-deletion mutations (indels). (A) HDR achieves precise insertion, deletion, or substitution of nucleotides using donor templates. (B) Green gene sequence, randomly deleted DNA sequences; yellow gene sequence, randomly inserted sequences; pink gene sequence, precise insertion, deletion, or substitution sequences. This figure was created using BioRender.com.

endogenous repair machinery, which mainly consists of either non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways. NHEJ introduces semi-random insertion-deletion mutations (indels) (Figure 1A); however, when a double-stranded or single-stranded donor template that has homology to the adjacent sequences surrounding the DSBs is present, the HDR pathway can be taken and follow the base sequence of the template to achieve the precise repair (Figure 1B).

CRISPR-Cas9-mediated HDR is the leading platform for precise gene editing and, importantly, holds tremendous application prospects. First, HDR can knockin an epitope tag or fluorescent protein fused with a specific protein to precisely monitor its subcellular localization, which is essential to understanding cellular processes.[11] Second, HDR can be used to create precise mutations *in vivo* and to establish models that mimic human disease.[12] Third, HDR is potentially promising for gene therapy, as inherited diseases caused by mutant genes could be corrected via HDR.[13] Furthermore, HDR can also enable precise insertion of therapeutic transgenes for permanent gene therapy.[14] Although these applications of HDR are very promising, limitations and challenges must be acknowledged. One major limitation is the low efficiency of CRISPR-Cas9-mediated HDR, particularly in non-dividing cells. Other issues such as the relatively high frequency of NHEJ and CRISPR-Cas9 off-target editing also hinder the adoption of CRISPR-Cas9-mediated HDR. These challenges are being addressed by a growing number of studies.

In this review, we aim to provide an overview of CRISPR-Cas9-mediated HDR. We briefly introduce mechanisms of DNA break repair, highlighting NHEJ and HDR as two distinct and competing outcomes. Next, we focus on current approaches for improving HDR efficiency, and on applications of precise gene editing in basic science and gene therapy. Although other Cas nuclease variants such as *Staphylococcus aureus* Cas9 (SaCas9), Cas12a, Cas12f, and Cas14 also enable gene editing, here we focus on Cas9, as it has been the subject of the majority of the relevant literature.

OVERVIEW OF CRISPR-CAS9-MEDIATED GENE EDITING

The CRISPR-Cas9 gene editing system employs a sgRNA to direct the Cas9 nuclease to the desired DNA target, inducing a DSB.[4] The sgRNA is derived from the mature tracrRNA:crRNA complex and

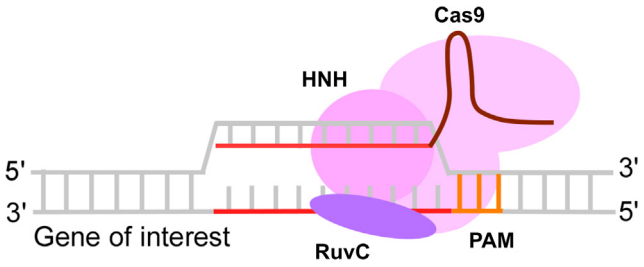

Figure 2. dsDNA resected through two domains of Cas9
Cas9 employs two distinct domains to cut both strands of DNA. The HNH domain targets and cleaves the DNA strand that pairs with the sgRNA, while the RuvC domain cuts the opposite strand.

contains both the 20 nucleotide target sequence to direct Cas9 to a specific genomic locus and the scaffold sequence necessary for Cas9 binding.[4] Cas9 recognizes the protospacer adjacent motif (PAM) and, subsequently, uses two different domains to cleave the two single-strand DNA (ssDNA) sequences. The HNH domain of Cas9 cleaves the DNA strand that is complementary to the sgRNA, while the RuvC domain is responsible for the cleavage of the remaining strand, resulting in a blunt-ended break (Figure 2).[4,15] While, DSBs with 5′ or 3′ overhangs have also been proposed.[16] In addition, considering that the two distinct domains of Cas9 interact with ssDNA, nickase Cas9 variants created by mutating the HNH (D10A) or RuvC (H840A) domains induce single-strand nicks instead of DSBs in the target DNA. By using a pair of these nickase CRISPR-Cas9 complexes to target opposite strands adjacent to the intended site, it is possible to achieve effects similar to DSBs but with 5′ overhangs, thereby reducing off-target frequencies.[17–19] Following cleavage, DSBs are mainly repaired via either the NHEJ or HDR DNA repair pathway.

NHEJ pathway

Although the resulting breaks can be repaired via either NHEJ or HDR pathway, NHEJ is the predominant DSB repair pathway. The first step of NHEJ is activation of the Ku protein complex, a heterodimeric protein composed of approximately 70- and 80-kDa subunits (Ku70 and Ku80),[20,21] which form a dyad-symmetrical molecule with a preformed ring encircling duplex DNA.[22] This ring recognizes and wraps the end of the broken DNA strand (Figure 3).[23]

There are three sub-pathways of the NHEJ repair pathway. The first is the blunt-end ligation-dependent Ku-XRCC4-DNA ligase IV sub-pathway, in which the Ku protein promotes the binding of X-ray repair cross-complementing protein 4 (XRCC4) and DNA ligase IV to the DNA ends. This Ku-XRCC4-DNA ligase IV complex catalyzes the reconstitution of broken double-strand DNA.[24] As CRISPR-Cas9 is thought to predominately produce blunt end DSBs,[4] this sub-pathway is particularly relevant in repairing DSBs caused by Cas9 (Figure 3A). The second is the nuclease-dependent sub-pathway, in which the Ku complex recruits DNA-dependent protein kinases (DNA-PKcs) to bind to DNA ends to form stable enzymatically active complexes.[25] DNA-PKcs then interact with and activate the endonuclease activity of Artemis, forming the Artemis:DNA-PKc complex,[26] which removes 5′ or 3′ overhangs, creating ends that can be ligated through the XRCC4-DNA ligase IV complex or combined with XRCC4-like factor and paralog of XRCC4 (Figures 3B and 3C).[27–30] The third sub-pathway is the polymerase-dependent sub-pathway, in which polymerase Pol μ and Pol λ are recruited to the DNA ends via interaction with the Ku-DNA complex.[28] Pol μ promotes the formation of terminal microhomology to stimulate the joining of two mismatched 3′ overhangs in a reaction that involves the Artemis:DNA-PKc complex (Figure 3D).[24] NHEJ does not require a homologous template for repair and is a critical pathway for maintaining genomic stability, preventing mutations, and cell death. NHEJ is not limited to a specific stage of the cell cycle and, therefore, CRISPR-Cas9-mediated NHEJ can function in any type of cell. Due to the action of the Artemis endonuclease and DNA polymerase, the NHEJ repair pathway will introduce or remove some bases, resulting in indels. However, recent studies have shown that the NHEJ pathway can also repair DSBs in an error-free manner. It has been demonstrated that deficiencies in Ku80 or Xrcc4, which are essential components of the NHEJ pathway, eliminate error-free repair events.[31] In addition, the repair of signal joints, which occurs through the direct ligation of blunt ends, is predominantly error free.[32,33]

HDR pathway

HDR is another pathway that repairs Cas9-mediated DSBs. It is an accurate mechanism due to the requirement of donor DNA templates. The most common form of HDR is homologous recombination (HR). Initially, the 5′-ended DSB is resected to provide short 3′ ssDNA overhangs, which are recognized and bound by a protein complex comprised of Mre1, Rad50, and Nbs1(MRN). The MRN complex recruits and activates the ataxia telangiectasia mutated (ATM) protein kinase[34,35] to phosphorylate and activate the C-terminal binding protein interacting protein (CtIP),[36] CtIP then combines with breast cancer-associated protein 1 (BRCA1) to create a BRCA1/MRN/CtIP complex.[37] Subsequently, the complex continues cleaving the 5′ end of the DSB site, exposing long 3′ ssDNA overhangs.[38–40] Replication protein A (RPA) then identifies and binds to the overhangs, protecting and stabilizing them.[41] RAD51 replaces RPA on the ssDNA by interacting with BRCA2 to form a presynaptic nucleoprotein filament complex, facilitating the search for endogenous and exogenous donor templates.[42,43] The donor templates invade the 3′ ssDNA overhangs, forming an intermediate displacement loop (D loop) and recruiting DNA polymerase δ (poly δ) to catalyze the synthesis of new strands, completing the DNA repair process.[44–46]

There are mainly two sub-pathways of HDR, including the classical double-strand break repair (DSBR) and synthesis-dependent strand annealing (SDSA).[47,48] In the DSBR sub-pathway, two intermediates with Holliday junctions (HJs) form when the 3′ ssDNA overhangs invade an intact homologous template. HJs are four-stranded branched structures accompanied by gap-filling DNA synthesis and ligation.[49] Each HJ resolution could happen on the crossing strand or on the non-crossing strand to produce crossover or non-crossover products (Figure 4A).[50] In the sub-pathway of SDSA, however, the

