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Advance trends in targeting homology-directed repair for accurate gene editing: An inclusive review of small molecules and modified CRISPR-Cas9 systems

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Abstract

Introduction: Clustered regularly interspaced short palindromic repeat and its associated protein (CRISPR-Cas)-based technologies generate targeted modifications in host genome by inducing site-specific double-strand breaks (DSBs) that can serve as a substrate for homology-directed repair (HDR) in both *in vitro* and *in vivo* models. HDR pathway could enhance incorporation of exogenous DNA templates into the CRISPR-Cas9-mediated DSB site. Owing to low rate of HDR pathway, the efficiency of accurate genome editing is diminished. Enhancing the efficiency of HDR can provide fast, easy, and accurate technologies based on CRISPR-Cas9 technologies.

Methods: The current study presents an overview of attempts conducted on the precise genome editing strategies based on small molecules and modified CRISPR-Cas9 systems.

Results: In order to increase HDR rate in targeted cells, several logical strategies have been introduced such as generating CRISPR effector chimeric proteins, anti-CRISPR proteins, modified Cas9 with donor template, and using validated synthetic or natural small molecules for either inhibiting non-homologous end joining (NHEJ), stimulating HDR, or synchronizing cell cycle. Recently, high-throughput screening methods have been applied for identification of small molecules which along with the CRISPR system can regulate precise genome editing through HDR.

Conclusion: The stimulation of HDR components or inhibiting NHEJ can increase the accuracy of CRISPR-Cas-mediated engineering systems. Generating chimeric programmable endonucleases provide this opportunity to direct DNA template close proximity of CRISPR-Cas-mediated DSB. Small molecules and their derivatives can also proficiently block or activate certain DNA repair pathways and bring up novel perspectives for increasing HDR efficiency, especially in human cells. Further, high throughput screening of small molecule libraries could result in more discoveries of promising chemicals that improve HDR efficiency and CRISPR-Cas9 systems.
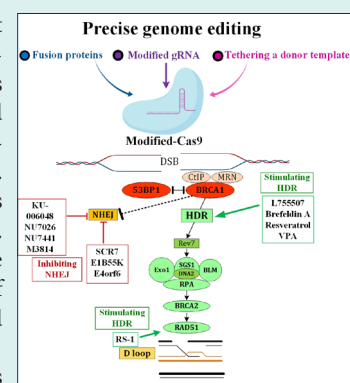
Precise genome editing

Introduction

The progress of programmable nucleases has considerably augmented the field of precision genome editing and provided a novel promising avenue for gene therapy approaches. Until now, four major classes of programmable nucleases have been established based on introducing a site-specific double-strand break (DSB); including i) meganucleases or homing endonucleases which are achieved from microbial mobile genetic compounds, ii) zinc finger (ZF) nucleases which are inspired by eukaryotic transcription factors, iii) transcription activator-like effector (TALE) nucleases which are originated from *Xanthomonas* bacteria, and iv) RNA-guided DNA endonucleases which are human codon-optimized form of archaeal and most bacterial adaptive immune systems, CRISPR-Cas.[1,2] CRISPR-Cas systems are categorized into two basic classes and six types. Because of the diversity and practicability of CRISPR-Cas9, which is categorized in class II-type 2, most attractions have been concentrated on this system and considered as an impressive genome editing technology in eukaryotic cells. In meganucleases, ZFN and TALEN, specific DNA binding is developed by protein-DNA interactions, while Cas9 is recruited to target DNA sequences by a single-guide RNA (sgRNA) molecule.[3,4] These four categories of genome editing tools have the common state of generating a site-specific DSB in the targeted genome (Fig. 1). Naturally occurred

DSBs are repaired by two distinct pathways, error-prone nonhomologous end-joining (NHEJ) or precise homology-directed repair (HDR).[5,6] Since NHEJ naturally is an error-prone repair pathway, by inhibiting the expression or function of essential components that are involved in this pathway, we could ameliorate the nuclease-mediated HDR efficiency.[7,8] In this regard, various small molecules have been recognized for modulating the efficiency of genome editing by either suppressing NHEJ or elevating the activity of HDR pathway. Using small molecules would develop a simple method that has several advantages for enhancing precision genome engineering. This review was focused on the mechanisms and effects of small molecules in DSB repair to progress the HDR pathway for improving the efficiency of precision genome editing in both *in vivo* and *in vitro* settings. Furthermore, we aimed to highlight the recent progress in enhancing HDR efficiency through using overlapping sequences and also applying novel Cas9 chimeric variants.

DNA repair pathways

By introducing a DSB, several factors such as BRCA1 (breast cancer type 1 susceptibility protein), 53BP1 (p53-binding protein1), and receptor-associated protein 80 (RAP80) are recruited to the damaged site and constitute ionizing radiation induced foci (IRIF). It is elucidated that a complex network of molecular interactions activating
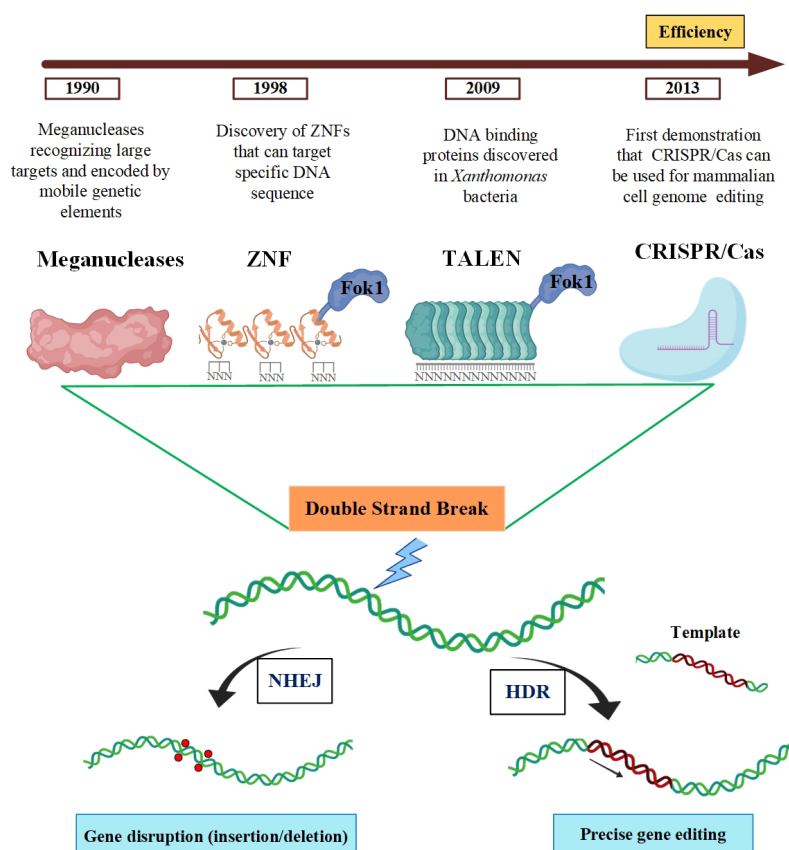

Fig. 1. The mechanisms and gene editing platforms for repairing DSB. DSBs which are induced by Meganucleases, ZFNs, TALENs, and CRISPR-Cas9 at specific sites could be repaired by NHEJ or HDR pathway. Figure was created with BiorRender (https://biorender.com).

