Molecular Therapy
Nucleic Acids
Review

Strategies for precise gene edits in mammalian cells

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CRISPR-Cas technologies have the potential to revolutionize genetic medicine. However, work is still needed to make this technology clinically efficient for gene correction. A barrier to making precise genetic edits in the human genome is controlling how CRISPR-Cas-induced DNA breaks are repaired by the cell. Since error-prone non-homologous end-joining is often the preferred cellular repair pathway, CRISPR-Cas-induced breaks often result in gene disruption. Homology-directed repair (HDR) makes precise genetic changes and is the clinically desired pathway, but this repair pathway requires a homology donor template and cycling cells. Newer editing strategies, such as base and prime editing, can affect precise repair for relatively small edits without requiring HDR and circumvent cell cycle dependence. However, these technologies have limitations in the extent of genetic editing and require the delivery of bulky cargo. Here, we discuss the pros and cons of precise gene correction using CRISPR-Cas-induced HDR, as well as base and prime editing for repairing small mutations. Finally, we consider emerging new technologies, such as recombination and transposases, which can circumvent both cell cycle and cellular DNA repair dependence for editing the genome.

INTRODUCTION

CRISPR-Cas technologies represent a major potential in genome editing. The developments in gene editing are moving at an exponential phase. However, a significant amount of molecular development is necessary for the full potential of CRISPR-Cas to be realized. Because active Cas proteins make double-stranded breaks (DSBs) in genomic DNA, the manner in which the break is repaired by the cell plays a major role in the final genomic edit. The mammalian cell has a variety of molecular pathways to repair DSBs, but the two major pathways are non-homologous end-joining (NHEJ) and homology-directed repair (HDR). While repair via HDR can lead to precise genome editing, Cas9-mediated DSB repair via NHEJ leads to mutagenic indels (insertions/deletions). While other repair pathways participate in the repair of Cas-induced DSBs, (e.g., microhomology-mediated end-joining [MMEJ], and single-strand annealing [SSA]), indel-generating repair and HDR represent the majority of Cas-induced DSB repairs, with repairs by indel-generating pathways (NHEJ and MMEJ) typically being the overwhelming majority. Base and prime editing are rapidly developing techniques that use a mutant Cas9 nickase (i.e., one that creates only a single-strand break or nick), fused to either a cytosine/adenine deaminase or a reverse transcriptase. These editing tools avoid indels generated by NHEJ/MMEJ but are limited to relatively minor changes. For larger genomic edits, a DSB followed by HDR remains the most precise and reliable way for genomic editing in human cells. HDR is more practical in instances where diseases result from a variety of different mutations in a gene; it is often impractical to generate base editing/prime editing repair strategies for each mutation. Increasing the efficiency of HDR editing after a Cas-induced DSB is a significant research thrust that many labs worldwide have been investigating. Editing mechanisms that circumvent this, such as CRISPR-guided transposons or recombinases that are currently only efficient in prokaryotes, are emerging technologies that have the potential to overcome the hurdles associated with HDR.

MOLECULAR REPAIR PATHWAYS

The major factors that represent the decision favoring HDR repair of DSBs are as follows: (1) end resectioning, (2) the availability of a homologous donor to inform the repair, and (3) cells in cycle (vs. quiescent cells). The molecular details of the processes of these pathways have been used to inform strategies to inhibit NHEJ and encourage HDR to achieve precise gene editing. Many previous articles and reviews have covered these pathways in detail,[1–5] so a brief summary will be presented here.

Non-homologous end-joining

In canonical NHEJ (cNHEJ), the DSB triggers a cellular signal to quickly ligate both ends of the DSB together with minimal end resectioning. This involves aligning both broken ends, end processing, and ligation. One of the first proteins recruited, 53BP1, binds to this repair complex to prevent resectioning and also inhibits the recruitment of BRCA1, which is a pro-HDR factor. The Ku70/Ku80 heterodimer recognizes and binds the broken ends, which signals repair fated for the NHEJ pathway. Once bound to the broken ends, end resectioning is prevented, which is necessary for homology-driven repair pathways.[4] DNA-dependent protein kinase (DNA-PK) is recruited, which may help hold the broken ends together,[6] and it recruits

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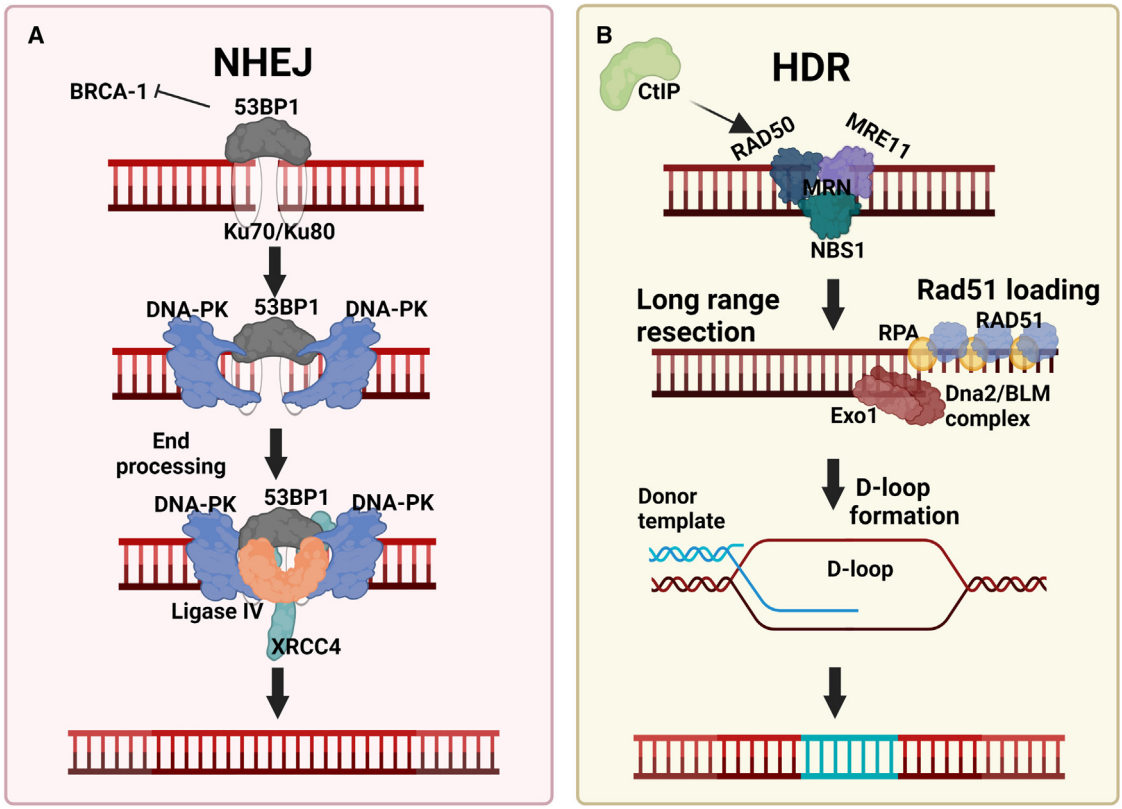

Figure 1. DNA repair pathways

Comparison of the molecular pathways for (A) non-homologous end-joining (NHEJ) and (B) homology-directed repair (HDR). Specific proteins discussed in the article are featured. See text and ref.[1–5] for a more detailed discussion of these pathways.

numerous other nucleases and polymerases to process the broken ends as needed to be bluntly ligated back together. After the ends have been suitably processed, XRCC4 and DNA ligase IV complete the ligation (Figure 1A). The modulation and inhibition of 53BP1 or other downstream NHEJ proteins has been a popular strategy to inhibit NHEJ repair after CRISPR-Cas9-mediated breaks.[4]

When NHEJ occurs naturally (e.g., DNA damage induced via cellular oxidative processes or UV irradiation), the breaks can often be repaired by NHEJ without incurring any genomic changes at the DSB site.[7] However, when the breaks are induced by CRISPR-Cas9, as long as Cas9 is active, it will continue to recut the targeted site until the protospacer adjacent motif (PAM; necessary for Cas9 to bind and cleave target DNA) is destroyed, making insertions/deletions at the DSB site obligatory. However, it is important to note that one can avoid continual cleavage by using a donor that, when incorporated into the repair, mutates the PAM or the gRNA target sequence (protospacer), inhibiting Cas9 from recognizing and cutting the site again.[8] Another complicating factor is that DSBs cut by Cas9 are created asymmetrically; the strand encoding the PAM is cleaved first, while the other end is cleaved sometime later.[9,10] This could allow one end to be exposed for end processing/resectioning, while the other end is shielded from repair enzymes from the still-bound Cas9. It is still unclear how this affects the DNA repair pathway choice in Cas9 gene editing.

On a cellular level, NHEJ is an active pathway in almost all phases of the cell cycle, and as such, it is often thought of as the "knee-jerk" reaction to a DSB. On the favorable side for the cell, NHEJ is one of the fastest repair mechanisms[11] and a repair mechanism that will take place whether a repair donor with homology is present or not, regardless of the cell cycle phase the cell is in. On the unfavorable side, NHEJ is often inaccurate, especially when extensive end processing needs to take place before ligation.[12] This may not be a problem if the goal of the edit is to ablate or dampen the expression of the genetic element; however, NHEJ is unsuitable for precise gene editing with CRISPR-Cas9. Because few diseases can be cured by disrupting a gene, precise repair is the preferred pathway for the majority of genetic edits. Therefore, a major goal of precise genome editing with CRISPR-Cas9-induced DSBs is to decrease the efficiency of NHEJ and increase the efficiency of HDR.