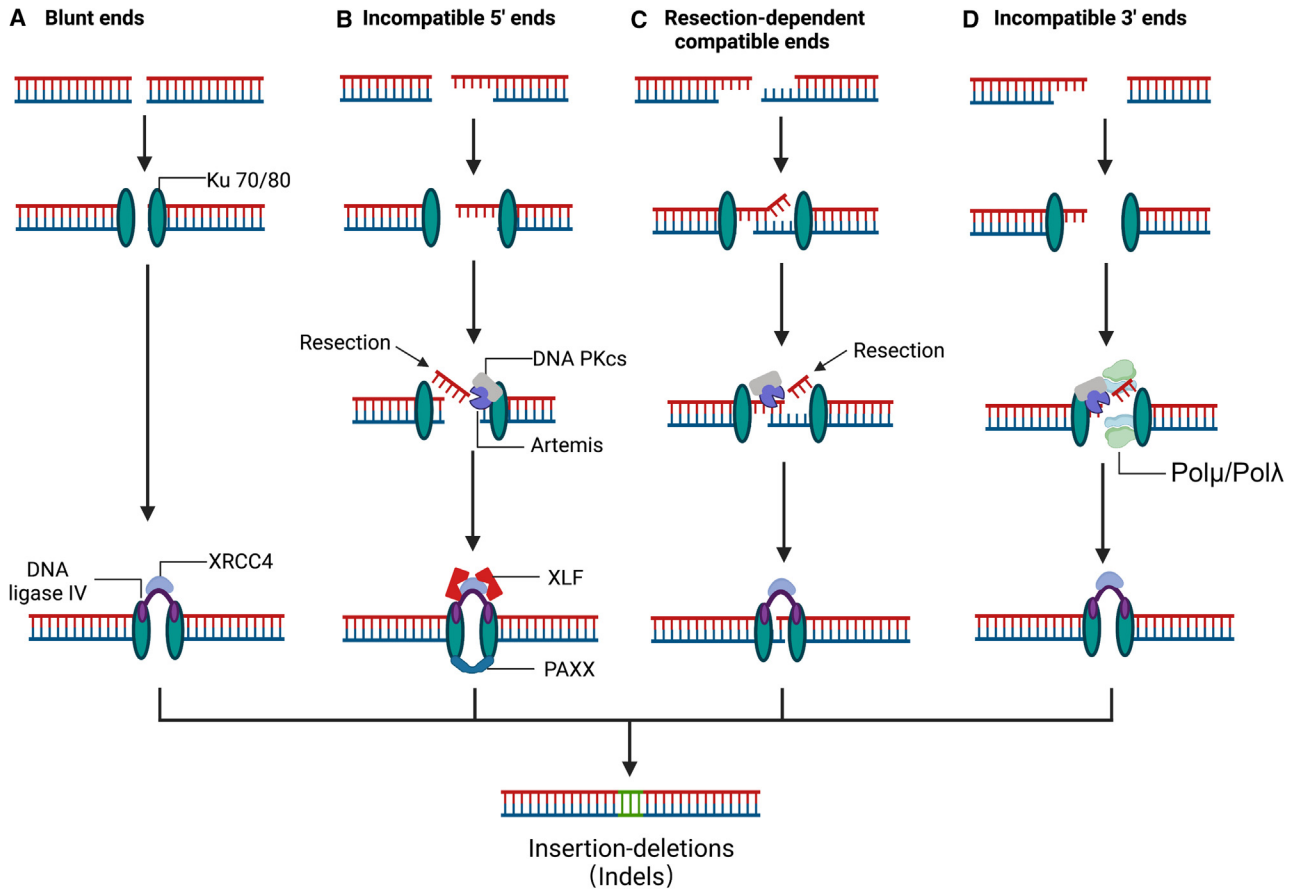

Figure 3. DSBs repaired through the NHEJ pathway

In the NHEJ pathway, DSBs are first recognized and wrapped by the Ku complex, and subsequently repaired through three main sub-pathways determined by the end structure of the DSB. Blunt-end DSBs will be repaired by the blunt-end ligation-dependent Ku-XRCC4-DNA ligase IV sub-pathway (A). DSBs with sticky-ends will be repaired by either the nuclease-dependent sub-pathway (B and C) or the polymerase-dependent sub-pathway, which depends on the presence of terminal microhomology to stimulate the joining of two mismatched 3′ overhangs (D). Green gene sequence, random insertion, deletion, or substitution sequences. XRCC4, X-ray repair cross-complementing protein 4; DNA-PKcs, DNA-dependent protein kinases; XLF, XRCC4-like factor; PAXX, paralog of XRCC4. This figure was created using BioRender.com.

invaded template strand separates from the D loop during fresh DNA synthesis.[51] After gap-filling DNA synthesis extends the two ends, the newly synthesized ssDNA anneals with the complementary ssDNA strand linked to the other DSB end. Therefore, this ligation produces only non-crossover products (Figure 4B).[50] Given that HDR uses donor templates to direct repair, it can be used to produce accurate DNA editing.

Under physiological conditions, the donor template is typically the sister chromatid, which is present during the S/G2 phases of the cell cycle. More importantly, the activation of the key protein ATM in the HDR pathway is cell-cycle dependent.[52] Therefore, HDR is restricted in the S/G2 phases of the mitotic cells.

Alternative DSB repair pathways

Besides the two canonical DSB repair pathways mentioned above, there are at least two additional repair pathways: alternative end joining (a-EJ) and single-strand annealing (SSA). Both the a-EJ and SSA are independent of the involvement of Ku, and the binding of Ku to DNA ends may need to be reduced for a-EJ and SSA to occur.[53] Similar to the HDR, the a-EJ and SSA pathways involve the 5′ to 3′ nucleolytic resection of broken ends. This process ends with 3′ ssDNA overhangs facilitated by CtIP and the MRN complex.[54–57] The a-EJ pathway relies on microhomologies ranging from 2 to 20 bp, whereas SSA requires more than 20 bp of homology.[58–61] In most eukaryotes, the a-EJ pathway is associated with DNA polymerase theta (Polθ) for annealing of microhomology.[62] Hence, a-EJ is often referred to as Polθ-mediated end joining (TMEJ).[63] Poly(ADP-ribose) polymerase 1 (PARP1) is another factor implicated in the a-EJ pathway. Studies have shown that the recruitment of Polθ to DSBs is diminished in cells lacking PARP1 or when cells are treated with PARP inhibitors.[64,65] In contrast, the SSA pathway depends on EXO1, Bloom syndrome RecQ-like helicase (BLM), or DNA replication helicase/nuclease 2 (DNA2) working together to create longer 3′ ssDNA tails, which are then coated by multiple copies of RPA.[38,66] Unlike

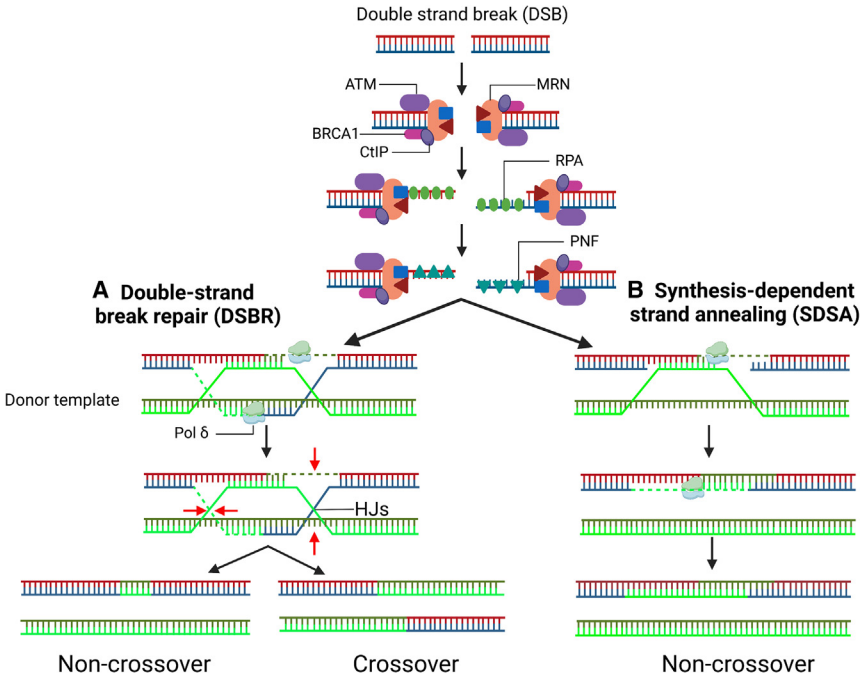

Figure 4. DSBs repaired through the HDR pathway
In the HDR pathway, DSBs are first recognized by the MRN complex, which continues recruiting other factors to form the BRCA1/MRN/CtIP complex. The complex then cleaves the 5′ end to expose long 3′ single-strand DNA (ssDNA) overhangs, which are bound by RPA. Eventually, RPA is replaced with the PNF complex. The DSB can be repaired via the DSB repair (DSBR) or the synthesis-dependent strand annealing (SDSA) sub-pathways. DSBR forms two intermediates with HJs, which are processed to generate either non-crossover or crossover products (A). In SDSA, an ssDNA 3′ end invades homologous dsDNA, which is used as a template for repair via the formation of a D loop structure. SDSA generates only non-crossover products (B). ATM, ataxia telangiectasia mutated protein kinase; MRN, Mre1-Rad50-Nbs1 complex; BRCA1, cancer-associated protein 1; CtIP, C-terminal binding protein interacting protein; RPA, replication protein A; PNF, presynaptic nucleoprotein filament complex; HJs, Holliday junctions.