BRCA1 or 53BP1 derives DSB to HDR or NHEJ repair pathway, respectively. These two antagonizing factors permanently are acting contrary to each other at the DSB site. In order to identify the effect of small molecules to increase HDR pathway for more efficient and precise gene editing, an overview of the DNA repair pathways and their key factors is presented.[9]

NHEJ pathway

Following the induction of DSB, the first reaction usually occurs through the NHEJ pathway. In mammalian cells, almost three quarter of DSBs are repaired via NHEJ and its defect results in various developmental disorders and enhances the rate of DSB-related chromosomal mutagenesis.[10] NHEJ is a broad term and commonly classified into two types:

1. Canonical NHEJ (c-NHEJ): it generally acts in end-joining and for a long time was characterized by its association with Ku, DNA ligase IV, and dependent factors.[10]

2. Non-canonical NHEJ: several homology-independent repairs triggered by the c-NHEJ dysfunction which need DNA Ligase I/III and is known as "alternative NHEJ" (alt-NHEJ/A-NHEJ), "microhomology-mediated end joining" (MMEJ), or "backup NHEJ".[10,11]

According to distinct DNA ends, NHEJ uses different strategies and is initiated by phosphorylation of 53BP1 at DSBs through protein kinase ataxia-telangiectasia mutated (ATM). 53BP1 is a chromatin-binding protein and an important part of DSB signaling repair in mammalian cells that during G1 promotes NHEJ. The basic effector proteins for 53BP1 are DNA polymerase zeta processivity subunit (REV7), PAX transactivation domain-interacting protein, and RAP1- interacting factor 1 (RIF1) (Fig. 2A).[12] RIF1 is acting together with REV7 for recruiting a large complex to DSB.[13] This large complex is called shieldin which consists of four components including REV7, SHLD1 (induces NHEJ while decreasing the HR by constraining DNA end resection), SHLD2 (an effector of REV7), and SHLD3.[14,15] Furthermore, the core NHEJ factor recognizes broken ends and keeps them next to each other so that the other processing factors can be activated.[16]

In c-NHEJ, predominantly Ku 80 interacts and promotes the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and creates a permanent complex that remains bound to the DSB ends.[16-18] DNA-PK can modulate the activity of different enzymes through autophosphorylation or phosphorylation and lead to DNA end processing by Artemis which cuts DNA overhangs for making blunt ends.[19] Then, DNA polymerase μ and λ can add missing nucleotides at the DSB ends.[16,20,21] The next step is ligation of blunt end that is accomplished by Ligase IV (Lig IV). Ligase IV usually makes a complex with X-ray repair cross-complementing 4 (XRCC4) and XRCC4-like factor (XLF) and induces related downstream pathways (Fig. 2B).[22]

Alt-NHEJ pathway is generally active during the S and G2 phases of the cell cycle and repairs DNA DSBs through microhomology (MH)-mediated end joining (MMEJ) (Fig. 2C).[23] With regard to the annealing of the flanking MHs, MMEJ is classified into three distinct steps: pre-annealing, annealing, and post-annealing.[24] The initial step starts from end resection to subject MHs flanking DSBs through joining of poly [ADP-ribose] polymerase 1 (PARP1) to DSB ends for facilitating the resection factors [BRCA1/CtIP and MRN complex].[25,26] Intermediate annealing is reclaimed by the XPF/ERCC1 structure-specific nuclease complex, which is similar to XRCC4 in c-NHEJ. Then, this complex cuts the non-homologous ends and generates 3'- hydroxyl overhang that is desirable for developing by DNA polymerase. Finally, the DNA ligation is catalyzed by Ligase II and III.[27]

Homology-directed repair (HDR) pathway

HDR, as a constant repair mechanism, comes into action in the S- or G2-phase and needs homologous DNA sequences. BRCA1 by suppressing 53BP1, is a pivotal initiating factor for HDR. Therefore, various inner and outer factors are involved in this case such as tudor-interacting repair regulator (TIRR) which is able to mask the H4K20me2 binding surface (a specific target for 53BP1).[28] It is reported that overexpression of TIRR leads to 53BP1 reduction at the DSB site through competing with RIF1 to bind 53BP1.[28,29] Furthermore, the special complex of MRN along with BRCA1 could identify dsDNA and create a 15–20 bp nick from the 5'-ends of the DSB.[30] MRE11 is an Mn^{2+} dependent endonuclease that nicks the DNA upstream from the break site and involves in DNA DSB repair homologous recombination for maintenance of telomere (Fig. 2A, D).[31,32] SCAI is a related protein to induce separation of 53BP1-RIF1. Dramatically the relation of SCAI-RIF1 leads to some of the BRCA1 activity. Hence, a great number of HR-factors such as BRCA1, MRN, CtIP, and so on are inhibited by knocking out of SCAI.[29,33] A deubiquitinating enzyme (DUB), POH1, increases the removal of 53BP1 from the damaged locus by preparing interaction between RAP80 and the BRCA1 BRCT domain.[34] This enzyme is a part of proteasome that can neutralize RNF8/RNF168 (E3 ubiquitin ligases)-dependent ubiquitination activity.[29,34,35] As above mentioned, the non-canonical NHEJ pathway is extremely complicated and needs numerous biochemical factors. Interestingly, HDR and A-NHEJ have a similar initial steps. However, it is not elucidated how these two repair pathways are segregated later.

The initiation of HDR is continued by a two-step end processing. First, it starts with the MRN complex and the CtIP nucleases that bind to the DNA DSB and second, in order to create longer 3'-ends, the second stage for deep resection is occurred by EXO1 and SGS1-DNA2 nucleases.[36,37] The impermanent single-stranded DNA

(ssDNA) overhang needs to be concealed by replication protein A (RPA) to be protected from exonuclease activities. Then, Rad51 replaces heterotrimeric RPA and for simplifying a quest for a homologous donor, generates a nucleoprotein presynaptic filament.[38,39] The 3′ DNA filament will be coated with many proteins, and then attacks homologous duplex DNA to develop the D-loop structure as an exchange intermediate. The D-loop structures are used for the synthesis of identical DNA sequences.[34,40,41] The second end joins the D-loop and starts the development of a double Holliday Junction (HJ) structure.[42] Indeed, 53BP1 and BRCA1- mediated mechanisms are complex systems that many factors and several antagonized points are involved in their inviolability. In this line, a plethora of targets exists for manipulating the efficacy of the HDR pathway.