Homology-directed repair

A hallmark of the HDR pathway is extensive end resectioning and the availability of a homologous DNA donor. In this pathway, the broken ends first go through a round of short end resectioning, starting with MRN complex (MRE11-RAD50-NBS1), which recruits CtIP.

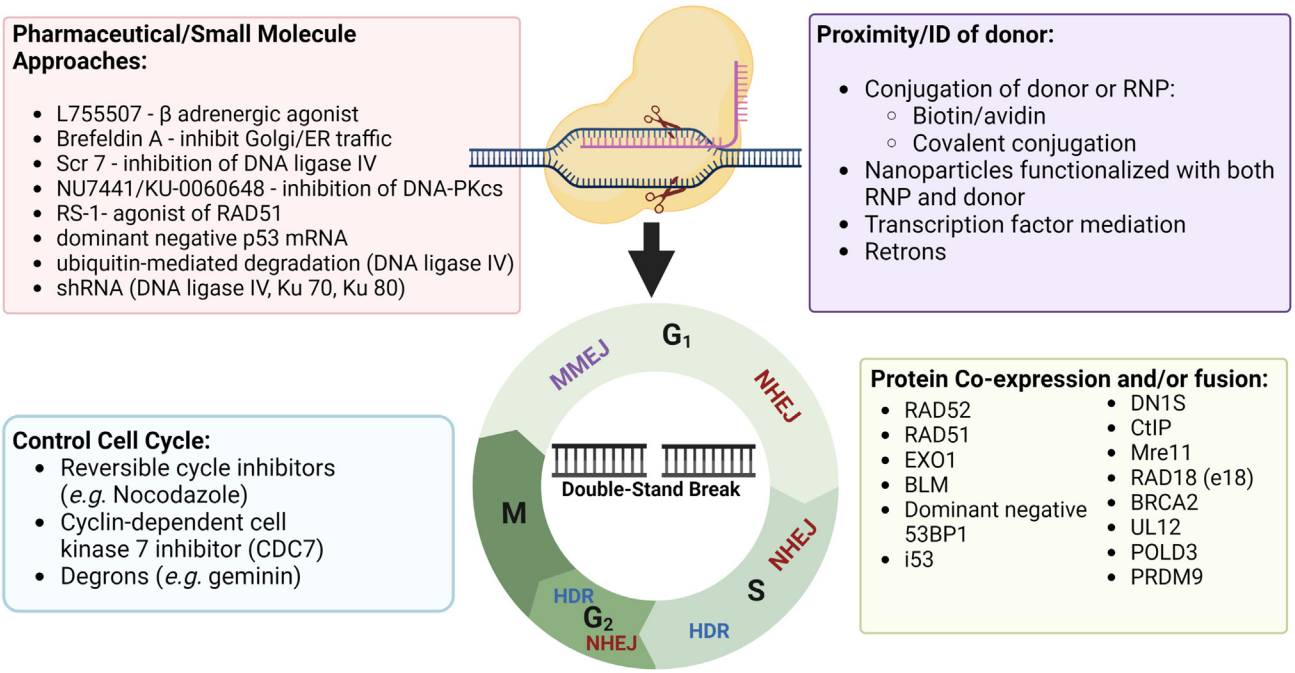

Figure 2. Summary of approaches to increase HDR after CRISPR-Cas-induced DSBs
Specific approaches discussed in the article are featured.

Afterward, long-range resectioning is performed by Exo1 and the Dna2/BLM complex, which results in 3′ overhangs. These overhangs are bound and protected by RPA, which is then replaced by RAD51. The RAD51/DNA complex generates nucleoprotein filaments that participate in the homology search for a sister chromatid (or another DNA molecule with enough homology to act as a homology donor) and initiate strand invasion. The invading strand generates a displacement loop (D-loop) and causes the formation of a Holliday junction, which is ultimately resolved with nickases and ligases to restore the original sequence (Figure 1B).

Knowledge of this pathway has inspired a number of approaches to increase its efficiency. For example, since HDR is primarily active in the G2/S phases of the cell cycle,[13] much work has been done in attempt to control cell cycle while Cas9 is active. Additionally, the design of the homology donor has been an intense area of research, as well as increasing the proximity of the donor to the break site to limit the search for a homology donor. Furthermore, the overexpression or fusion of DNA repair proteins to Cas9 has been used to increase HDR efficiency, with varying success. Fusion of these proteins to Cas9 is generally considered more beneficial (vs. overexpression) since it allows the repair protein to locate, both spatially and temporally, at the DSB with minimal disruptions to the global DNA repair process. However, there are critical questions about the functionality of these enzymes while fused to Cas9. For example, do structural constraints, while fused to Cas9, limit conformational changes necessary for enzyme action? Is there an ideal type, length, or flexibility of the linker between Cas9 and the protein? For further details regarding the fusion protein linkers—property, design, and advantages and disadvantages—the reader is referred to the excellent review by Chen et al.[14] Additionally, considerations must be given to the structure of the complexes as bound at the DSB to ensure that the fusion arrangement allows the enzyme to fully participate in the repair complexes.

ATTEMPTS TO INCREASE HDR LEVELS

Several molecular strategies have been developed to increase the efficiency of HDR repair for CRISPR-Cas9 genome engineering. Some of the earliest studies included pharmacological attempts to up- or down-regulate DNA repair pathways. Other strategies involve an attempt to control cell cycle to keep the cell in S/G2, where HDR is most active, or restrict Cas9 expression to cycling cells. Another division of research in the area involves bringing key biomolecules in close proximity to the Cas9 complex to affect the outcome of the DSB repair. It is important to note that attempts to increase precise editing via HDR have been undertaken by researchers in a vast variety of models (e.g., K562, HEK 293T, primary cells, iPSCs, embryos, and tissue models) in a diverse range of assays (e.g., fluorescent reporters and sequence-based assays) Additionally, different materials (Cas9/gRNA RNP, plasmids, mRNA) and transfer protocols (electroporation, transfection, microinjection, etc.) have different kinetics, which may not be directly comparable. In general, cell lines, especially HEK293 and K562 cells, demonstrate higher editing efficiency than iPSCs or primary cells. Furthermore, *in vitro* HDR results in primary hematopoietic stem and progenitor cells (HSPCs) are often not sustained at the same level *in vivo*. Here, we review key papers that have investigated these techniques. Figure 2 summarizes the approaches discussed below to increase HDR.

Pharmacological/small molecule approaches that globally reduce cellular NHEJ repair

Many previous studies have demonstrated that pharmacological agents active toward DNA repair proteins influence the outcome of CRISPR-Cas9-mediated genome editing. In a pioneering study in 2015, Lu et al. screened a library of 4,000 drugs and concluded that both L755507 and brefeldin A increased CRISPR-Cas9-induced HDR in mouse induced pluripotent stem cells (iPSCs).[15] Shortly after, both Maruyama et al. and Chu et al. published back-to-back studies.[16,17] Maruyama et al. demonstrated that inhibition of DNA ligase IV by Scr 7 increased HDR up to 19-fold in mammalian cell lines and mice. Chu et al. investigated the inhibition of DNA ligase IV via Scr 7 and ubiquitin-mediated degradation, as well as the effect of shRNA against DNA ligase IV, Ku 70, and Ku 80 in human and mouse cell lines. These efforts resulted in a 4- to 8-fold increase in HDR. Robert et al. pharmacologically inhibited DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) using NU7441 and KU-0060648 and found they could decrease NHEJ by 40% and increase HDR 2-fold in HEK cells.[18] Pinder et al. studied the effect of RS-1, an agonist of RAD51, and found the drug could increase HDR 2- to 3-fold in human cell lines.[19] Shortly after, Song et al. showed RS-1 increased HDR 3- to 5-fold in rabbit embryos, while Scr 7 did not appear to impact gene editing outcomes in their study.[20] In 2019, Jayavaradhan et al. studied the effect of Nu7441, Scr7, and RS-1 in human cell lines and human CD34+ HSPCs and reported severe toxicity among compounds that globally inhibit NHEJ.[21] Additionally, the HDR enhancer RS-1 was only found to increase HDR in cell lines, while CD34+ HSPCs were unaffected.

In 2019, Schiroli et al. suggested that sensing of the viral vector used to deliver gene editing molecules, along with the DSB itself, may trigger the activation of p53 in primary cells, resulting in toxicity.[22] This could be abrogated by transiently expressing a dominant-negative p53 mRNA.[22] These results suggest that attempts to use pharmaceutical approaches to increase HDR, at the expense of NHEJ, may have inherent toxicity, regardless of the drug chosen to do so. Furthermore, Ferrari et al. showed inhibition of this p53 response by transient expression of a dominant-negative p53 mutant protein (GSE56) together with adenoviral protein (Ad5-E4orf6/7) increased the HDR by 50% in CD90+ HSPCs.[23] While additional studies continue to investigate the effect of NHEJ inhibition,[24–26] in whole, because of the toxicity and lack of effect in clinically relevant cells, such as primary cells, especially hematopoietic stem cells (HSCs), researchers have moved toward other techniques to enhance HDR editing outcomes. DNA damage occurs naturally via oxidative radicals from endogenous metabolic reactions and replicative stress, and as often as 10^5 lesions per cell each day are repaired.[27] It is important to note that HSCs are exquisitely dependent upon NHEJ to repair the natural DSBs that occur,[28] and defective DNA repair in HSC results in their aging and accumulation of deleterious mutations. Hence, even temporary inhibition of NHEJ in HSCs with pharmacological reagents to increase HDR can be detrimental to its genomic integrity and can result in carry-forward of potentially oncogenic mutations.