HDR, where RAD51 replaces RPA to facilitate homology search and strand invasion, SSA relies on RAD52 and requires 3′ ssDNA tails with compatible sequence homology to form a stable annealing intermediate.[58,67] As a-EJ and SSA are involved in extensive resection, which is dependent on the cell cycle of cyclin-dependent kinases, these two pathways are favored in the S and G2 phases.[68,69]

Competition between NHEJ and HDR pathways

In the repair of DSBs, NHEJ and HDR are the predominant repair pathways, and the primary factor influencing pathway selection is the need for extensive DNA end resection.[70,71] The NHEJ pathway does not require extensive end resection, and the ends are protected by the binding of Ku70-Ku80. In addition, p53-binding protein 1 (53BP1) and the recently discovered Shieldin also play an important role in protection of DNA end.[72,73] While HDR requires MRN complex and CtIP for extensive 5′ to 3′ resection of regions of the duplex to generate stretches of ssDNA at DNA ends. NHEJ is a simple and efficient pathway that does not require an additional DNA template, only takes about 30 min[74] and, importantly, can occur throughout the cell cycle in a variety of cell types.[53] In comparison to NHEJ, HDR is a precise repair pathway with a complex mechanism. The entire HDR process takes at least 7 h or more,[47] requires endogenous or exogenous donor template and mainly occurs in the S/G2 phase of mitotic and meiotic cells.[1,75] The modification frequency of CRISPR-Cas9-mediated NHEJ on the mouse genome can reach up to 60%, while the modification efficiency of HDR is typically 0.5%–20%.[76] Given the benefits of HDR-mediated editing, methods to improve HDR efficiency are being actively pursued (Table 1).

STRATEGIES FOR BOOSTING HDR EFFICIENCY

There are a number of factors that may influence HDR efficiency. First, as the NHEJ pathway is the predominant mechanism of DSB repair, it has a major impact on HDR.[74] Second, the expression and activity of the many key HDR pathway proteins, such as BRCA1, BRCA2, and Rad51, are crucial. Third, the HDR pathway needs endogenous or exogenous donor templates, which can significantly affect editing efficiency.[77,78] Fourth, the delivery of Cas9/sgRNA reagents is another important factor, as it can also significantly affect HDR efficiency. Thus, strategies for enhancing HDR efficiency can be divided into four main categories: inhibition of the NHEJ DNA repair pathway, activation of the HDR DNA repair pathway, modification of the DNA donor templates, and delivery of Cas9/sgRNA reagents (Table 2). These approaches are discussed below.

Inhibition of NHEJ pathway

As discussed above, NHEJ is the predominant DNA repair pathway for DSBs, and multiple approaches have been developed to globally inhibit the cellular NHEJ repair pathway in order to boost HDR efficiency. These approaches include inhibition or depletion of NHEJ factors through small-molecule compounds, the ubiquitination of target proteins, and gene knockdown or silencing. Critical NHEJ factors that are often targeted include the Ku complex, DNA ligase IV, DNA-PKcs, or 53BP1.[20,21,25,72]

Several studies have reported the use of small-molecule compounds to inhibit key factors in the NHEJ pathway in order to enhance HDR efficiency. In one approach, a small-molecule inhibitor named Scr7, which interferes with the DNA binding domain of DNA ligase IV,

Table 1. Comparison between NHEJ and HDR repair pathway

The subset	NHEJ repair pathway	HDR repair pathway
Subtype repair pathway	blunt-end ligation dependent Ku-XRCC4-DNA ligase IV sub-pathway nuclease-dependent sub-pathway polymerase-dependent sub-pathway	double-strand break repair (DSBR) synthesis-dependent strand annealing (SDSA)
Duration time	about 0.5 h	over 7 h
Template requirement	no	yes
Key factors	Ku complex, XRCC4, DNA ligase IV, DNA-PKcs, et al.	MRN, Rad51, ATM, BRCA1, CtIP, RPA, et al.
Traits	throughout the cell cycle high efficiency predominant repair pathway	restricted in S/G2 phase of cell cycle low efficiency secondary repair pathway
Outcomes	error-free and semi-random insertion-deletion	precise substitution, insertion, or deletion
Usage	gene functional research gene knockout for gene therapy	protein labeling correction of mutant gene for gene therapy precisely mutated gene for mimicking disease precise insertion of therapeutic genes for permanent gene therapy

resulting in reduced affinity for DSBs and inhibited function, was used to induce improvements in HDR efficiency in both human and mouse cell lines. Impressively, Scr7 treatment boosted the efficiency of HDR by up to 19-fold.[76] In a different approach, several groups utilized various DNA-PKc inhibitors to efficiently increase HDR efficiency by up to 81% in multiple cell lines, such as 293T cells, K562 cells, primary $CD4^+$ T cells, and human induced pluripotent stem cells.[79,111–114] Given the role of TMEJ in DSB repair, simply inhibiting the NHEJ pathway may have a lack of consistent effect on HDR. To this end, two groups simultaneous inhibition of TMEJ with the Polθ inhibitor and NHEJ with DNA-PK inhibitor significantly improves HDR-mediated repair of Cas9-induced DSBs in both mouse and human cells.[115,116]

Ubiquitination is also used for the inhibition of proteins in the NHEJ pathway. Canny et al. screened a library of engineered ubiquitin variants for inhibitors of 53BP1, which is an essential regulator of DSB repair and functions to favor NHEJ over HDR via suppressing end resection. Expression of the inhibitor of 53BP1 in human and mouse cell lines improved the HDR efficiency with either dsDNA or single-strand oligonucleotide donors by up to 5.6-fold.[80] Weber et al. used adenovirus 4 to express E1B55K and E4orf6 proteins, which display ubiquitination and proteasomal degradation activity of DNA ligase IV. They co-expressed these two proteins with the CRISPR-Cas9 system and donor templates in both human and mouse cell lines. The results showed that the efficiency of HDR can be enhanced up to 8-fold.[81]

Gene silencing is another approach to boosting HDR efficiency through NHEJ inhibition. RNA interference (RNAi), which involves sequence-specific suppression of gene expression by double-strand RNA, is known for its precision, efficiency, and stability of gene

Table 2. Strategy for boosting HDR efficiency

The subset	Methods	Targets	Strengths and weakness	Reference
NHEJ pathway inhibition	small-molecule inhibition ubiquitination gene silencing	Ku complex DNA ligase IV DNA PKcs 53PB1	adverse for genome stability applied *in vitro*	Maruyama et al.[76]; Robert et al.[79]; Canny et al.[80]; Chu et al.[81]; Li et al.[82]; Zhao et al.[83]; Schiroli et al.[84]
HDR pathway activation	small-molecule activation fusion HDR factors to Cas protein new factor of HDR	Rad family MRN complex CtIP	adverse for genome stability applied *in vitro*	Song et al.[85]; Pinder et al.[86]; Reuven et al.[87]; Charpentier et al.[88]; Tran et al.[89]; Carusillo et al.[90]; Bashir et al.[91]; Nambiar et al.[92]
Donor template modification	improving concentration/numbers of dsDNA templates replaced as ssDNA templates optimize length of templates	concentration of templates structure of templates type of templates length of templates	widely applied *in vitro* and *in vivo*	Aird et al.[93]; Nguyen et al.[94]; Richardson et al.[95]; Sharon et al.[96]; Kong et al.[97]; Zhao et al.[98]; Zhang,[99]; Gutierrez-Triana et al.[100]; Yoshimi et al.[101]; Shy et al.[102]; Martin et al.[103]; Ishizu et al.[104]; Nishiyama et al.[105]; Zheng et al.[106]; Chen et al.[107]
Delivery of Cas9/sgRNA reagents	electroporation microinjection vector transfection	Cas9/sgRNA DNAs Cas9/sgRNA RNAs Cas9/sgRNA RNPs	widely applied *in vitro* and *in vivo*	De Caneva et al.[108]; Zheng et al.[106]; Yin et al.[109]; Schumann et al.[110]

suppression, and therefore is commonly used as a gene silencing technology.[117] Li et al. used siRNA to suppress the expression of the Ku70/Ku80 complex in pig fetal fibroblasts. They showed that the frequencies of multiple HDR pathways were promoted after downregulation of the complex, including HR, SSA, and single-stranded oligonucleotide-mediated DNA repair.[82] In another approach, Zhao et al. developed genome-wide high-throughput screening to search for genes suppressing HDR and identified the SHROOM1 gene. By using SHROOM1 siRNA, they significantly promoted precise gene editing in human and mouse cells, with the editing efficiency being improved by up to 10-fold.[83] Dominant negative inhibition, a mutant variant gene product that impairs the normal function of a wild-type gene product when co-expressed, was also used for gene silencing.[118] Studies have shown that Cas9-induced DSBs activate the P53 damage response and induce a transient arrest in the G1 phase of the cell cycle, which improves the NHEJ frequency.[119] In one approach, Schiroli et al. co-electroporated an mRNA encoding for a dominant negative p53 truncated form (GSE56)[120] when editing the *IL2RG* or *AAVS1* loci in hematopoietic stem and progenitor cells (HSPCs) with Cas9/sgRNA ribonucleoprotein (RNP) complexes, followed by the donor template. The result showed that the percentage of HDR-edited cells was higher among all different HSPC sub-populations.[84]