Single-strand annealing (SSA) is an important subtype of HDR because of its specific mechanism. This pathway is observed when DSBs occur between two repeat sequences (Fig. 2E).[43] SSA in terms of homological loci is similar to MMEJ. Although the applied mechanism is the same for both of them, the involving proteins in SSA are similar to HDR.[40,41]

Importance of small molecules in DNA repair pathways

Certain DNA repair pathways could be successfully activated or blocked by small-molecule compounds.[44] During the past decade, multiple studies have shown that small molecules are a straightforward strategy for increasing precision genome engineering.[7] Several advantages are mentioned for small molecules such as their high penetrant effects that lead to a rapid and controlled response. In addition, easy titration of small molecules provides optimal concentrations of inhibitors for delivering to the cell with extremely successful consequences.[45] The pharmacological approach to obtain a functional small-molecule usually contains: i) screening a library of chemical compounds to recognize lead scaffolds; ii) examining substitution places of the small molecule in terms of medicinal chemistry because replacements may result in modifications in specificity or sensitivity; and iii) deriving additional formatives for optimizing the efficacy of the small molecule. Generally, pharmacological procedures have been successful for recognizing various classes of potent inhibitor proteins such as proteases, nuclear hormone receptors, kinases, channels, and G protein-coupled receptors. Several
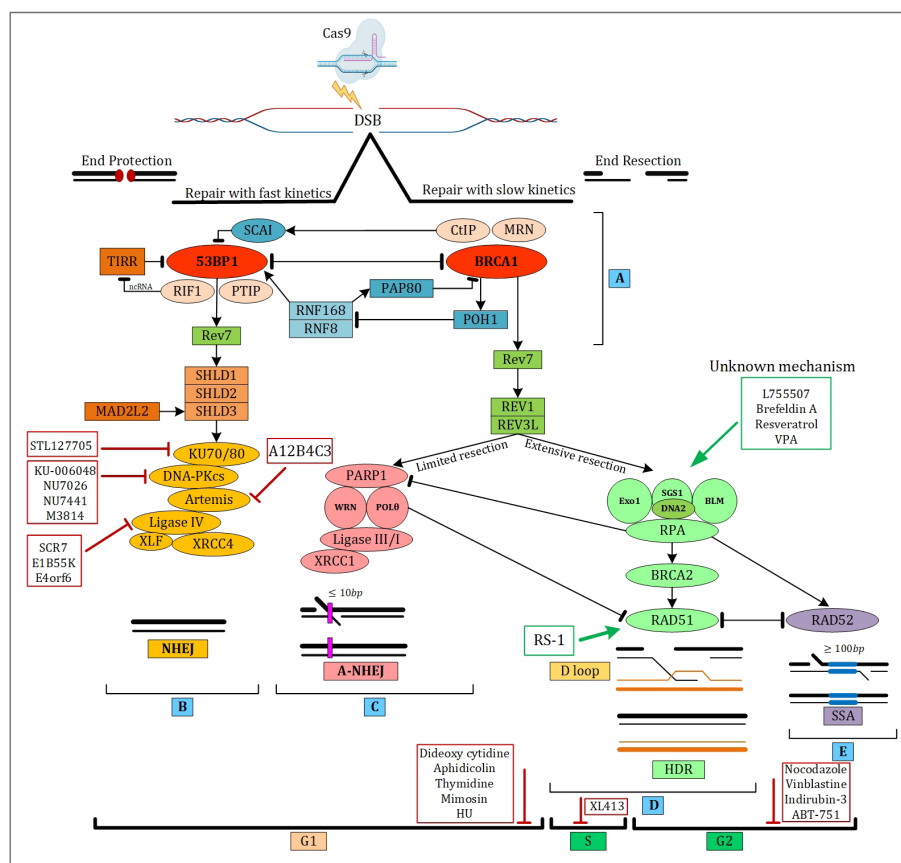
Fig. 2. The interaction of repair pathways at the DNA break site in mammals and comparison of c-NHEJ, a-NHEJ, HDR, and SSA mechanisms. (A) c-NHEJ key factor (53BP1) and HDR key factor (BRCA1) are motivated by complex interactions. (B) For DSB repair the first pathway choice in mammalian cells is NHEJ which is occurred in all cell cycle phases. When in DSB the terminuses are preserved from the incision and then ligated, NHEJ is promoted. (C) The microhomologous sequences which are adjacent to DSB are annealed in alt-NHEJ pathway. (D) HDR repair pathway employs a repair template such as sister chromatid to reliably amend the DSB. (E) SSA is a DSB repair pathway for fixing DSB by annealing lengthy homologous sequences at flanking sites. Figure was created with BiorRender (https://biorender.com).

efforts have been established to chemically scale up the efficiency of HDR pathway.[46] The nuclear domain knock-in screening that is described by multiple studies develops an idea that indicting simple means of quickly appraising small molecules that can elevate the efficiency of HDR is mediated by CRISPR-Cas9 technology.[7,45] For example, Yu et al applied a high-throughput chemical screening test based on the reporter system for exploiting HDR efficiency. It was reported that using the candidate small molecules could increased the efficiency of HDR for large fragments and point mutations 3- and 9-fold, respectively. L755507 and Brefeldin A were two small molecules whose positive effects were elucidated in this high-throughput chemical screening test. Moreover, many small molecules that inhibit HDR and could elevate indel mutations mediated by NHEJ were also reported.[7] Therefore,

research to screen other small molecule libraries has been going on and introduced a strategy that simplifies precise CRISPR-Cas gene editing for clinical applications and biomedical experiments.

Inhibition of key NHEJ factors
Recently numerous small molecules have been validated to enhance the efficiency of HDR, which is mediated by CRISPR-Cas9 in various cells.[46] Several studies have shown that DSBs, introduced by CRISPR-Cas9 system, are mostly repaired by NHEJ. Hence, it seems reasonable, by inhibiting the key enzymes of NHEJ, the HDR efficiency would be increased. A comprehensive list of small molecules with NHEJ inhibitory effects is summarized in Table 1.