Control of cell cycle

Because homologous recombination only occurs naturally in late S and G2 phases (when DNA synthesis is complete and a homologous donor is available as a sister chromatid), researchers realized the cell cycle could be modulated while Cas9 was expressed in an attempt to increase HDR. Lin et al. first attempted cell cycle modulation to improve HDR in 2014.[29] They used reversible chemical inhibitors to arrest the cell in G1, S, or M phase; then they released the cells to continue cycling before nucleofection with CRISPR-Cas9 ribonucleoproteins (RNPs). The delivery of RNP format, rather than Cas9-encoding DNA, allowed the gene editing tools to begin to work almost immediately after delivery.[30] They demonstrated that synchronization with nocodazole (at G2/M phase) could increase HDR up to 6-fold in HEK cells, but they also showed that the synchronization agent that best increased HDR varied with cell type. In 2016, Yang et al. studied the effect of nocodazole as well as ABT-751 (which also synchronizes cells at G2/M) in HSPCs and found that both increase HDR 3- to 6-fold.[31] Wienert et al. identified cyclin-dependent cell kinase 7 (CDC7) as a target that inhibits NHEJ when using a dsDNA donor.[32] XL413, a small molecule inhibitor of CDC7, was found to increase HDR up to 3.5-fold in K562 cells via a slowing of the S phase. While these cell cycle synchronizing agents can be useful in cell lines and immediate toxicity was not found in primary cells, long-term *in vivo* survival, self-renewing potential, or cell-fate has not been studied.

Charlesworth et al. found that stimulating HSPCs to enter the cell cycle and expanding them by plating at low densities before gene targeting significantly improves the HDR frequencies.[33] They also optimized donor delivery using recombinant adeno-associated virus serotype 6 (rAAV6). Shin et al. showed that editing CD34+ HSPCs leads to high levels of HDR in relatively differentiated subpopulations; however, HSPCs in G0 phase (which primarily comprise the long-term repopulating HSCs) almost entirely lose HDR alleles. Allowing HSPCs (CD34+) to enter the cell cycle briefly yields high levels of HDR; however, few quiescent cells, which are primarily long-term repopulating cells, undergo HDR. Shin et al. also found that quiescent stem cells use NHEJ, and stem cells that were stimulated to cycle and then forced to re-quiesce by medium composition change use both NHEJ and HDR and can be edited to increase HDR.[34]

Manipulating Cas9 to be available only in cycling cells was explored by fusing it to cell cycle degron proteins in an attempt to prevent NHEJ editing in non-cycling cells and promote HDR. In 2016, Gutschner et al. developed an innovative strategy to allow Cas9 expression only during the S/G2/M phases by fusing it to the first 110 amino acids of the degron geminin to create Cas9-hGem (1/110).[35] Geminin is a substrate of the APC/Cdh1 complex, which ubiquitinates the protein and targets it for degradation in late M and G1 phases of the cell cycle. They found this C-terminal geminin fusion to Cas9 increased HDR 1.87-fold in HEK cells. Furthermore, the authors studied the effect of this fusion along with cell synchronization using nocodazole; this combination increased HDR about 1.3-fold further. Later that year, Howden et al. also used this fusion

in human pluripotent stem cells and showed that while HDR levels remained unchanged, NHEJ levels were decreased about 2-fold.[36] Gerlach et al. tested the Cas9-geminin fusion in porcine fetal fibroblasts and found a 2-fold increase in HDR (while also showing no increase in HDR using Src 7).[37] Lomova et al. also investigated this geminin fusion in HSPCs, combined with synchronization with the drug RO-3306, which arrests cells at the G2/M transition.[38] Similar to results found in HSPCs by Howden et al.,[36] neither the geminin fusion nor RO-3306 significantly increased HDR alone, but both decreased NHEJ. The net effect of both the geminin fusion and RO-3306 increased the HDR/NHEJ ratio 4-fold by reducing NHEJ. Overall, the strategy of fusing Cas9 to a G0/G1 phase degron makes Cas9 unavailable in the G0/G1 phase of cell cycle, thereby reducing NHEJ and unintended indels.

Proximity and identity of homology donor

Another popular technique to boost HDR is to optimize the homology of the DNA donor template. The identity and type of the homology donor (i.e., single-stranded vs. double-stranded, chemical modifications, length of homology arms and their symmetry, plasmid vs. miniplasmid, recombinant AAV vs. non-integrating lentivirus vectors, etc.) has been explored, as well as increasing the proximity of the donor to the DSB site. Zhang et al. published an extensive review of studies that have explored how the identity of the DNA donor affects HDR rates, and the reader is referred to this excellent review for details.[39] In addition, more information on donor template design and its influence on HDR can be found in the study by Richardson et al.[40] Since donor type and design is a vast topic that merits an independent extensive review, we will focus on studies that have attempted to increase the proximity of the donor to the DSB site. It should be mentioned that approaches to co-localize the donor to the DSB site work best for linear DNA (<2,000 bp).

Some of the earliest work to bring the donor near a Cas9-induced DSB was based on a study by Ruff et al. in 2014.[41] They designed a bifunctional DNA donor molecule that contained a DNA aptamer that bound to the site-specific meganuclease I-SceI on the 5′ end and had homology to the break site on the 3′ end. The modified donor DNA template bound to I-SceI via the DNA aptamer, bringing the 3′ homology template in the proximity of the I-SceI-induced DSB break. Consequently, HEK 293T cells had up to a 16-fold increase in HDR compared to the donor without the aptamer. This large increase inspired several other strategies to co-localize donor DNA to the Cas9-induced break site.

The extremely high binding affinity between biotin and streptavidin has been a popular strategy to conjugate Cas9 and donor DNA. In 2017, Ma et al. fused Cas9 with avidin and biotinylated an ssDNA template, which assembled into the Cas9-Avidin-Biotin-ssDNA (CAB) system.[42] When delivered to mouse embryos, they demonstrated about 20% HDR frequency, even for insertions as large as nearly 1 kb. Later that year, Carlson-Stevermer et al. published a study with a gRNA designed with an RNA aptamer that binds streptavidin, which can then bind a biotinylated ssODN donor.[43] With this system, they showed a 1.8-fold increase in HDR in HEK 293T and HSPCs, but their genetic changes were mostly 18 bp or fewer. In 2018, Gu et al. also used a fusion between Cas9 and monomeric streptavidin in addition to a biotinylated linear dsDNA donor.[44] They also took advantage of using mouse embryos at the two-cell stage, where chromatin is much more open, and the cell cycle is in a prolonged G2 phase. Using this system, they achieved a 2-fold increase in HDR. Later that year, Roche et al. fused Cas9 to mono-avidin and used biotinylated dsDNA in human and mouse cells lines, which also resulted in a 2-fold increase in HDR.[45]

There have also been a number of studies that have more creatively attempted to co-localize Cas9 and donor DNA, which have largely demonstrated better HDR rates than biotin/avidin conjugations. In 2017, Lee et al. covalently fused the gRNA and linear ssDNA donor and then transfected the large complex into HEK 293T cells using a polycation.[46] With this system, they showed a 3-fold increase in HDR vs. gRNA and donor delivered separately. In 2018, Savic et al. used an SNAP-tag to covalently link an ssODN donor to the Cas9 protein.[47] This approach allowed them to demonstrate a 24-fold increase in HDR vs. the control without the donor linked. Later that year, Aird et al. fused an HUH endonuclease to Cas9.[48] Here, the HUH endonuclease can recognize a specific sequence in the ssDNA donor and covalently conjugates the donor to Cas9-HUH fusion protein via a phosphotyrosine linkage. With this arrangement, they demonstrated a 30-fold increase in HDR. In 2019, Shahbazi et al. generated gold nanoparticles functionalized with CRISPR-Cas9 machinery and an ssODN donor.[49] First, these nanoparticles were functionalized with crRNA-PEG via a semi-covalent gold-thiol interaction. Afterward, Cas9 was introduced to the functionalized nanoparticles, which associated with the crRNA to form RNPs on the surface. Then, the RNP-loaded nanoparticles were coated with polyethylenimine, which allowed further functionalization with a DNA donor via electrostatic interactions. Using this delivery method in HSPCs, they were able to show an 8- to 10-fold increase in HDR vs. naked CRISPR-Cas9 complexes electroporated with donor. In 2020, Ling et al. modified the amino acid sequence of Cas9 via genetic code expansion technology to include non-canonical amino acids containing azide moieties.[50] They followed this with covalent coupling to a small alkyne-modified ssODN molecule (via strain-promoted alkyne-azide cycloaddition), which could recruit an ssODN donor via base pairing. These complexes could increase HDR in HEK 293T cells 10-fold with a single mutation in Cas9 and an additional 2-fold when the Cas9 protein had two mutations, which they concluded was because the double-mutant could bind twice as much donor. In 2021, Li et al. fused Cas9 to the transcription factor THAP11, which recognizes DNA binding motifs designed into the donor DNA.[51] Using two copies of this binding motif of both ends of the linear dsDNA donor, they showed a 2-fold increase in HDR in HEK 293T cells. Overall, several modes of bringing the donor DNA template in proximity to the CRISPR-Cas9 complex have been utilized. However, the ease of generating this platform and increasing the efficiency of HDR in primary cells at a clinically meaningful range is necessary to move these technologies forward.