Although these methods are widely used to enhance HDR efficiency, there are several limitations. First, while inhibiting NHEJ at specific sites, these approaches also impair natural DSB repair. Given the key role of the NHEJ DNA repair pathway in genome maintenance, overall NHEJ inhibition strategies may have serious adverse consequences on genome integrity. Indeed, permanent global inhibition of NHEJ factors such as DNA ligase IV and DNA-PKcs in humans results in severe combined immune deficiencies, pancytopenia, and growth retardation.[121] To address this challenge, Jayavaradhan et al. developed a dominant negative version of 53BP1 (DN1S) that suppresses the accumulation of endogenous 53BP1 at the DSB sites. By fusion of DN1S to Cas9, DN1S is recruited to Cas9 target sites, resulting in improved HDR efficiencies, while NHEJ is not globally affected, thereby maintaining cell viability.[122] A second limitation of NHEJ inhibition strategies is that, currently, these methods are only widely used *in vitro*. Therefore, these approaches may be more suitable for *ex vivo* clinical gene therapy. As *ex vivo* gene therapy entails modifying cells outside the body to generate therapeutic factors, which are then transplanted back into the patient for treatment, and a range of cell types can be genetically engineered. *Ex vivo* gene therapy involved in HDR has been used in various cell types, such as HSPCs and T cells. However, as one of the most exciting applications of HDR-mediated precisely targeted gene editing in clinical gene therapy, adaptation of these methods for *in vivo* use is needed.

Activation of HDR pathway

Activation of the HDR pathway is another approach for improving HDR efficiency, as there are many proteins that play key roles in the HDR pathway. Therefore, activation of the HDR pathway can be achieved either by overexpression or stimulation of these proteins, such as factors that make up the Rad family, the MRN complex, and CtIP.

Small-molecule activators are widely used to stimulate the activity of target proteins. One such example, RAD51-stimulatory compound 1 (RS-1), can enhance the binding ability of Rad51 to ssDNA under various biochemical conditions, and stimulate Rad51 to mediate the formation of D loops between homologous strands, resulting in a 5- to 11-fold increase in HDR activity of early-passage neonatal human dermal fibroblasts.[123] Song et al. and Pinder et al. introduced RS-1 together with CRISPR-Cas9 components and in rabbit embryos and a variety of human cell lines, HDR editing efficiency was increased 3- to 6-fold.[85,86]

Some groups have attempted to improve HDR efficiency by fusion of Cas9 to a domain known to recruit HDR factors. To this end, Reuven et al. fused a 126-amino intrinsically disordered domain from HSV-1 alkaline nuclease (UL12) that recruits the MRN complex to the N- or C-terminal of spCas9. By transfecting with plasmid-encoded Cas9 or Cas9/sgRNA RNPs complexes, they improved the HDR efficiency 2-fold in HEK293 cells.[87] Charpentier et al. fused a minimal N-terminal fragment of CtIP to Cas9 and obtained a 2-fold or greater improvement in HDR efficiency compared with that observed with wild-type Cas9 in human cell lines, induced pluripotent stem cells (iPSCs), and rat zygotes.[88] Tran et al. investigated fusion proteins combining Cas9 nuclease with HDR effectors to improve precise genetic modifications and found that both Cas9-CtIP and MS2-CtIP systems increase the HDR/NHEJ ratio by 4.5- to 6-fold in human HEK293 cell lines.[89] Carusillo et al. developed a novel platform that combines Cas9 with a dominant negative ring finger protein 168 (dnRNF168) lacking the RING domain, which together inhibit NHEJ and promote HDR for precise repair of Cas-induced DSBs. This approach enhances error-free editing by 1.5- to 7-fold compared with standard CRISPR-Cas9 in primary human cells.[90] Bashir et al. found that co-expressing ubiquitin binding domain fusions from Rad18 or RNF169 with BRCA1 and DNA binding domains enhances HDR, reduces NHEJ, and increases the HDR/NHEJ ratio up to 6-fold in HEK293 cells.[91]

Alternatively, some studies have now focused on identifying new factors that are essential for the HDR. In one study, Nambiar et al. found that Rad18, a circular E3 ubiquitin ligase, can be recruited to DSBs and interacted with the recombinase Rad51C to promote HR.[124] Next, they screened an enhanced Rad18 variant, e18, and showed that e18 stimulated CRISPR-Cas9-mediated HDR using single-stranded oligodeoxynucleotides (ssODNs) donors up to 2.7- and 3-fold in HEK293 and HeLa cell lines.[92]

These approaches showed promise for boosting HDR efficiency; however, as discussed above, these methods are not targetable to specific cell populations, which may result in serious adverse consequences for genome stability. Hence, these approaches also suitable for *ex vivo* gene therapy.

Modification of DNA donor template

The entire process of HDR requires not only HDR pathway-related proteins but also a DNA donor template, which can be endogenous or exogenous in origin. Among these, endogenous templates mainly consist of sister chromatids, while extrachromosomal donor templates can come from a wide range of sources, such as plasmids, viral, and bacterial vectors, and artificially synthesized ssODNs. The effects of donor templates on HDR efficiency can be divided into several aspects: the concentration or availability of templates, the structure of templates, the type of templates, and the length of templates.

Concentration or availability of templates

Several studies have attempted to improve HDR efficiency by enhancing the concentration of templates. This strategy involves either enhancing the local concentration of donor templates at the DSBs or broadly introducing a large number of templates. In one approach, the ssODN template was covalently tethered to the Cas9/sgRNA RNP complex via a fused HUH endonuclease for localizing the template at DSBs, resulting in up to a 30-fold enhancement of HDR efficiency in HEK293 and U2-OS cells.[93] In a second approach, 16 bp truncated Cas9 target sequences (tCTs), which enable Cas9 binding but do not enable cutting, were added at the ends of the dsDNA donor template to interact with Cas9 RNPs to shuttle the template to the DSBs. By using this method, HDR efficiency was increased by 2- to 4-fold in different immune cell lines and induced pluripotent stem cell-derived HDPCs.[94] In a third approach, Richardson et al. found that dissociation of Cas9 from dsDNA substrate takes at least ~6 h, and initiates with Cas9 asymmetrically releasing the 3′ end of the cleaved nontarget strand. Therefore, they used a ssODN complementary to the non-target strand as the template and increased the HDR efficiency by up to 60% in HEK293 cells.[95]

In addition to concentrating the template at DSBs, increasing the number of templates may also improve HDR efficiency. Recently, Sharon et al. pursued this approach by using bacterial Retrons for template amplification.[96] Retrons are natural DNA elements that encode a reverse transcriptase, as well as a non-coding RNA (ncRNA) template on which the reverse transcriptase acts, to create a multi-copy single-stranded DNA (msDNA) product.[125,126] These msDNAs are covalently tethered to their template RNA. In this approach, the donor template is fused to a CRISPR sgRNA and transcribed. An exogenous reverse transcriptase recognizes the Retron RNA and produces donor template msDNA, which will be localized to the DSB site via linkage to the sgRNA.[127,128] By transforming Cas9-expressing yeast with Retron-carrying plasmids, the HDR-mediated insertion efficiency of short fragments in yeast reached almost 100%, while the insertion efficiency of long fragments (e.g., GFP gene, 765 bp) reached 92%.[96] More recently, Kong et al. modified this strategy for use in mammalian cell lines. By co-expression of Cas9-RT fusion and Retron ncRNA-sgRNA fusion in HEK293T cells, they demonstrated a rate of Retron editing-mediated HDR efficiency at endogenous genomic loci up to 10%.[97] Zhao et al. also used this strategy in HEK293T and K562 cells and achieved HDR efficiencies up to 11.4%.[98]

Structure of templates

The process of HDR requires invasion of the donor template, making the template structure an important determinant of HDR efficiency. Therefore, various approaches have been explored to modify the template structure and context. In one such approach, the plasmid vector was engineered to contain a donor template with flanking sites homologous to the sgRNA target sequence. Consequently, Cas9 would not only cut the target DNA sequence but also cleave the plasmid vector, creating a linear donor template. This method increased HDR efficiency in 293T cells and human iPSCs by 2- to 5-fold.[99] Injected (linear) dsDNA and the high activity of NHEJ re-ligating CRISPR-Cas9-mediated DSBs can lead to the multimerization of donor templates, thereby reducing HDR.[100] To address this, the 5′ end of the long dsDNA donor template was modified using "bulky" moieties such as biotin, amino-dT (A-dT), and carbon spacers to prevent its multimerization. By utilizing this modified donor template, 9.5% of the injected and surviving zygotes exhibited precise HDR-mediated single-copy integration. Specifically, 15.8% of the injected zygotes expressed GFP and, among these, 60% demonstrated precise single-copy integration.[100]