Table 1. Small molecules involved in inhibition of NHEJ and stimulation of HDR

Targeted protein	Small molecule	Cell type	Locus/Gene	Dose of substance	Study method	Observed effects	Ref.
				Inhibiting NHEJ			
DNA-PK	Nu7026	iPSC	CALD1 KATNA1 SLITRK1	20 μM	*In vitro*	1.5 2.6 2.5-fold increase in HDR	46
	Nu7026	HEK293	HPRT	20 μM	*In vitro*	3.0-fold increase in HDR	46
	Nu7026	K562	HPRT	20 μM	*In vitro*	4.0-fold increase in HDR	46
	Nu7026	CD4+ T	HPRT	20 μM	*In vitro*	3.0-fold increase in HDR	46
	Nu7026	CD34+ progenitor cells	HPRT	20 μM	*In vitro*	1.7-fold increase in HDR	46
	Nu7026	HEK293	GFP	30 μM	*In vitro*	2.5 -fold increase in HDR	47
	Nu7026	HepG2-1.1merHBV	HBV genotype D	7.5 μM	*In vitro*	Increased A-NHEJ	48
	NU7026	K562	GFP	3 μM	*In vitro*	Modest increase in HDR (1.1-fold)	49
	NU7441	K562	GFP	3 μM	*In vitro*	2.4-fold increase in HDR (8.6% to 21.5%)	49
	NU7441	HSPC	PTPRC	3 μM	*In vitro*	2-fold increase in HDR (12 to 24%)	49
	NU7441	iPSCs	CTNNB1	2 μM	*In vivo*	Modest increase in HDR 1.2(16% vs. 13% in control)	50
	NU7441	HEK293/ TRL	GFP	2 μM	*In vitro*	2-fold increase in HDR	51
	NU7441	MEFs	TP53	2 μM	*In vitro*	10- fold increase in HDR	51
	KU-0060648	HEK293/ TRL	GFP	250 nM	*In vitro*	2.1-fold increase in HDR	51
	M3814	409B2 hiPSC[1]	FRMD7	2 μM	*In vitro*	Increased in HDR (18% to 81%)	52
Ku complex	STL127705	SF-767 cells		2.5 μM	*In vitro*	Not tested	53
Ku complex	STL127705	PrEC cells		2.5 μM	*In vitro*	Not tested	53
ATR[2]	VE-822	hiPSC	OCT4 ALBUMIN	1 μM	*In vitro*	4-fold increase in HDR by CRISPR-Cas9 and CRISPR-Cpf1- mediated targeting 3.5-fold increase in HDR by CRISPR-Cpf1 5-fold increase by CRISPR-Cas-mediated targeting	54
ATM	Trichostatin A	iPSCs	CALD1 KATNA1 SLITRK1	0.01 μM	*In vitro*	1.5 2.2 1.8-fold increase in HDR	46
CRISPY mix	Trichostatin A Nu7026	iPSCs	CALD1 KATNA1 SLITRK1	0.01 μM 20μM	*In vitro*	1.8 2.5 3.1-fold increase in HDR	46
Ligation	SCR-7	HCT-116 cells	AAVS1	10 μM	*In vitro* *In vivo*	1.7-fold increase in β-catenin gene (14.6% vs. 8.4% in control %)	55
	SCR-7	Mouse embryos	lgkc	1000 μM	*In vitro*	4.5- fold increase in HDR (22.7% vs. 5% in control)	8

Table 1. Continued

Targeted protein	Small molecule	Cell type	Locus/Gene	Dose of substance	Study method	Observed effects	Ref.
	SCR-7	Mouse embryos	Kell	1000 µM	*In vitro*	2.2-fold increase in HDR (59.3 % vs. 26.6% in control)	8
	SCR-7	CHO cells	COSMC FUT8	0.1–20 µM + 10 mM LiCl	*In vitro*	None	56
	SCR7	iPSCs	CALD1 KATNA1 SLITRK1	1 µM	*In vitro*	None	46
	SCR-7	Mouse ESCs[3]	Actb	1 µM	*In vitro*	Promoted Tild-CRISPR-mediated knock-in	57
	SCR-7	HEK293T cells	MALAT1	1-10 µM	*In vitro*	Modest fold increase in HDR (13.6% vs. 12.5 in control %)	58
	SCR-7	Murine zygotes	Tex15	50 µM	*In vivo*	9.7-fold increase in HDR (56.2% vs. 5.8% in control)	59
	SCR-7	HEK293A	LMNA	1µM	*In vitro*	Modest increase in HDR (11.7% vs. 9.9% in control)	45
	SCR-7	Porcine fetal fibroblasts	GFP	200,50 µM	*In vitro*	2-fold increase in HDR (11.2% vs. 5.6% in control)	60
	SCR-7	Porcine fetal fibroblasts	ROSA26	100 µM	*In vitro*	1.9-fold increase in HDR with neomycin selection (49.7% vs.26.2% in control)	60
	SCR-7	Zebrafish embryos	Ybx1^{S82A}	20µM	*In vivo*	3.6 -fold increase in HDR (55% vs. 15% in control)	61
	SCR-7	COS-7 cells	PAH	15 µM	*In vitro*	2.5-fold increase in HDR (22.1% vs. 8.8% in control)	62
	SCR-7	HEK293T cells	GFP	1 µM	*In vitro*	1.8- fold increase in HDR	51
CRISPY mix	SCR-7 KU-0060648	HEK293T cells	GFP	1 µM 250 µM	*In vitro*	2.9- fold increase in HDR	51
CRISPY mix	SCR-7 NU7441	HEK293T cells	GFP	1 µM 2 µM	*In vitro*	3- fold increase in HDR	51
Stimulating HDR							
RAD51	RS-1	K562	GFP	3 µM	*In vitro*	2.2-fold increase in HDR (17.6% vs. 8.6% in control)	49
	RS-1	Zebrafish embryos	eBFP2	30 µM	*In vivo*	1.5-fold increase in HDR by Cas9-mediated	63
	RS-1	U2OS	LMNA	10 µM	*In vitro*	Modest increase in HDR (2.5% vs. 1.9 in control %)	45
	RS-1	HEK293 A cells	LMNA	10 µM	*In vitro*	6-fold increase in HDR (21% vs. 3.5 in control %)	45
	RS-1	HEK293 A cells	PML	10 µM	*In vitro*	4- fold increase in HDR(40% vs. 10% in control %)	45
	RS-1	Zebrafish embryos	Ybx1^{S82A}	20 µM	*In vivo*	1.6- fold increase in HDR (24% vs. 15% in control %)	61
	RS-1	iPSCs	CALD1 KATNA1 SLITRK1	1 µM	*In vitro*	None	46
	RS-1	iPSCs	CTNNB1 PRDM14	10 µM	*In vivo*	None	50
	RS-1	Bovine zygotes	XbaI	7.5 µM	*In vitro*	2.1-fold increase in HDR (53% vs. 25% in control %)	64

[1] Human iPS cells; [2] ATM and Rad3-related; [3]Embryonic stem cells.