The use of retrons to produce multiple copies of single-stranded donor DNA inside of the cells (as an alternative to producing the donor exogenously and then delivering the donor to cells) has seen some recent popularity. Retrons are bacterial genetic elements that reverse transcribe RNA, which are then used to produce multi-copy single-stranded DNA (msDNA). A pioneering study in 1993 demonstrated that retrons could synthesize intracellular DNA with a defined, non-native sequence.[52] Retrons became more immediately relevant to gene editing applications when Sharon et al. introduced CRISPEY (Cas9 retron precise parallel editing via homology)[53] This study used retrons to produce msDNA in yeast that were then used as donors in CRISPR-Cas9 editing. Limited studies have indicated that some retron systems can be used to produce msDNA in eukaryotic cells.[54,55] However, these studies have shown that the retrons are capable of producing many copies of the donor and that production of the donor using retrons increases HDR vs. donor that is provided exogenously.[54–56] The use of retrons is an emerging technology that bypasses the need to generate an ssDNA donor via nucleotide synthesis or other means and the toxicity inherent in delivering them to cells. However, the editing efficiency remains relatively modest (~10% HDR),[55] and the delivery of the retron itself may outweigh the benefits of the simple delivery of an ssODN donor. A major benefit of the retron system seems to be the ability to produce a higher number of donor copies vs. what could be delivered exogenously. However, potential toxicity from an excess msDNA causing innate cellular immune responses or off-target integrations remain to be studied. Future work in the development of this field may be very relevant to increasing precise gene editing outcomes.

Co-expression and/or fusion of DNA repair proteins

In 2017, newer techniques were used to increase HDR rates by the co/overexpression of proteins involved in DSB repair pathways. Pioneering work in this area began with RAD52, which coats the ends of the DSB and promotes annealing to a homologous donor.[57] In 2017, Wang et al. overexpressed yeast RAD52 with Cas9 editing tools in a chicken embryo fibroblast cell line and observed a 3-fold increase in HDR.[58] Shao et al. also co-expressed, and created a protein fusion between, Cas9 and yeast RAD52. They showed that the protein increased HDR up to 3-fold both when co-expressed and when fused to the N terminus of Cas9.[59] Paulsen et al. investigated the co-expression of key proteins involved in repair via HDR alongside human RAD52 (including human RAD51, EXO1, and BLM and a dominant-negative version of mouse 53BP1 [dn53PB1]) in human cell lines and human iPCSs.[60] They found that when all of these proteins were co-expressed with Cas9, HDR increased approximately 2.5-fold in HEK cells (from about 15% to about 38%), but only expression of both RAD52 and dn53BP1 in combination was needed to achieve this level. Notably, they found that dn53BP1 alone did not increase HDR.

Regardless, the utility of inhibiting 53BP1 found interest in multiple research labs. Because 53BP1 is one of the first proteins recruited for NHEJ at the site of a DSB, and is responsible for recruiting downstream NHEJ factors, inhibiting the binding of 53PB1 could be a very useful way to inhibit NHEJ. Canny et al. found an engineered ubiquitin variant that inhibited 53BP1 (i53).[61] Co-expression of i53 in human cell lines increased HDR up to 5.6-fold. Jayavaradhan et al. developed their own dominant-negative version of 53BP (termed DN1S), which they tested in human cell lines and patient-derived cells.[62] By fusing DN1S to Cas9, they attempted to inhibit NHEJ, not globally, but only at Cas9-induced DSBs. Their results, in contrast to results reported by Paulsen et al.,[60] demonstrated that a dominant-negative version of 53BP1 alone could significantly increase HDR. The Cas9-DN1S fusion increased HDR about 2-fold in cell lines and patient-derived B lymphocytes, and it showed no toxicity compared with Cas9 alone, but unfused DN1S demonstrated significant toxicity in hematopoietic cells. This discrepancy between the findings of Paulsen et al.[60] and Jayavaradhan et al.[62] may be due to the mouse vs. human version of the protein or the fact that DN1S was fused to Cas9, while dn53BP1 was not.

CtIP is another protein that has gained much attention as a tool to increase HDR. CtIP is one of the first proteins to be recruited to the DSB for repair via HDR; therefore, placing CtIP near the DSB could help to recruit pro-HDR factors and increase HDR rates. Charpentier et al. first investigated the fusion of both full-length CtIP and a minimal N-terminal fragment of CtIP to Cas9 in human cell lines, iPSCs, and rat zygotes. They observed a 2-fold increase in HDR in human cell lines.[63] Tran et al. also investigated CtIP, along with RAD52, MRE11, and RAD51C as fusion proteins, in HEK cells.[64] They also found that CtIP increased HDR up to 2-fold, along with RAD52 and MRE11 but not RAD51C.

In 2019, Nambiar et al. published a broad-sweeping study of 204 proteins involved in DNA damage response and used high-throughput analysis to identify candidates that increase Cas9-induced DSB repair via HDR.[65] They found that RAD18 was an outstanding candidate with both ssODN and dsDNA donor templates and then engineered an enhanced RAD18 variant (e18) that maximizes HDR in human cell lines (up to 2-fold) while minimizing the size of the protein. Their mechanistic data also demonstrated that e18 enhances HDR by inhibiting the localization of 53BP1 to the DSB. While this study did not attempt to fuse e18 to Cas9, this remains an interesting further step to explore.

RAD51 has been investigated in several studies because of its interesting role in strand invasion and its role in repairing Cas-induced DSBs and nicks. Two Cas9 nickases, which represent Cas9 mutants of either of the endonuclease domains, are often used: Cas9 (D10A) inactivates the RuvC domain and only cleaves the target strand (paired with the gRNA), while Cas9 (H840A) inactivates the HNH domain and only cleaves the non-target strand. It has also been shown that precise editing can be achieved when using a nickase and a donor template.[66] Although it is not as efficient as using fully active Cas9, the rate of indels is much less when attempting genome editing with nicks vs. DSBs.[67] While RAD51 is necessary for HDR of a DSB, studies have shown that inhibition of RAD51 actually increases precise editing of a nick (depending on which strand is nicked),[66,68] which implies the use of alternate HDR pathways for repair of a nick.[69] In 2019, Rees et al.

showed that the fusion of a human RAD51 mutant (that prevents BRCA2 binding) to Cas9 (D10A) nickase increased HDR 3.5-fold in human cell lines.[67] WT RAD51 has also been shown to increase HDR after Cas9-mediated DSBs. In 2020, Kurihara et al. tested the overexpression of RAD51 with *in utero* electroporation of plasmid DNA (pDNA) expressing fully active Cas9 and RAD51 into embryonic mouse neurons and found overexpression of RAD51 increased HDR 2.5-fold.[70] Later that year, Ma et al. published a study that fused a 36-amino-acid motif of BRCA2 that binds to RAD51 to fully active *Staphylococcus pyogenes* Cas9 (SpCas9) to aid repair of DSBs that increased HDR 2- to 3-fold.[71] These studies indicate that RAD51 could be a versatile agent to use with the repair of both DSBs and nicks.

A few other notable studies have explored protein candidates to increase HDR. In 2018, Reuven et al. studied UL12, a recombinase from human simplex virus-1, which can mediate recombination. They chose a small (126 AAs) intrinsically disordered portion of the N terminus of UL12, which can recruit the MRN complex, and created a fusion to Cas9.[72] Their results showed that this fusion protein could increase HDR up to 2-fold in human cell lines. In 2021, Hackley published a study regarding the fusion of hExoI to the N terminus of Cas9.[73] He proposed that the rate-limiting step of HDR is long-range resection that commits repair of the DSB to HDR and, as such, demonstrated that hExoI-Cas9 fusion increased HDR in human cell lines by 2- to 2.5-fold. Reint et al. screened the fusion of 450 DNA repair proteins with Cas9 for those that increase HDR in HEK293T green fluorescent protein (GFP) reporter cells.[74] They found approximately 31 fusions improved HDR, with many belonging to the replication fork machinery, including several replicative polymerases. They further studied the seven top proteins and found that the fusion performance was strongly dependent on individual loci and cell types, with POLD3 outperforming the other fusions, increasing HDR by ~2 fold.[68] They performed affinity purification mass spectrometry to show the interaction of the POLD3-Cas fusion with proteins known to have chromatin remodeling and helicase activity. From these results, they proposed that these proteins dislodge Cas9 from the targeted DSB site, making ends available for the cellular DNA repair machinery, thereby promoting HDR. They then compared POLD3 to other Cas9-fusions described above and showed that increase in HDR was dependent on cell-type, locus-targeted, and gRNA sequence, as well as likely dependent on chromatin architecture. More recently, Chen et al. attempted to alter chromatin histone marks at the cut site by fusing Cas9 with four different histone methyltransferases, since histone marks H3K36me3 and H3K4me3 are necessary for homologous recombination.[75] They showed that Cas9 fusion to PRDM9, a chromatin remodeling factor that deposits histone methylations H3K36me3 and H3K4me3, increases HDR efficiency by 3-fold. However, the effect was highly dependent on both endogenous and newly acquired histone marks, as well as the locus targeted. Similarly, Benitez et al. compared various Cas9 fusions with DNA repair proteins or cell cycle degrons and showed that although the fusions altered cellular DNA repair outcomes, there were locus- and cell-specific effects, with the best effect seen after restricting Cas9 expression to cycling cells.[76] Fusion of DNA repair proteins to Cas9 can elegantly skew repair toward gene correction only at the Cas9 target site, without perturbing the overall cellular repair machinery. However, these fusions need to be carefully selected with respect to the specific cell type and target locus for optimal increase in HDR.