Type of templates

Studies employing CRISPR-mediated HDR have primarily used two types of donor templates: dsDNA and ssDNA. dsDNA donor templates can be carried by plasmids, viruses such as lentivirus, and bacterial vectors, while ssDNA donor templates can be artificially synthesized ssODNs, or viruses such as adeno-associated virus (AAV). A growing body of evidence has shown that HDR strategies utilizing ssDNA donor templates are more efficient than those with dsDNA donor templates.[77,78,129–136] Therefore, strategies utilizing ssDNA as donor template are becoming increasingly common. Artificially synthesized ssODNs as donor templates were widely used in *in vitro* applications due to their ease of production and operation. For instance, in one approach, a 1-kb ssODN co-injected with sgRNA and Cas9 mRNA produce efficient GFP-KI at the *Thy1* locus into rat zygotes.[101] In addition, ssDNA donors have reduced toxicity. The group of Marson found that a high concentration of dsDNA donor templates flanked with the Cas9 target sequence could be toxic to primary cells, whereas an ssDNA template flanked by dsDNA Cas9 target sequences exhibited reduced toxicity and enhanced knockin efficiency across various target loci in various human cell types, including primary human T cell subsets, B cells, natural killer cells, and CD34+ cells, achieving HDR efficiencies of up to 80%–90%.[94,102]

Interestingly, the AAV genome is initially ssDNA and only converts to dsDNA upon entering the host cell nucleus.[137] AAV demonstrates high transduction efficiency in both mitotic and postmitotic cells, possesses an attractive safety profile, and has the ability to selectively transduce various tissues through different serotypes.[137,138] Consequently, AAV serves as an ideal vector for delivering ssDNA donor templates in gene editing applications, suitable for both *in vivo* and *in vitro* experiments. For instance, in one approach, a donor template delivered by serotype 6 (AAV6) along with Cas9/sgRNA RNPs was introduced into human iPSCs, resulting in highly efficient and

bi-allelic integration frequencies across multiple loci, including precise editing frequencies of up to 94%.[103] In another approach, AAV6 was utilized to deliver sgRNA and the donor template to cultured Cas9-expressing primary mouse cardiomyocytes, achieving an HDR editing efficiency of approximately 25%.[104] In addition, Nishiyama et al. employed AAV9 to deliver sgRNA and a homologous template, administering AAV9 systemically into Cas9-expressing mice. The results demonstrated that *in vivo* HDR efficiency in neurons can reach up to ∼15%.[105] Recently, our team employed AAV9 to deliver sgRNAs and DNA repair templates to Cas9-expressing mice, observing that the HDR efficiency in cardiomyocytes reached up to ∼45% of transduced cells.[106] Chen et al. investigated the use of self-complementary AAV (scAAV), a type of dsDNA AAV, to carry the donor template. Intriguingly, when the donor vector and Cas9/sgRNA RNPs were introduced into mouse zygotes, scAAV efficiently mediated HDR, outperforming standard AAV by ∼17%.[107] This outcome could possibly be attributed to increased stability, the presence of two copies of the donor template in opposite polarities, or the potential for double crossover events.[139] Nonetheless, the mechanism by which AAV participates in HDR as a donor template warrants further investigation.

Lentivirus is a stable and adaptable method for expressing Cas9 and/or gRNA, making it a valuable tool in CRISPR-Cas9 applications. Unlike AAV vectors, which have a limited packaging capacity of about 4.5 kb, lentivirus vectors can carry a larger genetic payload, approximately 8 kb. This greater capacity makes lentivirus a popular choice for genome-wide CRISPR-Cas9 screens. However, producing lentiviral particles requires packaging and envelope plasmids, which provide the essential components for the process. In terms of HDR, research has shown that, by combining efficient expression of engineered nucleases with the delivery of donor templates via integration-defective lentiviral vectors, highly efficient HDR can be achieved, as demonstrated in the WA09 human embryonic stem cell (ESC) line.[140] Another study introduced a method using a single integration-defective lentiviral vector that includes a Cas9 off switch. This one-vector system effectively supports knockin of a full-length EGFP gene sequence with efficiency rates of up to 80%.[141]

Adenovirus vectors is another powerful tool for highly efficient gene delivery, both *in vitro* and *in vivo*. This efficiency is largely due to the widespread expression of primary adenovirus receptors and secondary integrin receptors on most cells. Studies have shown that adenoviral CRISPR vectors can achieve knockout efficiencies of up to 80%.[142] However, using adenoviruses *in vivo* poses challenges, as they can trigger strong immune responses and inflammation. In addition, producing adenoviruses is labor-intensive, and there are limited reports on their ability to enhance HDR efficiency.

Length of templates

In classical HR, the BRCA1/MRN/CtIP complex resects the 5′ ends at the DSB site to expose long 3′ ssDNA overhangs, which require a long sequence of homology to facilitate precise repair.[38,40] Hence, the length of the donor template may also be a key factor for HDR efficiency. The length of the donor template can range from ∼50 to ∼1 kb in each homology arm. Typically, ssODNs carry short-length homology arms from ∼50 to ∼100 bp due to the difficulty of synthesizing long ssODNs,[101] while vectors such as AAV are typically used to deliver much longer homology arms, such as 400–1,000 bp.[103,106,143] Interestingly, there is no evidence showing that longer homology arms are favored in Cas9-mediated HDR. Given that long homology arms are essential for canonical HR, it is possible that these short ssDNA homology arms participate in alternative HDR pathways. Indeed, one recent report demonstrated that Cas9-induced DSBs are repaired by single-strand template repair, which requires the Fanconi anemia pathway in human cells.[144]

Donor templates are essential to the HDR process and play a crucial role in determining its efficiency. Moreover, ssDNA donor templates, including ssODNs and AAV, or dsDNA templates such as plasmids, do not typically induce severe side effects in cells due to their lack of *cis*-regulatory and protein coding sequences. Therefore, optimizing the donor template represents a safer strategy for enhancing HDR efficiency compared with alternative approaches such as inhibiting the NHEJ pathway or promoting the HDR pathway. This method of donor template optimization is widely utilized in both *in vivo* and *in vitro* applications, including preclinical studies. Thus, enhancing HDR efficiency by modifying donor template characteristics or concentration holds significant promise.

Delivery of CRISPR-Cas9 reagents

Safe and efficient delivery of the CRISPR-Cas9 genome-editing system to target cells or tissues is a crucial step for successful genome editing. There are three primary CRISPR-Cas9 delivery strategies for genome editing: delivery of Cas9/sgRNA DNAs, RNAs, and RNPs.[145]

Delivering Cas9 and sgRNA DNA components

The method of delivering Cas9 and sgRNA DNA components through vectors such as plasmids or recombinant viruses is commonly utilized both *in vitro* and *in vivo* due to its simplicity and versatility. However, one primary limitation of plasmid delivery is its variable transfection efficiency across different cell types, which restricts its use mainly to *in vitro* applications. In contrast, viral vectors, including adenovirus, AAV, and lentivirus, offer several advantages. Among them, AAV stands out as the preferred choice due to its ability to achieve high transfection efficiency both *in vitro* and *in vivo*, coupled with stable transgene expression, low immunogenicity, and serotype-specific targeting capabilities.[137] For example, De Caneva et al. demonstrated a significant increase in targeting efficiency by injecting neonatal mice with two AAVs: one expressing SaCas9 and sgRNA, and the other containing a promoterless cDNA flanked by albumin homology regions. This approach resulted in a remarkable 26-fold enhancement in targeting efficiency for an EGFP reporter cDNA, with up to 24% of EGFP-positive hepatocytes.[108] Similarly, our team employed the dual AAV strategy to target cardiomyocytes in neonatal mice, achieving up to 45% HDR efficiency.[106] However, delivering Cas9 and sgRNA DNA components via AAV has its limitations, notably the constant expression of Cas9, which can trigger a