DNA-PK

The first component of NHEJ for recognizing and binding to DSBs is DNA-PK. This holoenzyme is a compound of a 460 kD catalytic subunit, DNA-PKcs, Ku70, and Ku80 subunits (regulatory heterodimer). It has been revealed that recruiting libraries of these compounds could develop several molecules for inhibiting DNA-PK activity. Since NHEJ repair relies on DNA-PK activity, detrimental genetic mutations or using small molecules with inhibitory effects on DNA-PK could result in an increase in HDR occurrence. Targeting the ATP binding site of DNA-PK by small molecules is the most successful procedure for inhibiting this pivotal factor in NHEJ. For the phosphate transfer reaction by irreversible alkylation, lysine 802 in DNA-PKcs active site could be targeted by Wortmannin which is a small molecule with an inhibitory effect on PI3 kinases. Wortmannin naturally originated from a furanosteroid metabolite from *Penicillium funiculosum*. Although the experimental efficacy of wortmannin has been proven, multiple obstacles such as poor solubility, lack of specificity, and *in vivo* toxicity restrict its clinical application.[65] LY294002 is a competitive inhibitor of DNA-PK and PI3 kinase. It is reversibly able to interact with the kinase domain. This small molecule is originated from the plant flavonoid as a morpholine derivative. LY294002 leads to a delay in DSB repair, which might attribute largely to inhibition of DNA-PK. Because of fast-metabolic clearance and *in vivo* toxicity, it is impossible to clinically evaluate the effects of LY294002. However, LY294002 has been demonstrated to be a productive and leading compound which by acquiring biochemical alterations, a series of recombinant compounds with more appropriate features would be established. A modified form of LY294002 is NU7026 which is more selective for DNA-PK compared to other PI3 kinases such as ATM and ATR that had an IC50 of 13μM for PI3K, 0.23μM for DNA-PK, and >100μM for ATR or ATM. Nonetheless, for obtaining potent and selective DNA-PK inhibitors, the substitution of 2-morpholin-4-yl and alteration of thiopyran-4-ones or pyran-4-ones is proposed.[66] NU7441 is another agent that originates from LY294002 with remarkably improved potency compared to NU7026. It strongly inhibits DNA-PK with an IC50 of 0.3 μM in cell lines. Tavecchio et al reported that in the presence of ionizing radiation (IR) and NU7026, the induced DSBs continued for a long time and the activeness of HR enhanced moderately.[67] Development of NU7441 continues and leads to the identification of KU-0060648, which has greater solubility against DNA-PK and is a binary inhibitor of PI-3K and DNA-PK *in vitro*. Munck et al showed that the inhibitory effect of KU-0060648 on DNA-PK in MCF7 is approximately 8-fold higher than in SW620 cells.[68] Other non-toxic compounds based on the LY294002 structure, such as IC86621, IC87102, and IC87361 have expanded the application of compounds derived from this small molecul.[66] NU7026, NU7441, and KU-0060648 provide high efficiency for improving the HDR rate in genome editing compared to other DNA-PK inhibitors. Previously it was demonstrated that the efficiency of knock-in is augmented by NU7026 in hiPSCs. Likewise, this small molecule is a crucial complex in the NHEJ pathway. Riesenberg et al reported that NU7026 was the only small molecule that clearly increased the efficiency of targeted gene fragment insertion in HEK293 (by 3-fold), K562 cells (by 4-fold), CD4+ T cell (by 3-fold), and CD34+ progenitor cells (by 1.7-fold).[46] Besides, Robert et al described that treating HEK293 TLR with NU7441 and KU-0060648 leads to, respectively, a 3- and 4-fold increase in HDR efficiency, and an approximately 2-fold reduction in the NHEJ repair. They also demonstrated that oligonucleotide-mediated HDR, as a repair template, at the endogenous site could be stimulated by both NU7441 and KU-0060648. Therefore the results of using additional DNA-PK inhibitors lead to compatibility with the Cas9 editing system.[51] Moreover, combining proteins or siRNA along with small molecules could be more effective. They showed that Adenovirus 4 (Ad4), E1B55K and E4orf6 proteins with KU-0060648 or NU7441 could induce HDR approximately up to 5-fold in HEK293T cells.[51] Aksoy et al reported the initial application of NU7441 as a powerful HDR enhancer with an increase in HDR efficiency up to 13.4-fold in zebrafish embryos genome-edited by CRISPR-Cas9 system.[63] Recently, M3814 was introduced as a new selective pharmacological inhibitor of DNA-PK which has not been applied in genome editing before. M3814 is a highly potent molecule that showed acceptable activity in preclinical models. This molecule was also introduced as a practical therapeutic strategy in cancer treatment.[69] For instance, in combination with cisplatin and etoposide, M3814 has represented promising activity in lung cancer *in vivo* models.[70] On the other hand, Sun et al showed that the repair of radiation-induced DSBs could fruitfully be shut off by M3814. Furthermore, this small molecule can efficiently increase activation and phosphorylation of p53.[71] Additionally, M3814 was used in phase Ib/II clinical trials for the treatment of several cancers such as rectal cancer (NCT03770689) and small cell lung cancer (NCT03116971). Riesenberg et al used M3814 in genome editing for the first time. It was used to transiently inhibit NHEJ and increase HDR from 18% to 81% in K562 cells while demonstrating moderate toxicity.[52] In line with these premises, M3814 could be applied in gene therapy, where high HDR performances may be required to achieve therapeutic goals. VE-822, a specific inhibitor of ATR, that was recently used by Ma et al enhances the efficiency of HDR efficiency in combination with a plasmid donor and a ssODN donor by 5.9-fold and 3-fold, respectively. Furthermore, combining AZD-7762, a specific inhibitor of checkpoint kinase CHEK1 and the ATR inhibitor could remarkably boost the specificity of CRISPR-Cas9 genome editing.[54]

Ku70/80 complex

The heterodimeric Ku complex is the most logical choice for inhibition of the entire NHEJ process. This protein shows a great binding affinity for dsDNA termini and has a ring-like shape which upon binding DNA, cannot tolerate any substantial structural variation. This ring-like structure leads to the interaction of Ku protein with DNA that is crucial for the activation of kinase and in this regard, it is also an appropriate target for intervention.[65] Although in the initiation of the NHEJ pathway, Ku has a key role, limited studies have addressed this protein and only one inhibitor of Ku protein was obtained (STL127705).[44] For the first time, Weterings et al in the low micro-molar range, confirmed the compound with Ku-inhibitory activity. However, the ability of this molecule to block NHEJ is not well documented.[53] Recently, Gavande et al represented novel small molecule inhibitors that bind to the Ku–DNA protein to block the protein–DNA interaction. These specific Ku–DNA binding inhibitors (Ku-DBi's) block Ku-DNA interaction, the activity of DNA-PK kinase, and in vitro NHEJ by directly binding to Ku protein. Moreover, Ku-DBi's increase the cellular activity of radiomimetic agents and IR.[72]

DNA-end processing enzymes

The DNA-end processing step in NHEJ is an attractive issue for studying and various nucleic acid enzymes involved in this step are recognized. However, no remarkable effort has been made to validate these enzymes as potential targets. Because of the hardness in purifying the proteins and the requirements for complex assay, no inhibiting agent has been identified for Artemis as DNA-PK dependent endonuclease. Polynucleotide kinase/phosphatase (PNKP) can bind to DNA 5'-end and dephosphorylate DNA 3'-end in the NHEJ pathway. Furthermore, PNKP is essential for both single- and DSB repairs. Therefore identifying small molecule inhibitors for this enzyme seems very useful to regulate NHEJ.[65] Five compounds with remarkable inhibitory effect on PNKP phosphatase activity was recognized by Freschauf et al.[73] A12B4C3 is one of these compounds with IC50 value of 0.06 Mmol/L, showing the highest noncompetitive inhibitory effect on phosphatase activity of PNKP by obstructing its secondary structure.[73] There is no more information about the effect of this inhibitor on NHEJ pathway. Therefore, identifying and introducing additional small molecules for inhibition of DNA-end processing enzyme is required.