It should also be pointed out that the fusion of a nuclear localization sequence (NLS) to the Cas protein is necessary to promote nuclear entry of the RNP. In some cases, the identity and number of these NLSs may be optimized to further increase HDR. For example, Wu et al. demonstrated that adding an additional NLS on the N terminus of Cas9 (to a construct already containing two NLSs on the C terminus) increased gene editing levels, although the dosage and delivery conditions may need to be optimized to control toxicity.[77] Cas9-protein fusions, if combined with other approaches (i.e., to control cell cycle and to proximate the donor template), may greatly advance HDR repair rates.

EMERGING PRECISE GENE CORRECTION TECHNOLOGIES

While established CRISPR-Cas gene editing is an intense research focus, there are still drawbacks that have inspired other researchers to discover and develop alternative technologies. While it has been relatively simple to efficiently target CRISPR to a sequence of choice, the cellular DNA damage response, the cell's choice of DNA repair, and its dependency on its cell cycle phase have been significant hurdles for effective clinical translation. Some of these newer technologies still draw on the utilization of Cas9 editing machinery and/or a programmable RNA. Still others, however, have discovered alternative systems that do not use the popular RNA-guided nuclease but are interesting candidates in future precise human genome editing techniques. Here we will review some of the most popular and interesting emerging alternative technologies capable of making precise genomic edits that either utilize a non-DSB based strategy (i.e., base editing and prime editing) or are independent of the cellular DNA damage/repair response (recombinases and RNA-guided transposons). Since emerging precise gene correction technologies are a fast progressing field, we refer to the reader to current specialized reviews for these topics as the field advances.

Base editing

The first popular strategy to avoid DSBs inherent in CRISPR-Cas genome editing was the development of base editors (BEs). Base editing, described first by Komor et al. in 2016, generates point mutations in genomic DNA without directly generating DSBs, requiring a DNA donor template, or relying on cellular HDR. The basic structure of a BE is a Cas9-nickase fused to a nucleotide deaminase.[78] DNA BEs are categorized as cytosine base editors (CBEs) or adenine base editors (ABEs) (Figure 3).

A CBE was the first DNA base editing method described for gene correction without generating DSBs.[78] CBEs convert a C·G base pair into a T·A base pair by deaminating the exocyclic amine of the target cytosine to generate uracil. The first generation of BEs

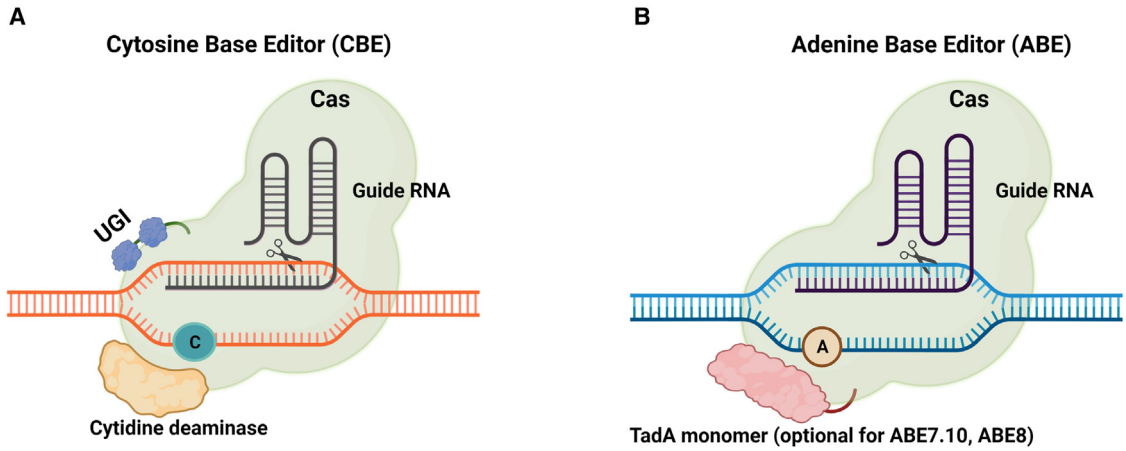

Figure 3. Schematic representation of cytosine and adenine base editors

(A) Cytosine base editors (CBEs) are composed of a Cas9 nickase fused to a cytosine deaminase and one or two uracil glycosylase inhibitors (UGIs). CBEs convert C·G into T·A base pairs. (B) Adenine base editors (ABEs) are composed of a Cas9 nickase fused to a wild-type or mutant tRNA (tRNA) adenosine deaminase (e.g., TadA). ABEs convert A·T into G·C base pairs.

(CBE1) was a fusion of APOBEC1 (an ssDNA-specific cytosine deaminase) to the catalytically dead version of SpCas9 (dSpCas9, containing D10A and H840A mutations).[78] Mechanistically, the dSpCas9/gRNA binds to the protospacer and then locally denatures the DNA duplex. An R loop is exposed when gRNA binds to the protospacer, exposing ssDNA on the non-target DNA strand, which can be deaminated by APOBEC1.[78] In addition, insertion of a 16-residue XTEN linker between dCas9 and APOBEC1 proteins significantly improves the efficiency.[78]

Although CBE1 was efficient *in vitro*, it was not efficient in human cells (deamination efficiency dropped from 25% to 40% *in vitro* to 0.8%–7.7% in cells). This low efficiency was partially due to high cellular repair of the newly generated U·G base pair by uracil DNA glycosylase (UDG), a base excision repair (BER) enzyme.[78] UDG catalyzes the removal of uracil in DNA to initiate the BER pathway, which usually results in reversion to the original C·G base pair.[79] In order to block UDG activity, uracil DNA glycosylase inhibitor (UGI, from *Bacillus subtilis* bacteriophage) was fused to the C terminus of dCas9 in the CBE1. This resulted in a second-generation CBE: CBE2 (APOBEC-XTEN linker-dCas9-UGI).[80] CBE2 showed 3-fold efficiency enhancement compared with CBE1.[78] Since dCas9 fusions do not cause a DSB, indel formation rates were $\leq$0.1% with CBE1 and CBE2 base editors.

CBE2, however, was only able to edit one strand of DNA, thereby increasing the chance of the cellular DNA repair machinery reverting the edit of the complementary to the unedited strand. Therefore, CBE3 was created by substituting a Cas9 nickase for dCas9. This could trigger the cellular DNA repair machinery to correct the G present in the non-edited strand.[78] Cas9 nickase in these constructs, Cas9 (D10A), inactivates the RuvC domain but retains activity in the HNH domain.[78] Cas9 nickase nicks the DNA backbone of the unedited DNA strand, which biases the cellular repair of the U·G mismatch to favor a U·A outcome. CBE3 (APOBEC1-XTEN linker-Cas9 nickase-UGI) increased the editing efficiency by 2- to 6-fold compared with CBE2 in mammalian cells.[78]

Later, Nishisa et al. engineered a synthetic complex called "Target-AID" that contained an activation-induced cytidine deaminase (AID) ortholog, PmCDA1, as well as the Cas9 (D10A) nickase. AID triggers immunoglobulin hypermutation, recombination, and gene conversion by producing U·G mismatches in DNA.[81] The mutational efficiency of Target-AID in the CBE was highly dependent on the relative position of the target cytidine within the genomic sequence. Although it was highly effective in yeast, it induced indels in mammalian cells. Target-AID showed a different activity window compared with CBE3, which may be attributed to the enzymatic characteristics of PmCDA1 compared with APOBEC1, or the C-terminal vs. N-terminal fusion to Cas9.[78,81] In 2018, Li et al. developed a CBE strategy in which APOBEC1 was fused to a catalytically inactive version of Cpf1 from *Lachnospiraceae bacterium* (also known as Cas12a). Catalytically inactive/dead Cpf1 (dCpf1) fused to CBE induced fewer indels, non-C-to-T substitutions and recognized AT-rich PAM sequence (TTTV) rather than the SpCas9 NGG PAM sequence used in CBE3 and Target-AID.[82] A further development, BE-PLUS (BE-programming larger C to U scope) generated by the SunTag amplification system, contains the GCN4 peptide recognized by single chain variable fragment (scFv) antibody.[83] To avoid protein aggregation, a small binding domain of protein G was fused to the C terminus of scFv. The fusion of 10 copies of 19-amino acid-GCN4 peptide to Cas9 nickase recruits scFv-APOBEC-UGI-GB1 to the target sites and induces C-to-T conversions. BE-PLUS showed a broader editing window and higher fidelity compared with CBE3.[83] In order to improve the low precision of CBEs (i.e., specificity for the target cytidine vs. other nearby cytidines), another CBE strategy was suggested by Tan et al. The authors developed high-precision base editing by engineering CDA1 (an AID homolog) fused to Cas9

(D10A) nickase that preferentially edits a cytosine 18 bases upstream relative to the NGG PAM sequence.[84] Further, an engineered A3A deaminase showed high-precision CBEs that predominantly edit position C15 or C16 with high editing efficiency.[84] While the CBE3 editors showed much improvement, even more development to this system was published.