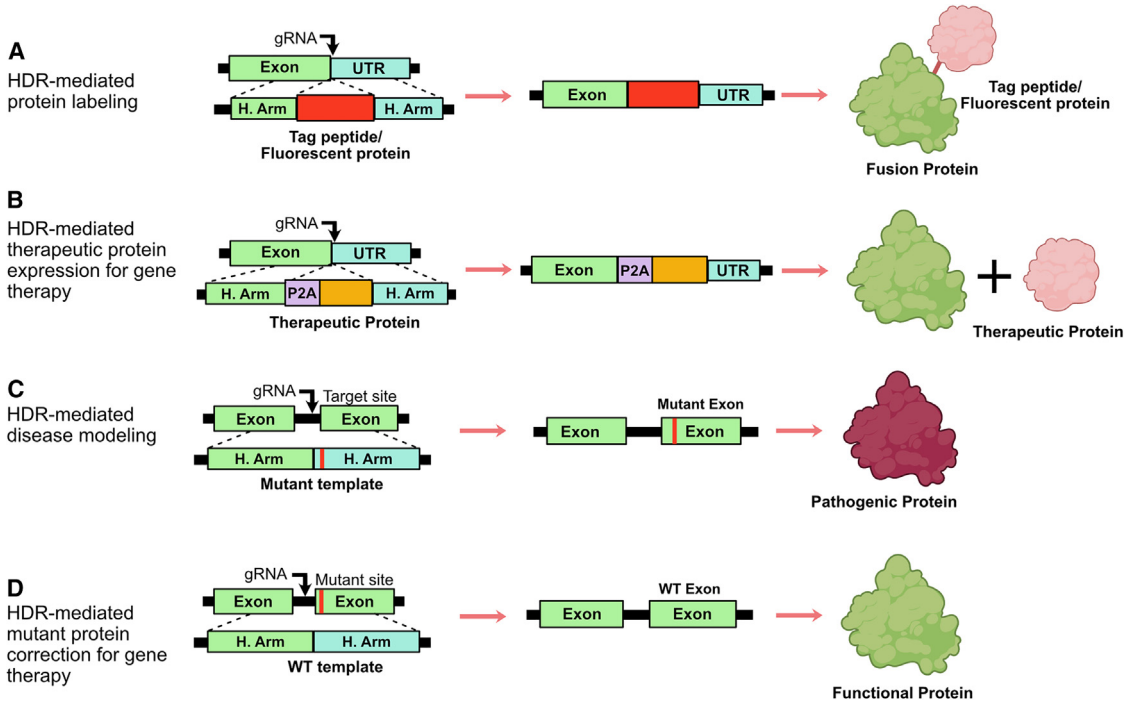

Figure 5. Types of HDR applications

In HDR-mediated protein labeling, sequences encoding tag peptides or fluorescent proteins are precisely integrated into the N- or C-terminal of the target gene to create a fusion protein (A). For HDR-mediated therapeutic protein expression in gene therapy, therapeutic proteins are precisely integrated into a safe harbor locus for permanent gene therapy (B). In HDR-mediated disease modeling, specific disease-causing mutations are introduced (C). Lastly, HDR-mediated correction of mutant proteins for gene therapy involves replacing the mutant sequence with the wild-type sequence, thereby restoring normal protein function (D).

p53-mediated DNA damage response, potentially increasing off-target risks.[119]

Delivering mixture of Cas9 mRNA and the sgRNA

The delivery of Cas9 and sgRNA through RNA-based methods offers several compelling advantages, including their smaller molecular size, rapid onset of action, and the potential to mitigate off-target effects by maintaining continuous expression of Cas9 and sgRNAs.[146] Methods for delivering Cas9 mRNA and sgRNA encompass various approaches, such as microinjection or electroporation of RNA mixtures, as well as the utilization of non-viral vectors such as lipid nanoparticles (LNPs)[147] and recently developed retrovirus-like proteins,[148] combined LNPs encapsulating SpCas9 mRNA with AAV encoding a sgRNA, and a repair template to correct the Fah mutation in hepatocytes of $Fah^{mut/mut}$ mice. The results demonstrated a correction efficiency of >6% in hepatocytes, with a low *in vivo* off-target lesion rate observed for viral sgRNA in conjunction with non-viral mRNA delivery of SpCas9.[109] However, it is essential to address the challenge of low transfection efficiency and targeting for further improvement in these methods.

Delivering Cas9/sgRNA RNPs

Compared with DNA and RNA forms of CRISPR-Cas9 delivery, RNP-based delivery offers the swiftest gene editing by bypassing the need to synthesize proteins and sgRNAs within cells. In addition, Cas9/sgRNA RNP transfection circumvents DNA integration into the genome, thereby minimizing the risk of off-target effects associated with persistent CRISPR component expression. Delivery of Cas9/sgRNA RNPs can be achieved through direct microinjection or electroporation of RNPs, or facilitated by vectors such as LNPs,[149] virus-like particles,[150] and the recently developed extracellular contractile injection systems.[151] For example, Schumann et al. utilized electroporation to deliver Cas9/sgRNA RNPs and a donor template, achieving precise DNA replacements at CXCR4 and PD-1 loci in T cells with HDR efficiency reaching approximately 20%.[110] This led to enhanced T cell effector function. Despite the potential benefits, efficient delivery of Cas9/sgRNA RNPs remains challenging. Cas9 proteins and sgRNAs are inherently unstable, and the large size and low endosomal escape efficiency of Cas9/sgRNA RNPs hinder their effective delivery.

APPLICATIONS OF HDR

CRISPR-Cas9-mediated HDR holds immense utility and promise in both basic science and medicine, with its applications primarily concentrated in three key areas. Firstly, it enables precise transgene integration for various purposes, such as protein labeling through the creation of fusion proteins with tag peptides or fluorescent proteins (Figure 5A), or the insertion of therapeutic genes for permanent gene therapy (Figure 5B). Secondly, it facilitates the generation of

specific genetic mutations essential for disease modeling (Figure 5C). Lastly, CRISPR-Cas9-mediated HDR allows for the correction of mutations, offering prospects for advanced gene therapy (Figure 5D).

Application of HDR for labeling proteins

The subcellular localization of proteins plays a pivotal role in cellular function and regulation. Firstly, proteins function optimally within specific subcellular compartments, where their proper localization facilitates interactions with targets and the execution of biological roles.[152] Secondly, subcellular localization contributes to the spatial organization of cellular processes by enabling the compartmentalization of biochemical reactions, thereby facilitating efficient coordination and regulation of cellular activities.[153] In addition, dysregulation of protein localization has been implicated in numerous diseases, including cancer, neurodegenerative disorders, and metabolic syndromes. Aberrant subcellular localization of proteins can disrupt normal cellular processes, leading to pathological conditions and disease progression.[153] Therefore, elucidating the subcellular location of proteins provides invaluable insights into their functions, cellular dynamics, and roles in health and disease. It serves as the foundation for understanding fundamental cellular processes and holds significant implications for biomedical research and therapeutic development.

Traditionally, methods such as immunostaining and overexpression of proteins fused with epitope tags or fluorescent proteins have been extensively used to study protein subcellular localization. However, immunostaining often encounters challenges due to the lack of specific antibodies tailored to the target protein. Moreover, it may struggle to distinguish between wild-type and mutant proteins, especially when the mutant protein carries minor alterations such as small point mutations or indels. Overexpression of tagged proteins presents similar limitations, including the potential for cellular protein targeting mechanisms to be overwhelmed by elevated levels of exogenous protein, resulting in a subcellular localization profile that differs significantly from that of the endogenous protein.[154] For instance, when fluorophore tags are present in high concentrations within a cell, they can cause the proteins to which they are attached to form unwanted complexes, potentially leading to these proteins being located in incorrect areas within the cell.[155] To address these challenges, precise gene editing via HDR can be employed to insert epitope tags or fluorescent proteins at endogenous loci, both *in vivo* within somatic tissues and *in vitro*. This approach offers the advantage of accurately reflecting the native expression levels and subcellular localization patterns of the target protein, thereby providing more reliable insights into its biological functions and regulatory mechanisms.

In a study conducted by Mikuni et al., a technique called single-cell labeling of endogenous proteins by CRISPR-Cas9-mediated HDR (SLENDR) was developed.[11] In this study, they successfully employed this technology to insert a sequence encoding an epitope tag or a fluorescent protein at the N or C terminus of a gene of interest. They achieved this by delivering the editing machinery to dividing neuronal progenitors through *in utero* electroporation. This approach proved effective in various cell types, regions, and ages in the brain.[11] In a second study, the hemagglutinin epitope tag was inserted into the endogenous *Nlgn1* gene using CRISPR-Cas9-mediated HDR. This study demonstrated that Nlgn1 is enriched at synapses between parallel fibers and molecular layer interneurons, as well as in the glomeruli.[156] Recently, our team performed systemic injection of AAV9 to deliver a donor template to Cas9-expressing mice. We successfully inserted the red fluorescent protein mScarlet into the endogenous *TTN* and *PLN* loci, allowing for visualization of their respective localization patterns.[106] Nevertheless, inserting epitope tags or fluorescent proteins at endogenous proteins via HDR may also cause dysfunction due to the increased size of the epitope tags or fluorescent proteins, or the epitope tags or fluorescent proteins might obstruct the protein's function by covering the active site or interfering with interactions.

Cas9-mediated HDR can also be used to determine the localization of mutant proteins. However, this approach requires both the creation of mutations and epitope tag insertions using the same donor template, meaning the mutation site must be close to either the N or C terminus. This method also allows for the creation and tagging of frameshift mutations that result in nearby premature stop codons, adjacent to mutations close to the native termini. It should be noted that target alleles undergoing NHEJ but not HDR may experience some loss of function. Even though these alleles will not be tagged, the localization of the tagged allele may change due to indels causing function disruption. These confounding effects can often be mitigated by designing sgRNAs to cleave nearby introns or untranslated regions instead of targeting coding sequences.

Application of HDR for generation of genetic models

Conventionally, there are two approaches for creating a mouse model of human disease. The first involves pronuclear microinjection of transgene DNA directly into zygotes,[157] and the second entails the injection of edited ESCs into blastocysts.[158] However, these methods have inherent issues. Pronuclear injection results in the semi-random insertion of DNA at one or more sites, often leading to the concatemerization of transgenes and the formation of integrations with varying copy numbers. This integration process may disrupt host genes, potentially causing cancer or other dysfunctions.[159–163] Meanwhile, blastocyst injection necessitates the use of edited ESCs, typically achieved through the inefficient natural HR process.[164] By inducing CRISPR-Cas9-mediated DSBs at the recombination target site, the efficiency of HDR-mediated precise editing for creating mutations is significantly enhanced compared with natural HR.