Ligation process enzymes

The ligation process of NHEJ particularly the DNA ligase enzyme is an attractive target for regulating NHEJ. L189 was the first recognized compound by Chen et al for inhibiting the DNA ligase.[74] Although this molecule had a poor specificity, it represented a promising inhibitory effect on Ligase I, III, and IV.[74] One of the L189 derivatives, SCR7, was synthesized as a more specific and putative

inhibitor of NHEJ. This compound blocks end-joining through intervening with the connection of Ligase IV to DNA, consequently, the accumulation of DSBs within the cells and increasing cytotoxicity were accrued.[75] Most investigations showed a considerable dose-dependent decline of NHEJ in various models, both in vitro and in vivo. Some groups demonstrated that a modest increasing efficiency of HDR-mediated gene insertion could be achieved by combining SCR7 with enhancing genome engineering techniques such as synchronization or optimized Cas9 delivery.[76,77] The SCR7 effects in CRISPR-Cas9 experiments and subsequently the increased HDR rate was demonstrated in various cells such as HEK293T (by 2-fold),[51] A549 (by 3-fold), MelJuSo (by 19-fold),[8] and HEK293 (by 5-fold).[77] However, complete inefficiencies of Scr7 or slightly increasing the HDR rate were reported in cells such as human embryonic stem cells,[78] iPSCs,[50] Porcine fetal fibroblasts,[79] rabbit embryos,[80] HEK293A,[45] and CHO cells.[56] Srivastava et al demonstrated that the reason for these inconsistency effects of SCR7 is related to different expressions of LigIV in various cell cultures, and the cells with high expression level of LigIV are more sensitive to SCR7.[40, 75] Contrary to the results for SCR7 reported by Raghavan et al, others discovered several differences in the original structure of SCR7.[75, 81] Greco et al performed a broad investigation on structural determination of SCR7 and confirmed the structure of SCR7-R and its closely related derivative, SCR7-G, that is generated by the synthesis protocol described by Raghavan (Fig. 3).[81] They found that commercially available SCR7 (SCR7-X) structurally is similar to SCR7-G. Indeed, both SCR7-G and SCR7-R have weak inhibitory effects on LigIV while providing stronger activity with regard to LigI and LigIII/XRCC1. Hence, they suggested that the effect of increasing HDR by SCR7, particularly at a low concentration (about <200 uM) is occurred by other mechanisms.[81] Despite these differences, it is confirmed that at the defined concentration of SCR7-X/SCR7-G (1 µM), the efficiency of CRISPR-Cas9 gene editing could be enhanced.[75,81] By focusing on embryos, it was observed that SCR7 increases HDR efficiency in the mouse embryos,[8,82] but not in the rabbit embryos.[80] Likewise, Singh et al reported the increased rate of HDR (by 10-fold) in murine zygotes[82] and Maruyama et al also reported correcting homozygous editing in addition to increased HDR efficiency.[8] In order to improve the efficiency of SCR7, combining this small molecule with other NHEJ inhibitors is a logical suggestion. Chu et al showed that using Scr7 simultaneously with XRCC6 and LIG4 knock-out models resulted in 5-fold increase in HDR efficiency,[77] while others have not observed such increasing effects.[83] Moreover, it was determined that a combination of SCR7 with KU-0060648 or NU7441 in HEK293 cell (CRISPY mix) could stimulate HDR efficiency by 3-fold.[51] In general, inhibiting the key factors of NHEJ causes the unrepaired DSB accumulation in the cell, as a result,
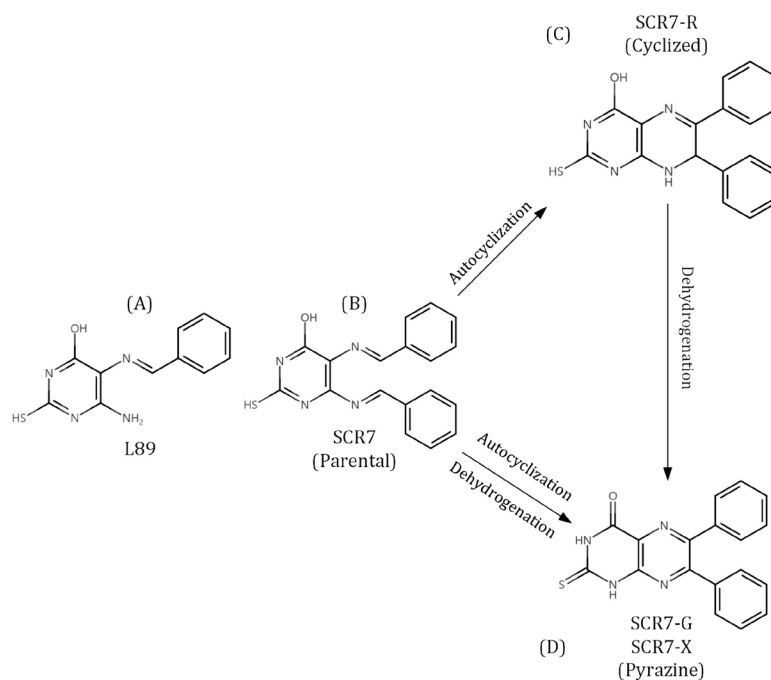
Fig. 3. Different structures of the Ligase IV inhibitor, SCR7. (A) L189 as a human DNA ligase inhibitor. (B) Less stable parental SCR7 reported by Srivastava et al can get autocyclized into a more stable form (SCR7-R). (C) Having the same molecular weight, number of protons, molecular mass, and melting point. Pending dehydrogenation, SCR7-R gets converted into SCR7-G (D), the compound sold as SCR7 by XcessBio (SCR7-X), possessing a different molecular weight, number of protons, molecular mass, and melting point.

apoptosis and cell death would be increased. In this line, this approach could increase cytotoxicity and should be applied with precaution.

Supporting HDR key factors

HDR is a substantial pathway for certifying the precise repair of nicks and DSBs. Therefore, another way for changing DNA repair from NHEJ to HDR is instigation of HDR key components.[84] A comprehensive list of small molecules, which are involved in stimulation of HDR, is represented in Table 1.