CBE4 was generated to reduce the undesired C·G or C·A conversions that were reported with previous CBEs. Komor et al. discovered that these by-products resulted from excision by uracil N-glycosylase (UNG) during BER, and adding a second UNG inhibitor decreased undesired editing.[78] In addition, the linker (32-amino acids) between APOBEC1 and Cas9 (D10A) nickase increased product purity.[78]

Further generation of SpCas9-based CBE4 and *Staphylococcus aureus* Cas9 (SaCas9)-based SaCBE4 increased editing efficiency by 50%.[78] Furthermore, a fusion of Gam protein (a bacteriophage Mu protein) to the N termini of CBE4, SaCBE4, CBE3, and SaCBE3 reduced the frequencies of indels and non-C·T edits.[78] Additionally, with the modification of nuclear localization signals and codon usage of CBE4, CBEmax was generated, which further improved the editing efficiency.[78] Recently, Chen et al. showed base editing (TAG to TAA) of 33 target sites (out of the 47) via a single transfection.[85] Several other studies improved base editing by narrowing the editing window, enhancing DNA specificity, and developing different PAM compatibilities, using a different variant of APOBEC1 and small molecule dependence.[86–94]

Another type of BE is ABE, developed by Gaudelli et al. by fusing dCas9, or Cas9-nickase, to adenine deaminase, which converts an A·T base pair into a G·C base pair. ABEs have the potential to correct ~47% of disease-associated point mutations.[86] ABEs consist of a mutant tRNA (tRNA) adenosine deaminase (TadA), a Cas9 nickase, and a sgRNA. The first ABE, designed in 2017, used TadA from *Escherichia coli* that is able to deaminate adenosines in DNA.[95] The mutant TadA, fused to dCas9, (TadA*–dCas9) converts a deoxyadenosine to a deoxyinosine, which is further repaired by the mismatched repair pathway to a deoxyguanine. After extensive directed evolution (structural and protein engineering), the seventh generation of ABE (ABE7.10) was developed that edits A·T to G·C in human cells at approximately 50% efficiency with <0.1% indels.[86]

ABE7.10 contains 14 amino acid substitutions and performs conversion within an editing window of protospacer positions ~4–7, counting the PAM as positions 21–23.[86] Furthermore, improvements in optimizing the NLS and codon usage led to the generation of ABEmax by replacement of SV40 NLS in ABE7.10 with bis-bpNLS (bpNLS ABE7.10 ABEmax). Editing efficiencies were increased 1.5- to 2-fold in HEK293T cells with ABEmax.[96] To further increase the capability of ABE, alternative-PAM ABEmax variants and circularly permuted Cas9 (cpCas9) variants were used, which increased the editing window from ~4–5 nucleotides to ~8–9 nucleotides and reduced byproduct formation.[97] Together, base editing enables efficient C·G to T·A base pair conversion in bacteria, yeast, rice, zebrafish, mammalian cells, mice, and human embryos.[86–93,98]

The biggest advantage of BE technologies is high editing efficiency, ability to edit in non-dividing cells, and avoidance of DSBs. CBEs and ABEs can collectively mediate all transition mutations (C·T or T·C, A·G or G·A) in cells with high efficiency. Additionally, off-target indels may not be as problematic with BEs as on-target undesirable base edits or off-target base edits. However, BEs are unable to catalyze transversion mutations. Other drawbacks included limitations in targetable sites, unanticipated off-target editing, and confinement to specific target sequences that do not have additional cytosines or adenines surrounding the targeted base. Hence BEs are applicable to diseases caused by a single point mutation (e.g., sickle cell disease). For diseases caused by many mutations (e.g., thalassemia, severe congenital neutropenia from ELANE mutations, CGD, etc.), the entire gene would need to be targeted for clinical translatability to all patients with that disease. Here, gene replacement via HDR or other newer strategies may be the best option.

Prime editing

Prime editing is a precise genome editing method that edits all 12 types of point mutations without directly forming DSBs or requiring a donor DNA template.[99] Prime editing consists of a Cas9-nickase fused to reverse transcriptase (RT). The reverse transcriptase RNA template (RTT; which serves as the donor/correction template after reverse transcription) and an RT primer binding site (PBS) are fused to the guide RNA to generate a prime editing guide RNA (pegRNA) (Figure 4). The initial version of pegRNA contained a programmable RTT, PBS, and a protospacer sequence for directing prime editing to the genomic target sites.[99]

During genome editing, the PBS allows the 3′ end of the nicked DNA to hybridize to the pegRNA extension, and the RTT serves as a template for synthesizing edited genetic information. DNA polymerization leads to the generation of a 3′ DNA flap that contains the newly synthesized sequence and a 5′ flap that contains an unedited DNA sequence. Next, excision of the displaced 5′ flap allows ligation of the 3′ flap, which results in a hetero-duplex DNA composed of an edited DNA strand and an unedited DNA strand. The cellular DNA repair machinery resolves this to install the edit by repairing the unedited strand.

In 2019, Anzalone et al. generated the first prime editor (PE) by fusing SpCas9 (H840A) nickase to the wild-type RT from Moloney murine leukemia virus (MMLV).[99] In this report, this PE (termed PE1) showed less than 5% efficiency; therefore, to improve the editing efficiency, PE2 was generated by introducing a pentamutant MMLV RT (D200N, L603W, T330P, T306K, W313F). Mutated RT increased thermostability, processivity, and DNA:RNA substrate affinity that inactivates RNaseH activity, which led to a 1.6- to 5.1-fold editing efficiency in PE2 compared with PE1.[99] Despite the increased efficiency of PE2, it still relied on the endogenous cellular repair process to copy the edited DNA strand to the complementary strand.

PE3 was designed by adding an additional sgRNA that matched the edited sequence (pegRNA) and directed a Cas9 nickase to nick the

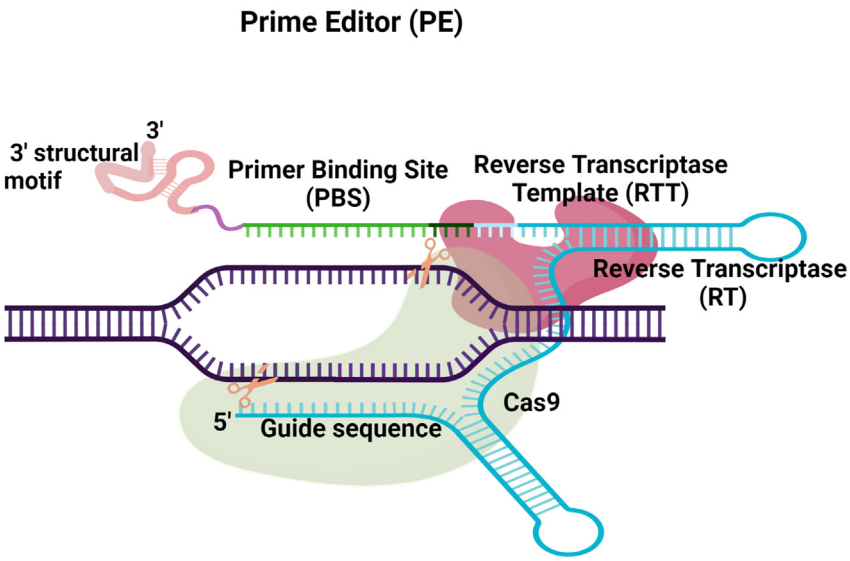

Figure 4. Schematic representation of a prime editor (PE)

A PE consists of a fusion of Cas9 nickase and reverse transcriptase (RT). This complex is coupled with a prime editing guide RNA (pegRNA), which consists of the spacer, scaffold, reverse transcriptase template (RTT), primer binding sites (PBS), linker, and 3′ structural motifs (optional).

unedited strand so that the cellular repair is biased toward copying the edited strand. Although PE3 improved the editing efficiency by 1.5- to 4.2-fold, it also increased indels compared with PE2, likely from the presence of simultaneous nicks on both DNA strands.[99] PE4 and PE5, which were generated based on PE2 and PE3, respectively, transiently inhibited mismatched repair (MMR), resulting in an improvement of the editing outcome by 7.7- and 2.0-fold. In these systems, indels were minimized.[100]

A further study of prime editing by Nelson et al. showed that degradation of 3′ end of the pegRNA reduces the activity of a PE system.[101] Therefore, several groups engineered/enhanced pegRNAs to epegRNA by introducing 3′ structural RNA motifs that enhance the stability of the 3′ end of pegRNA and avoid its degradation.[101–103] This epegRNA improved the prime editing efficiency 3- to 4-fold in cells and primary human fibroblast without increasing the off-target editing activity.[101] Generally, prime editing allows insertions up to ~44 bp and deletions up to ~80 bp.[99] Other strategies (e.g., flip and extension scaffold, an 8-nt linker between 3′ structural motifs and PBS, installation of silent mutations, optimization of nuclear localization signals, and Cas9 mutations) improved prime editing efficiency further, which led to the development of the PE max architecture, PE2*, CMP-PE, and hyPE2.[99,100,104–106]

Although "traditional" prime editing mediates efficient editing to increase the size of insertion/deletion, additional developments have been published: twinPE, BiPE, PRIME-Del, GRAND, PEDAR, and dual-pegRNA. TwinPE contains two sets of pegRNA, which bind Cas9-RT and nick on opposite strands of DNA, and the newly synthesized genomic flaps are complementary to each other. This allows the insertion of over 100 bp and deletions of around 800 bp.[99,107] BiPE, PRIME-Del, GRAND, and PEDAR are also similar systems to twinPE. In BiPE and PRIME-Del, the opposite strand of DNA is not only complementary to each other but also complementary to the genomic sequence upstream of the nick on the opposite DNA strand. PRIME-Del achieved deletion up to 10 kb with 1%–30% editing efficiency.[108] PE-Cas9-based deletion and repair (PEDAR) consists of a Cas9 nuclease instead of Cas9 nickase. PEDAR removed 1.38 kb pathogenic insertion within the Fah gene and precisely repaired the deletion junction to restore FAH expression in the liver of a tyrosinemia mouse model.[109] In 2022, Zhuang et al. reported an approach named the homologous 3′ extension mediated prime editor (HOPE) in which they used a pair of pegRNAs (sense and anti-sense) encoding the same edits to target both DNA strands.[110] The sequence between two nicks was not deleted in HOPE and dual-pegRNA systems. Although in twinPE and GRAND, the sequence between two nicks is deleted and replaced with a new edited sequence. GRAND showed the ability to insert ranging from 20 bp to 1 kb at the target sites with editing efficiency up to 63.0% for 150 bp and 28.4% for 250 bp.[111] However, efficiency remains low in fragments larger than 400 bp. Overall, the initial PE systems were of limited efficiency and utility over BEs, but the newer systems are expected to allow scientists to perform longer genomic edits.