In one study, a mixture of sgRNA, ssODN with a mutant Mpl^{S504N} donor template, and Cas9 protein was injected into one-cell stage mouse embryos. Among the 16 pups that were screened, 2 founder mice harbored the mutation and displayed the myeloproliferative neoplasm phenotype.[165] In a second study, the $Kras^{G12D}$ mutation was introduced via Cas9-mediated HDR, combined with *p53* and *Lkb1* loss-of-function mutations via Cas9-mediated NHEJ in a Cas9-expressing mouse line, resulting in significant pathological

Table 3. CRISPR-Cas9-HDR-mediated gene therapy

The subset	Advantages	Limitations	Examples	Reference
Ex vivo	easy manipulation, high gene editing efficiency, well-targeted	problematic for differentiated cells poor transplantation of cells off-target effects	correction of CYBB gene for treating X-CGD correction of HBB gene for treating SCD CAR-T for treating tumor	De Ravin et al.[177]; Dever et al.[178]; Wilkinson et al.[179]; Eyquem et al.[180]; Chang et al.[181]
In vivo	suitable for more diseases, simultaneously target a variety of tissue types	low HDR efficiency off-target effects	correction of OTC gene for treating OTCD correction of *LDLR* gene for treating HF insertion of therapeutic transgene for treating OTCD	Wang et al.[14]; Yang et al.[182]; Zhao et al.[183]

changes in lung adenocarcinoma.[166] In another study, Paquet et al. established a Cas9-based genome-editing workflow that allows for the selective introduction of mono- and bi-allelic mutations. Homozygous mutation introduction required sgRNA targeting close to the intended mutant site, while heterozygous mutations were introduced either by distance-dependent suboptimal mutation incorporation or by using mixed repair templates. Using this approach, the group generated human iPSCs with heterozygous and homozygous dominant early-onset Alzheimer's disease-causing mutations in APP^{Swe} and $PSEN1^{M146V}$ and derived cortical neurons, which displayed genotype-dependent disease-associated phenotypes.[167] In addition to small animal disease modeling construction, Yan et al. established engineered pig models mimicking the selective neurodegeneration seen in patients with Huntington's disease by replacing expanded CAG repeats in the mutant HTT allele with a normal CAG repeat using CRISPR-Cas9-mediated HDR.[168]

Application of HDR for gene therapy

Among the approximately 25,000 annotated genes in the human genome, more than 3,000 mutations are associated with diseases, and additional disease-relevant genetic variations are being uncovered.[169] Conventional treatments exhibit limited efficacy and offer only partial relief from clinical symptoms for a subset of these diseases. Therefore, there is an urgent need for gene therapy approaches that are both effective and safe, aiming to achieve a comprehensive cure for genetic diseases. Before the advent of gene editing technology, two conventional methods of gene therapy have been widely used: exogenous normal protein expressed to replace the defective endogenous genes by using recombinant viruses (such as adenovirus, AAV, and lentivirus)[170,171] and RNAi to degrade target mRNA to suppress the expression of defective genes.[172,173] These methods, however, have some limitations. For example, the introduction of therapeutic genes via viral vectors may induce new mutations during the viral entry process into the body, leading to the dysregulation of endogenous gene expression.[174] RNAi may encounter challenges such as modest inhibitory effects and inadequate specificity.[175,176]

In contrast, gene editing via the CRISPR-Cas-mediated HDR has shown great advantages in gene therapy via correction of mutant genes or insertion of therapeutic genes at safe harbor loci. HDR-mediated gene therapy can be performed by either *in vivo* or *ex vivo* pathways. In the *ex vivo* gene therapy, the mutant genes within target cells, obtained from patients, undergo correction through HDR. Subsequently, these cells are cultured, expanded, and reintroduced to the patients, ensuring a sufficient population expressing the corrected gene *in vitro*. In the context of *in vivo* gene therapy, the crucial elements of HDR are administered directly into the body using RNPs, LNPs, or viral vectors. This process is employed to trigger edits in the genome of somatic cells (Table 3).[169]

HDR-mediated ex vivo *gene therapy*

Ex vivo gene therapy offers several advantages. First, *ex vivo* approaches allow for precise genetic modifications in a controlled environment. Target cells are isolated, modified, and carefully selected before reintroduction, ensuring a more specific and accurate therapeutic outcome.[184] Second, since the genetic modifications occur outside the patient's body, *ex vivo* gene therapy reduces the risk of unintended consequences or off-target effects that may arise with *in vivo* approaches.[185] Third, *In vitro* settings provide a controlled environment for efficient genome editing. Techniques such as CRISPR-Cas9 and HDR can be optimized *ex vivo*, increasing the likelihood of successful genetic corrections. Moreover, by isolating and modifying cells outside the body, the potential for systemic side effects of *ex vivo* therapy is minimized in comparison to *in vivo* therapy.[185]

X-linked chronic granulomatous disease (X-CGD), a rare and life-threatening primary immunodeficiency without ethnic preference, arises from mutations in the *CYBB* gene.[186] This gene encodes $gp91^{phox}$, which is the catalytic center of DANPH oxidase 2 (NOX2).[187] In this disorder, restoration of NOX2 to ~10% to ~15% of neutrophils would provide significant benefit. In one approach, CD34+ HSPCs from patients with X-CGD were transfected with Cas9 mRNA, sgRNA, and ssODNs *CYBB* correction donor template. Sequencing results showed that more than 20% of HSPCs from X-CGD patients had restoration of NADPH oxidase function and superoxide radical production. These gene-corrected X-CGD HSPCs were then transplanted into the X-CGD disease model mice resulting in efficient engraftment and production of functional mature human lymphoid and myeloid cells for up to 5 months. Whole-exome sequencing did not detect any indels outside of the CYBB gene after gene correction.[177] Another case demonstrating the promise of *ex vivo* precise editing relates to sickle cell disease (SCD), which can be caused by a missense Glu6Val mutation in the β-globin coding gene *HBB*.[178] Recently, Wilkinson et al. studied Cas9-AAV6-mediated HBB-correction in a humanized SCD mouse

model that sought to generate functional hematopoietic stem cells (HSCs) for the purpose of autologous transplantation. This strategy resulted in long-term multipotent HSCs that were gene corrected *ex vivo* and displayed stable hemoglobin A *in vivo* following autologous transplantation.[179]

In addition, CRISPR-Cas9-mediated HDR can also be applied for chimeric antigen receptor (CAR)-T cell therapy. Presently, CARs are commonly introduced into T cells through the utilization of γ-retroviral vectors or other vectors capable of random integration.[188–190] Nevertheless, employing these vectors can lead to various concerns such as clonal expansion, oncogenic transformation, variegated transgene expression, and transcriptional silencing.[191–193] CRISPR-Cas9-mediated HDR enabling efficient sequence-specific insertion of CARs could avoid the above limitations and have tremendous application prospects. In one approach, Eyquem et al. applied CRISPR-Cas9-mediated HDR to achieve high efficiency and precision of gene targeting CAR-T cells by co-electroporating Cas9 mRNA, sgRNA, and AAV containing CAR cDNA and donor template of T cell receptor α constant locus in T cells. They showed that the edited CAR-T cells vastly outperformed conventionally generated cells in a mouse model of acute lymphoblastic leukemia.[180] In another approach, to address the limitations of CAR-T cell therapy in glioblastoma (GBM), Chang et al. designed and screened anti-GBM chlorotoxin-CAR constructs with neutrophil-specific signaling domains by using HDR to insert them into the *AAVS1* safe harbor locus of human iPSCs to develop an optimized CAR for neutrophil-mediated tumor-killing. The resulting stable CAR-expressing human iPSCs were then differentiated into CAR neutrophils, which kept an anti-tumor N1 phenotype and exhibited enhanced anti-GBM activities within the hypoxic tumor microenvironment in female tumor-bearing mice.[181]

While *ex vivo* gene therapy has shown promise, it also comes with certain limitations and challenges. First, *ex vivo* gene therapy involves multiple steps, including cell isolation, genetic modification, and cell expansion. This complexity increases the overall cost of the procedure, making it resource-intensive and potentially less accessible for widespread use.[169] Another limitation is that *ex vivo* gene therapy is typically suitable for disorders where the target cells can be easily isolated, modified, and reintroduced. It may be less applicable for conditions involving complex multicellular interactions or systemic effects.[169]

HDR-mediated in vivo *gene therapy*

Compared with *ex vivo* gene therapy, there are several advantages to *in vivo* gene therapies. First, *in vivo* gene therapy is versatile and applicable to a wide range of diseases, including genetic disorders, cancers, and acquired diseases. It allows for the treatment of conditions affecting multiple tissues or organs simultaneously. Second, *in vivo* gene therapy, being more convenient and expedited than *ex vivo* methods, can be administered directly in a clinical setting, bypassing elaborate laboratory processes and enhancing accessibility for patients.