Rad51, a factor for exchanging DNA strands, is the central and target point for HDR, and by the accumulation of other proteins on ssDNA, it forms presynaptic filaments.[85] Indeed, it has an extensive role in the formation of a RAD51–ssDNA filament by replacing RPA, and interactingwith ssDNA. In order to enhance RAD51 binding to DNA, Jayathilaka et al developed a high-throughput microplate-based assay for evaluation of the filament formation of hRAD51 on ssDNA.[86] By applying this approach, a library with 10,000 compounds was screened that resulted in the recognition of a small molecule called RAD51-stimulatory compound 1 (RS-1).[87] In different biochemical conditions, RAD51 binding could be enhanced by RS-1. After the ultrastructural assay of developed filaments on ssDNA, it was determined that RS-1 could increase protein–DNA complex lengths and the pitch of helical filament turns. Moreover, the experiment of RS-1 by salt titration demonstrated increasing filament stability. According to experiments,

RS-1 depending on the condition can stimulate the activity of RAD51-mediated homologous strand (D-loop) at least 5- to 11-fold.[86] Actually, the organization of active presynaptic filaments is boosted by RS-1 that can increase the homologous recombination activity of RAD51 by about 2-fold.[86] Therefore, Jayathilaka et al showed that RS-1 could motivate the performance of homologous recombination repair; thereby, it is functional in both medical and research settings.[86] In research, RS-1 in HEK293A could increase the HDR rate up to 6-fold and 4-fold by using Cas9 and Cas9 nickase, respectively.[88] Song et al reported impressive results in both *in vivo* and *in vitro*. They indicated that RS-1 enhanced the HDR rate by 6-fold for the ROSA26-like locus (RLL) *in vitro* and by 2.4-fold for the CFTR gene in rabbit embryos.[80] Combinatorial treatment using RS-1 and NU7441 in zebrafish embryos[63] or applying both SCR7 and RS-1 with/without L755507 in HEK293 cells did not show a significant increase in HDR efficiency compared to RS1 alone.[45] Similar results were also observed when L755507, SCR7, and RS-1 were used together in stem cells.[46] However, in one report it was revealed that the combination of SCR7 and RS1 increased HDR efficiency up to 74% in zebrafish embryos.[61] Unfortunately, contradictory outcomes have been achieved for both small drugs, SCR7 and RS-1, in various cell types. In this regard, the highest efficiency of RS1 was observed in bovine zygotes.[64] In addition to RS-1, MLN4924 is another small molecule for stimulating HDR. It is a Nedd8 activating enzyme (NAE) inhibitor that can block the neddylation of CtIP and result in a rise in the

extent of DNA end cutting at strand breaks. MLN4924 through inhibition of neddylation artificially increases the CtIP expression level which in turn promotes HDR efficiency.[46] Although the effect of some small molecules was unknown or was observed in different stages of DNA repair (multiple effects), they could provide increasing HDR efficiency. A comprehensive list of small molecules with multiple or unknown effects is summarized in Table 2.

Synchronizing cell-cycle

HDR and NHEJ are dominant repair pathways at different phases of cell-cycle. Synchronization of cells is reversible and provides to be widely used while it does not affect the pluripotency of stem cells. One approach for increasing HDR efficiency in CRISPR-Cas9 genome-edited cells is using small molecules for cell-cycle synchronization. In this line, various small molecules have been introduced for arresting cell cycle at different phases. For example, Lovastatin hampers cell cycle at early G1 and partly at G2/M phase; L-mimosine, Hydroxyurea (HU), Aphidicolin, Dideoxycytidine (ddC), and Thymidine could apprehend cells at G1-S frontier before starting the DNA replication; and antineoplastic microtubule polymerization inhibitor (Nocodazole), Indirubin-3-monoxime, Vinblastine, and ABT block cell cycle at G2/M phase. Since HDR is restricted to the S and G2 phases, synchronizing the cell cycle in these two stages might be beneficial for increasing the HDR rate. Some small molecules arrest cells at the G1-S border before the onset of DNA replication by inhibiting the effective enzyme of A-NHEJ pathways. Some molecules arrest cells at the G2/M phase, thereby providing modulation of HDR-mediated Cas9 genome-editing through cell cycle synchronization.

Arrest at the G1-S border

Aphidicolin is a reversible chemical inhibitor that inhibits the DNA polymerase-α, δ, and blocks cell cycle in S phase.[89] Several reports have estimated that Aphidicolin increases HDR rate in HCT116 cells, embryonic stem cells (ESCs), and primary neonatal fibroblasts by 3-fold, 1.6-fold, and 1.3-fold, respectively.[90,91] Furthermore, the frequency of HDR was enhanced with thymidine treatment in H9 human embryonic stem cells (hES) and human primary neonatal fibroblasts (neoFB).[91] It is determined that HU arrests cell cycle in S-phase by inhibiting ribonucleotide reductase enzyme. Tsakraklides et al reported that cells, by using HU before transformation process in five yeast strains, were synchronized in S-phase and the efficiency of gene targeting was increased.[92] 2′,3′-dideoxycytidine is another promising small molecule that slows down the replication fork movement and results in S-phase extension. Brachman et al reported that this small molecule develops the S phase up to 70% and increases HDR rate by 3-fold in DLD-1 cells.[93] Recently, XL413, a new small molecule targeting cell division cycle 7(CDC7) plays an important

role during initiation of DNA replication, arrests primary T cells in S phase, and enhances the HDR efficiency up to 3.5-fold.[94] According to these observations, designing experiments for identifying the most optimal small molecules is necessary because different small molecules that intervene in cell synchronization have varied effects on various cells.

Arrest at G2/M

G2/M phase is another crucial hot spot in cell cycle for being regulated by small molecules to increase HDR efficiency. Lin et al reported that Nocodazole is able to arrest cell cycle at specific phases and increase HDR rate with CRISPR-Cas9 by 1.4-6-fold in HEK293T cells.[91] By applying Nocodazole or ABT-751, Yang et al could successfully enhance HDR-mediated knock-ins by 3- to 6-fold.[78] The treatment with Nocodazole reverses the synchronization of cell cycle in different cell models such as iPSCs and human pluripotent stem cells (hPSCs),[50,78,91] albeit not observed in ESCs and primary neonatal fibroblasts.[91] Combinatorial treatment with CCND1, a cell cycle regulatory subunit in G1/S transition, and Nocodazole increased the HDR efficiency up to 30% in iPSCs.[50] Vinblastine is another useful small molecule for cell cycle synchronization in the G2/M phases through binding to tubulins and blocking microtubule dynamics.[95] It is elucidated that Vinblastine increases HDR rate by 6- to 10-fold in HeLa, U-2OS, and HT-1080 cell lines. Likewise, Indirubins by inhibition of several cyclin-dependent kinases, leads to cell-cycle prevention in G1/S or G2/M in different cell models.[96] Rahman et al reported that by utilizing meganuclease I-SceI and ZFNs together with indirubin-3′-monoxime, the HDR rate increased by 2- to 5-fold in Hela, U-2OS, and HT-1080 cell lines. Incredibly, indirubin-3′-monoxime results in an enhancement in HDR by 10-fold in primary umbilical cord–derived mesenchymal stromal cells (UC-MSCs). Finally, it was concluded that indirubin-3′-monoxime is a promising small molecule for enhancing adeno-associated virus/ ZFN-mediated gene editing especially in UC-MSCs for clinical approaches.[97]