Recombinase systems

Site-specific recombinases catalyze the exchange of two ds DNA sequences by recognizing an attachment DNA sequence (e.g., attP, attB, sometimes referred to as a "landing pad") at the site of insertion. These recombinases have been repurposed from their original bacterial and viral hosts for bioengineering tasks such as insertions, deletions, and inversions in other genomes. Serine recombinases derived from phages (e.g., Bxb1 and phiC31) can commit irreversible recombination in human cells to swap "cargo" DNA sequences into genomes if the genomes are pre-installed with the unique attachment sites (attP/attB, i.e., landing pads) in the desired place in the genome. This can allow the installation of very large insertion sequences (up to 33kb).[112,113] Tyrosine recombinases (e.g., Cre and Flp) have also been used to insert sequences, although the efficiency remains lower, and the insertion is reversible. However, tyrosine recombinases have found utility in knockout studies.[114,115] While these molecular tools have seen much use, they suffer from overall low editing efficiency and lack of precise programmability.

However, this field is now becoming more accessible to precise gene editing. In 2021, Durrent et al. analyzed the sequences of nearly

200,000 bacterial genomes for attachment sites used by these enzymes and used the data to predict their recombination specificity.[116] They tested the recombination activity in human cells and found 7-fold higher recombination than the often-compared Bxb1 recombinase, opening the field to a pool of new recombinases to be developed. In 2022, Blanch-Asensio et al. demonstrated the use of STRAIGHT-IN (serine and tyrosine recombinase-assisted integration of genes for high-throughput investigation) in hiPSCs.[113] This requires three steps: (1) the pre-installment of an attP landing pad using traditional CRISPR-Cas editing and subsequent selection to generate a clone of cells with the inserted sequence; (2) the delivery of pDNA vectors expressing a serine recombinase and a donor containing the DNA payload, which results in the insertion of the entire pDNA donor, and further enrichment via antibiotic selection; and (3) the removal of unnecessary "auxiliary" sequences (e.g., sequences of the pDNA backbone, which may contribute to genotoxicity). The authors of these studies were able to show the incorporation of very large DNA payloads (>100 kb) with very high efficiency (90%) after selection steps in human iPSCs.

These recombination techniques, if developed to become feasible in primary cells without selection, are very attractive for several reasons. First, they do not leave an exposed DSB that is subject to the DNA repair pathways of the cell. This removes the potential for indels at the edit site and requirements of cell cycle or cellular DNA repair. Additionally, the size of the insertion does not yet have an upper limit, with the authors of STRAIGHT-IN demonstrating the insertion of a 173-kb bacterial artificial chromosome into hiPSCs.[113] Also, this method is not prone to off-target effects, provided the pre-installed landing pad is inserted precisely on target into the selected genomic target. However, much development needs to be done to make this technology relevant for therapeutic gene editing use. First, many steps need to be taken to reach the final desired edit, which may not be clinically useful in delicate primary cells. Further, the efficiency of this technology is relatively very low without the enrichment steps since recombination happens in less than 1% of cells containing the landing pad.[113]

Just recently, a new system was developed that combines aspects of PEs and recombinase technology. Yarnall et al. developed a new method called PASTE (programmable addition via site-specific targeting elements), which consists of a Cas9 nickase fused to RT and a serine integrase.[117] PASTE can integrate large sequences in human cell lines, primary T cells, and non-dividing primary human hepatocytes with efficiencies between 5% and 60%.[117] PASTEv1 was designed by incorporating the ∼46-bp *attB* landing site of serine integrases into pegRNA, referred to as attachment site-containing guide RNA (atgRNA). The authors tested PASTEv1 with a single transfection of the PE vector, atgRNA, nicked sgRNA for another strand, and a mammalian expression vector for the corresponding integrase or recombinase and a 969-bp minicircle DNA cargo encoding GFP. They found BxbINT integrates at the ACTB locus with the highest integration rate (15%).[117] Overall, PASTE efficiency and atgRNA landing site insertion was improved by modification of the scaffold design (atgRNAv2). This modification increased the stabilization and expression from RNA polymerase III promoters. In addition, several protein modifications (i.e., adding XTEN linker between Cas9 and RT, fusing of MMuLV RT to the Sto7d DNA binding domain, or mutation of RT [L139P]) improved PASTE integration efficiency.[117]

Further modifications were made to the PASTEv1 system. PASTEv2 was generated by combining the above modifications with a GGS6 linker between the RT and BxbINT, which led to ∼30% gene integration. Furthermore, the combination of PASTEv2 with atgRNAv2 generated PASTEv3, which allowed the integration of ∼36-kb DNA donor with 10%–20% integration efficiency. Integration activity was further improved in PASTEv3 by using mutant *attP* (vs. wild-type *attP)* at *ACTB* and *LMNB1* target sites. PASTEv4 was generated by fusing BceINTa integrase (from *Bacillus cereus)* to SpCas9-MLV-RT(L139P), which improved PASTE efficiency.[117] Building on prime editing and twinPE, the authors introduced PASTE-Replace, which requires two atgRNAs (PBS and *attB* sequence) with the optional inclusion of RT to bridge the deletion. The integrase efficiency was improved using PASTv3 and longer Bxb1 *attB* and *attP* lengths.[117]

As with many of the base and prime editing systems reviewed in this paper, using recombination systems for gene editing applications is seeing a growth spurt. It is expected that many more systems will be developed as the technique of recombination is further developed to be used for genome engineering. While the currently developed recombination methods show relatively high efficiency and can deliver large payloads, a major obstacle is the ability to pre-install the landing pad/attachment sites in the targeted cell type.

RNA-guided transposon systems

Transposons are a natural mechanism to move genetic elements in organisms and have been studied intensively in evolutionary contexts owing to their ancient origins. In brief, the transposon cargo is removed from one genetic area by transposases that recognize flanking nucleic acid sequences and are inserted into another area. This differentiates their activity from recombinases since DNA is not swapped for a different sequence; it is only integrated. Transposons have been widely studied and used for numerous biomedical applications, with notable examples such as the PiggyBac and Sleeping Beauty transposon systems. However, the lack of programmability in established transposon systems and the ability to "hop" around the genome have been major obstacles in the development of transposon systems for precise genome editing.

CRISPR-guided transposon systems have recently been discovered that have generated excitement as an alternative to established CRISPR-Cas gene editing. The intrigue in a gene editing context is that therapeutic donor sequences could be used as the genetic mobile cargo, allowing relatively large genetic inserts, i.e., 10 kb or more.[118] RNA-guided transposon systems could be an efficient way to insert a large genetic payload into a programmable site in the genome without requiring the cell or DNA repair machinery to resolve a DSB. Additionally, previous studies have reported that the on-target

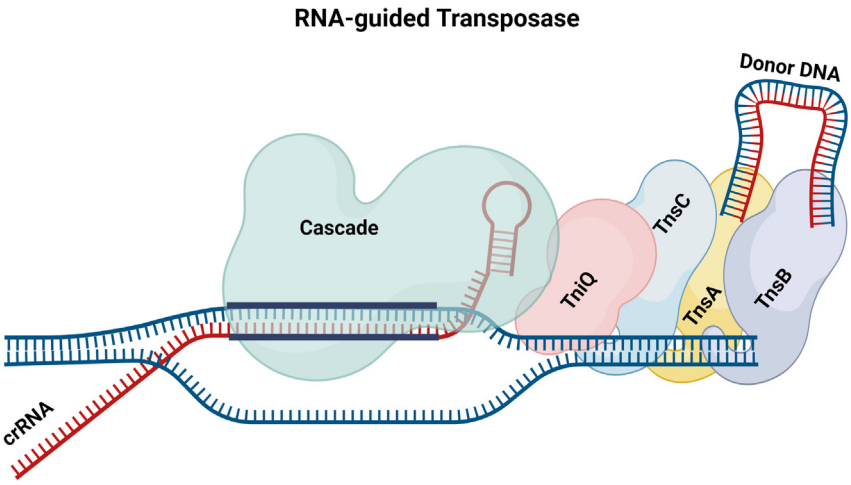

Figure 5. Schematic of Tn7-like RNA-guided transposon systems

A Cascade/crRNA/TniQ complex is guided to the target site for insertion by the crRNA. TnsC and TnsA/B bind to the complex and increase the fidelity for the target site. Enzymatic integration of the donor DNA into the genome is mediated by TnsA/B.

activity of these systems is much higher than traditional SpCas9 systems,[119] which would increase the safety and efficacy of this potential therapeutic tool.