X-linked ornithine carbamoyltransferase disease (OTCD) is caused by a mutation in the *OTC* gene, which encodes ornithine carbamoyltransferase.[194] Yang et al. intravenously injected two AAV vectors, one expressing Cas9 and the other expressing sgRNA and donor template of *OTC*, into newborn mice with OTCD. This strategy corrected 6.7%–20.1% of hepatocyte mutations and improved survival in mice with a high-protein diet, which exacerbated disease.[182] Mutations in *LDLR* will cause familial hypercholesterolemia (FH).[195] Two AAV vectors, one expressing Cas9 and the other expressing sgRNA and donor template of *LDLR*, were subcutaneously injected into newborn mice with FH. Treated mice displayed reductions in total cholesterol, total triglycerides, and LDL cholesterol in the serum, whereas the aorta had smaller atherosclerotic plaques and a lower degree of macrophage infiltration. Sequencing results indicated that ∼6.7% of the *LDLR* alleles were corrected.[183]

In addition to the correction of mutant genes, HDR-mediated gene therapy can also be used to insert a therapeutic transgene at an endogenous locus for permanent gene therapy. In this manner, delivery of a single wild-type gene can correct deficiencies caused by a large variety of patient-specific mutations. Furthermore, in contrast with many standard gene therapies, the donor template can often be engineered to lack *cis*-regulatory elements, reducing the risk of oncogenic insertions. In an effort to develop a gene therapy for OTCD, which is caused by mutation of the *OTC* gene, Wang et al. attempted delivery of a therapeutic transgene to an OTCD mouse model. Two AAVs were injected, with the first expressing Cas9, while the other expressed sgRNA and a donor template that contained the liver-specific promoter TBG and a *mini-OTC* gene. This strategy resulted in 25%–35% of OTC-expressed hepatocytes at 3 and 8 weeks in mice and displayed efficient, sustained, and beneficial gene targeting in the liver in the absence of any selective growth advantage for OTC-positive cells.[14]

While there have been advancements in *in vivo* gene therapy utilizing CRISPR-Cas9-mediated HDR, it still faces several challenges. First, the delivery of DNA CRISPR-Cas9 into the body may result in unintended effects on non-target tissues, leading to off-target and potential adverse events. Moreover, the introduction of therapeutic vectors may trigger immune responses, leading to the neutralization or elimination of the delivered genes, reducing the therapy's effectiveness, and potentially causing safety concerns. Despite these challenges, ongoing research and advancements in technology aim to address these disadvantages and enhance the safety and efficacy of *in vivo* gene therapy for a wider range of genetic and acquired diseases.

DISCUSSION AND PROSPECTS ON FUTURE DIRECTIONS

CRISPR-Cas9-mediated HDR is a powerful genome editing tool that can introduce precise changes to the DNA sequence of cells or organisms. The ability to manipulate the genome with such precision has many potential applications in various fields of research and medicine. However, as with any new technology, there are still limitations and challenges that need to be addressed. First, HDR is a less-efficient

DNA repair pathway compared with NHEJ. The efficiency of HDR repair depends on several factors, such as the NHEJ and HDR pathways, donor template, and the location of the target site. Moreover, the donor template must be delivered to the target cells or organisms with high efficiency, which can be a challenge for some cell types. A second challenge is that, although CRISPR-Cas9 is highly specific, it can still cause off-target mutations. Therefore, efforts to reduce or even eliminate the impact of off-target effects is currently a hot spot in CRISPR research. One approach for reducing off-target effects is screening the sgRNAs by using next-generation sequencing and choosing the optimal sgRNA. Another method is sgRNA modification and engineering, such as truncated sgRNA and chemical modification of sgRNA.[196,197] In addition, newly developed Cas variants with lower rates of off-target editing have been reported, including a novel subtype of Cas12f known as enAsCas12f, which exhibited lower off-target effects compared with Cas9.[198] A third limitation relates to the availability of the cellular machinery involved in HDR. HDR repair is typically restricted to the S and G2 phases of the cell cycle. The cell-cycle dependence of HDR repair can limit the efficiency of genome editing, particularly in cells with a short S/G2 phase or *in vivo* applications. Moreover, the effective delivery of CRISPR-Cas9 reagents to target cells or tissues is a pivotal requirement for achieving successful genome editing. The efficiency of delivery relies on factors such as the specific cell or tissue type, the chosen method of delivery, and the stability of the reagents within the *in vivo* environment. Unfortunately, effectively delivering components to numerous tissues *in vivo* continues to pose significant challenges.

To address these issues, there have been attempts to develop alternative methods to replace HDR-mediated precise gene editing. One of the methods is homology-independent targeted integration (HITI), which is a relatively new gene editing technology that allows for the targeted integration of exogenous DNA sequences into the genome without the need for HDR.[199–202] Another approach is base editing, which can directly convert one DNA base to another without the need for DSBs.[203,204] Base editing has been used to generate precise point mutations and correct disease-causing mutations with high efficiency and specificity. The third method is prime editing, which is a novel genome editing technology that can directly write new genetic information into a specified DNA site using a fusion protein of Cas9 and reverse transcriptase.[205] While these technologies offer promising alternatives to HDR, each has its own limitations and challenges. For example, although HITI-mediated insertion can work in both dividing and non-dividing cells, its precision and off-target integration need to be further characterized.[200] Both base editing and prime editing are only suited for point mutation, small deletions, or insertions, while base editing has high specificity requirements for the sgRNA.[206,207] Therefore, these approaches currently are unable to replace HDR-based editing.

Currently, the majority of HDR studies are focused on spCas9 or saCas9. New Cas variants, however, are constantly being developed, such as Cas12a,[208] Cas12e,[209] Cas12f,[210,211] Cas12j,[212,213] Cas12n,[214] IscB,[215] and TnpB.[216] One advantage is that some new Cas variants have been developed to improve the efficiency of HDR-mediated precise gene editing or to reduce the rates of off-target editing. For example, recently, Chen et al. developed cas12a-mediated staggered cuts that can increase both the efficiency of gene knockout by NHEJ and insertion of exogenous donor templates by HDR.[208] The second advantage is that these new Cas variants usually have a small molecular weight. Cas9 is typically >1,000 amino acids, approaching the packaging limit in a single AAV vector (<4.7 kb), which consequently hinders its delivery in clinical applications. Cas12f is considered to be among the most compact Cas variants, typically comprising approximately 400–700 amino acids.[210] Several groups have developed a series of Cas12f proteins, which showed efficient gene editing.[198,217,218] This makes it possible to package all Cas and HDR DNA elements into a single AAV vector. However, these Cas variants generally have less frequently occurring PAM sequences, and their gene editing efficiency needs further improvement.[217,218] Currently, these limitations hinder their application, ensuring that interest in Cas9 remains strong.

In addition, the ethical and legal implications of CRISPR-Cas9-mediated gene editing, including those associated with HDR, must be carefully evaluated. The phenomenon of gene drive, which can increase the inheritance rate of the CRISPR-Cas9-edited locus and enable its rapid spread throughout a population,[206] raises significant concerns. If edited genes and traits are accidentally released, they could have unpredictable consequences on human health and the ecological environment. While the therapeutic and human gene editing applications of CRISPR are particularly promising and captivating, they also come with significant risks. In recent years, CRISPR-Cas9-mediated therapies have been developed for conditions such as Duchenne muscular dystrophy,[219] herpetic stromal keratitis,[220] and SCD and β-thalassemia.[221] However, in somatic applications, the primary risk remains off-target editing. Researchers and clinicians must carefully balance the potential risks and benefits for patients. The concerns extend beyond just health and biological issues, such as inaccurate or incomplete editing and unforeseen editing effects. There are also societal and ethical questions to consider.[222] The irreversibility of genetic edits once introduced into a population could lead to unintended consequences through reproduction.[222] Furthermore, edited individuals might face challenges to their sense of self-identity as human beings, potentially impacting social stability and structure.

Overall, despite the great potential of CRISPR-Cas-mediated HDR, therapeutic adoption of the technique faces many challenges. In the future, we expect a surge of novel strategies to address these challenges, resulting in HDR-mediated editing with improved efficiency, fidelity, and delivery mechanisms, for both the nuclear and mitochondrial genomes. One of the major challenges of HDR is its low efficiency, particularly in non-dividing cells. More strategies, such as optimizing the delivery of donor templates and the length of homology arms, and modulating the activity of HDR regulators, need to be developed for boosting HDR efficiency. HDR is traditionally considered to occur only in the S/G2 phases of the cell cycle. However, we and other teams have found that postmitotic cells can also repair

DSBs through HDR.[104–106] More importantly, we found that HDR efficiency in postmitotic cells is considerable and can be comparable with mitotic cells.[106] This will certainly expand the applications of HDR. However, future research is needed to fully understand the mechanisms underlying HDR in postmitotic cells. Overall, CRISPR-Cas9-mediated HDR has already made significant contributions to the field of genome editing, and ongoing research efforts will likely continue to improve the technology and expand its range of applications in the years to come.

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AUTHOR CONTRIBUTIONS

H.L. and J.W. contributed equally to this manuscript. H.L. and J.W. completed literature review, investigation, wrote the original draft, and designed the figures. Y.Z. and Y.L. conceived the study, revised the draft, and provided funding acquisition and supervision. N.J.V.D. reviewed the manuscript. All the authors approved the submission.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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