In 2017, Peters et al.[120] published the discovery of the first Tn7-like transposons that encoded CRISPR-Cas systems, and in 2019, experimental evidence published by Klompe et al.[121] demonstrated that these transposons used RNA to guide the transposon to the target site. These transposon systems typically are associated with a catalytically inactive Cas protein (or protein complex) and other genes of the transposon system that excise the cargo from one genetic area and integrate it into another. In a series of papers describing the molecular structure and mechanism of this system,[119,122–127] it was found that these Tn7-like systems encode protein-transcribing genes that rely on intermolecular interactions to complex and perform CRISPR-guided integration of the DNA cargo. From this published evidence, the following molecular mechanism has been postulated: (1) TnsA and TnsB interact to form a complex that binds to the ends of the DNA sequence to be inserted. (2) Separately, the Cascade/RNA complex associated with this transposon binds to TniQ, and this multisubunit complex searches the genome for the target of the insertion. This is done by using both the RNA and specificity of the Cascade complex for the target sequence. It has been found that these Cas proteins do have a transposon-associated motif, which is analogous to a PAM in established CRISPR-Cas editing. In this searching step, the RNA-guided Cascade/TniQ is relatively promiscuous in its binding to the genome. (3) TnsC binds the Cascade/RNA/TniQ complex and further increases the specificity of the complex. (4) TnsC acts as an adaptor to bind the TnsA/TnsB/cargo DNA complex. The fully loaded complex (Cascade, TniQ, TnsC, TnsA, TnsB) has been shown to have very high specificity and very low off-target binding to non-target sequences.[119] (5) Finally, the TnsA/TnsB complex has the enzymatic ability to insert this sequence into the target DNA site (Figure 5).

While these transposon systems have generated excitement in the field, they currently are only capable of performing gene editing in bacteria, and much work is still necessary to advance this technology to a feasible gene editing technology for human cells. It remains to be seen if the proteins involved in the Tn7-like transposon system can be modified to perform transposition in human cells. Additionally, because of the inherent mobility of transposon systems, the permanence of the gene-edited product in human cells would need to be evaluated. Finally, because the system uses multiple proteins, an efficient delivery method to deliver all the components necessary must be developed and optimized. If these obstacles are overcome, CRISPR-guided transposon systems may be an attractive alternative to HDR in non-cycling cells.

CRITICAL HURDLES AND OPPORTUNITIES OF PRECISE GENE CORRECTION

While HDR has great potential for gene editing and targeted genome modifications, there are several critical hurdles associated with this technology: (1) regarding efficiency, HDR efficiency can vary depending on the cell cycle, cellular DNA repair machinery, cell type, delivery method, and the length/complexity of the DNA template. (2) Regarding off-target effects, DSBs to enable HDR can occur in off-target sites, leading to an unintended mutation in the genome and/or chromosomal translocation from two DSBs (i.e., on-target and off-target DSBs). (3) HDR requires the delivery of a repair template, which can be challenging in certain cell types (e.g., heart and liver). BEs and PEs can overcome dependence on DSBs but have limitations. These methods only allow nucleotide edits, small insertions (less than ~50 nucleotides), or short deletions (less than ~80 nucleotides). They cannot introduce or replace large segments of DNA. Paired-guide prime editing methods that utilize two pegRNAs with complementary reverse transcription template regions bias the repair process toward the edited strands, enabling the insertion of large DNA sequences. However, the efficiency of this method decreases in the range of 1–5.6 kb. Moreover, they introduce staggered DSBs, which may be prone to chromosomal translocations. Thus far, only NHEJ-mediated CRISPR technologies have moved into the clinic and shown success.[128] HDR-, BE-, and PE-based CRISPR technologies are either published as proof-of-concept studies or are in various stages of preclinical development, of which BE is closest to clinical translation. Furthermore, all of these CRISPR technologies rely on host cell DNA repair. Newer technologies (i.e., recombinases and RNA-guided transposases) may be able to shore up these deficiencies by circumventing DSBs and DNA repair and have the flexibility of large edits.

Table 1. Comparison of technologies to achieve precise gene editing mentioned in this review, including features, pros, and cons

	HDR	Base editor (BE)	Prime editor (PE)	Recombinase[b]	PASTE	Transposon
Gene editing components	Cas9 site-specific nuclease[a], gRNA, homology donor/DNA template	Cas9-deaminase-UG-I fusion protein, gRNA	Cas9-reverse transcriptase (RT) fusion protein, prime-editing-gRNA (pegRNA)	"landing pad" (in genome), serine/tyrosine recombinase, DNA donor template	Cas9-RT-serine recombinase fusion protein, attachment site carrying gRNA (atgRNA), DNA donor	Cascade-TniQ, TnsA/TnsB and TnsC, gRNA and DNA donor
Scope of genetic modification	point mutations up to multiple kb, replacements/insertions	point mutations or 3–5bp changes in the BE window	~40 bp, deletions of up to 700 bp with twinPE	wide range (1–100 kb)	wide range (up to 35 kb)	wide range (1–100 kb)
Cell cycle dependence	yes, requires cycling cells	no	no	no	no	no
Cellular DNA repair dependence	yes	yes	yes	no	yes	no
Gene editing cargo size	medium–small RNP or pDNA/mRNA delivery	relatively bulky fusion complex	relatively bulky fusion complex	relatively bulky fusion complex	bulky fusion complex	bulky protein complex
Cellular toxicity	DNA DSB-associated cellular toxicity	minimal cellular toxicity	minimal cellular toxicity	no toxicity	minimal cellular toxicity	not currently used in mammalian cells
Genotoxicity	on-target indels (NHEJ), off-target indels	minimal indels; on-target and off-target undesirable edits	minimal indels with PE1, PE2, and PE4; more indels with PE3, PE5, and twinPE	off-target edits due to natural landing pads, albeit low	combined effects of PE and recombinase	not established in mammalian cells
Efficiency in mammalian cells	modest efficiency	high efficiency	modest efficiency	low efficiency	low efficiency	not tested

[a]Note that other site-specific nucleases (e.g., homing endonucleases/meganucleases, zinc finger nucleases or TALENS) are not included in this review.
[b]Recombinases featured here do not use a CRISPR-Cas nuclease system.

However, they are currently only used in biological models (i.e., prokaryotic cells or mammalian cell lines), do not show efficiencies required for clinical application thus far, and have not been tested in primary cells or developed far enough yet to be useful in clinical settings. We summarize the pros and cons of the precise editing technologies in Table 1.

SUMMARY AND FUTURE DIRECTIONS

There are many potential strategies that could elevate CRISPR-Cas genome editing to clinically relevant cures for genetic diseases by increasing cellular repair rates of HDR, employing base or prime editing, or using emerging technologies that involve recombinases/transposases. Table 1 lists the features, pros, and cons of each of these technologies. While small molecules and other pharmaceuticals have shown improvements in HDR rates, overall, this strategy tends to be cytotoxic and ineffective in clinically relevant cell types, such as primary cells and stem cells, and it needs rigorous *in vivo* testing in relevant model systems. Nevertheless, this review has presented several studies that have made progress toward increasing HDR rates at clinically relevant levels with reduced or acceptable toxicity profiles. Controlling cell cycle so that editing only occurs when the cells employ HDR machinery is a promising option, but it is not as relevant in quiescent/non-cycling cells; this may be a limiting factor in editing quiescent HSCs or post-mitotic cells such as hepatocytes, cardiomyocytes, etc. Optimizing the DNA repair template and aiding HDR via delivery of HDR-boosting (or NHEJ-inhibiting) proteins have been shown to be more promising. Future work could attempt to boost HDR with delivery of HDR-boosting proteins as well as controlling cell cycle (i.e., simultaneous use of a Cas9-DNIS fusion with a degron such as geminin).

Furthermore, while base and prime editing are exciting developments in the field, these tools are not able to correct all genetic defects (e.g., insertions of large edits or correction of multiple bases in a large locus). Base editing is restricted to editing mutations and creating transitional base changes at the single-base level. Prime editing can result in any type of base change but still is practically confined to small edits. However, these technologies can circumvent the need for cycling cells, and they are more clinically relevant in editing quiescent cells *ex vivo*, retaining their engraftability, or editing post-mitotic cells *in vivo*. HDR remains the preferred strategy to make larger edits and is desirable for diseases where there are many mutational hotspots in the mutated gene. Gene editing systems such as recombinases and RNA-guided transposases are being refined for efficient mammalian genome editing and will complement or replace HDR when ready for "prime time." The biggest advantage of these RNA-guided transposases/recombinases/integrases is the circumvention of both cell cycle dependence and cellular DNA repair; when optimized, they may become clinically desirable. However, the size of the editing machinery and efficient delivery may become rate limiting. Therefore, work

in this field is still important to realize the full potential of genome editing technologies.

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AUTHOR CONTRIBUTIONS

K.M.F., T.S., and P.M. performed background research and the manuscript.

DECLARATION OF INTERESTS

P.M. has patents and royalties with CSL Behring and Aruvant Sciences.

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