The effective strategy of modified CRISPR-Cas Systems for boosting HDR activity

HDR pathway leads to the precise insertion of the donor template at the DSB site in the presence of a homologous donor strand. Induction of the HDR pathway for repairing DSBs which is introduced by CRISPR-Cas9 is a suitable approach for increasing the accuracy of knocking in approaches. This procedure provides plenty of technical capabilities for researchers and industries such as developing insertions or deletions models, inserting sequences for epitope tags, generating SNPs, and inserting whole genes into target site to produce modified organisms. Although the HDR pathway along with CRISPR-Cas9 is an easy way to introduce a site-specific rectification, HDR

Table 2. Small molecules with multiple or unknown effects

Targeted point	Small molecule	Cell type	Locus/ Gene	Dose of substance	Study method	Observed effects	Ref.
β3-adrenergic receptor agonist	L755507	K562	GFP	3 μM	In vitro	1.5-fold increase in HDR	49
	L755507	HeLa	ACTA2	5 μM	In vitro	1.5-fold increase in HDR (5.1% vs. 3.5% in control)	7
	L755507	hiPSCs	SOD1	5 μM	In vitro	8.9-fold increase in HDR (3.13% vs. 0.35% in control)	7
	L755507	Mouse ESCs	NANOG	5 μM	In vitro	1.9-fold fold increase in HDR (33.3% vs. 17.7% in control)	7
	L755507	HUVEC	ACTA2	5 μM	In vitro	2-fold increase in HDR (1.8% vs. 0.9% in control)	7
	L755507	K562	ACTA2	5 μM	In vitro	1.6-fold increase in HDR (1.3% vs. 0.8% in control)	7
	L755507	Fibroblast CRL-2097	ACTA2	5 μM	In vitro	1.7-fold increase in HDR (6.0% vs. 3.5% in control)	7
	L755507	Human ES cell-derived (neural stem cells)	ACTA2	5 μM	In vitro	1.3-fold increase in HDR (1.0% vs. 0.8% in control)	7
	L755507	iPSCs	CTNNB1 PRDM14	5 μM	In vivo	None	50
	L755507	Porcine fetal fibroblasts	GFP	40μM	In vitro	1.9- fold increase in HDR (10.9% vs. 5.6% in control)	60
	L755507	Porcine fetal fibroblasts	ROSA26	40μM	In vitro	2- fold increase in HDR with neomycin selection (51.6% vs. 26.2% in control)	60
	L755507	Porcine fetal fibroblasts	GFP	20μM	In vitro	1.6 fold increase in HDR (9% vs. 5.6% in control)	60
	L755507	HEK293A	LMNA	5 μM	In vitro	Modest increase in HDR (10.1% vs. 9.9% in control)	45
	L755507 +RS-1	HEK293A	LMNA	5 μM + 10μM	In vitro	1.7 fold increase in HDR (17.1% vs. 9.9% in control)	45
	L755507 +RS-1+SCR-7	HEK293A	LMNA	5 μM + 10μM+1μM	In vitro	1.4 fold increase in HDR (14% vs. 9.9% in control)	45
Inhibition of intracellular transport from ER to Golgi	Brefeldin A	Mouse ES cells	Nanog	0.1 μM	In vitro	2-fold increase in HDR (27.2% vs. 17.7% in control)	7
	Brefeldin A	iPSCs	CTNNB1	0.1 μM	In vivo	1.3-fold increase in HDR (17% vs. 13% in control)	50
	Brefeldin A	iPSCs	PRDM14	0.1 μM	In vivo	Modest increase in HDR (8.5% vs. 7% in control)	50
Broad range of biological activities	Resveratrol	Porcine fetal fibroblasts	GFP	50μM	In vitro	2.7-fold increase in HDR (15% vs. 5.6% in control)	60
	Resveratrol	Porcine fetal fibroblasts	GFP	200μM	In vitro	2.3-fold increase in HDR (13% vs. 5.6% in control)	60
	Resveratrol	Porcine fetal fibroblasts	ROSA26	50μM	In vitro	1.8-fold increase in HDR with neomycin selection (46% vs. 26.2% in control)	60
	Resveratrol	iPSCs	CALD1 KATNA1 SLITRK1	1 μM	In vitro	None	46

activity is very low that has a negative and direct effect on precision genome engineering functions.[98,99] Enhancing the efficiency of HDR provides fast, easy, and accurate technologies based on CRISPR-Cas9.[44,100] In this regard, engineering strategies are required for inducing the HDR pathway for accurate gene modification.

HDR-fusion Cas9

According to previous findings, each of NHEJ and HDR pathways is dominated in different cell-cycle phases separately.[40,101] In this line, precisely activating Cas9 in S and G2 phases results in an increased HDR rate. The main studied fusion motifs include CtIP, Rad52, DN1S, mSA, and Geminin. These engineered variants usually enhance knock-in occurrence in both *in vivo* and *in vitro* (Fig. 4). It is reported that by fusing a N-terminal fragment of CtIP, which is a key protein in HDR initiation, to Cas9 protein, the CtIP was forced to DSB site that increasing the efficiency of HDR by 2-folds. Moreover, when comparing the patterns of indels introduced by wild-type Cas9 and engineered Cas9, it was speculated that existence of different patterns could originate from a different balance of DSB repair pathways.[102] According to this observation, recently Tran et al designed an experiment to establish Cas9 fusion with other factors which are involved in HDR pathway. They reported that similar to CtIP, fusing Cas9 to Mre11 and Rad52 but not Rad51C, could scale up the efficiency of HDR by 2-fold. Moreover, it was indicated that fusing CtIP to gRNA through MS2 binding loops resembling Cas9-CtIP variant is able to extend the ratio of HDR/NHEJ approximately by 6-folds (Fig. 4A). Remarkably, when simultaneously using Cas9-CtIP with MS2-CtIP, the ratio of HDR/NHEJ boosted up to 14.9-fold.[103] However, there are several limitations in using these chimeric proteins broadly. For example, Mre11 and CtIP have complex post-translational regulation and the prediction of protein interactions is confusing. This phenomenon could not be profitable because it originates from endogenous cellular components.[104] 53BP1 plays a key role in the initiation of NHEJ. In this line, by inhibiting this protein the efficiency of HDR would be increased (Fig. 4B). It is indicated that fusing Cas9 to a truncated piece of p53 named DN1S, a dominant-negative mutant of 53BP1, could significantly reduce NHEJ and increase the efficiency of HDR up to 86% especially at DSB sites introduced by CRISPR-Cas9 system in different human cell lines.[105] A challenging limitation for using chimeric Cas9 proteins for increasing HDR efficiency is their cellular toxicity. It is indicated that Cas9-DNS1, in reality, increases cellular toxicity by about 10%. Finally, it should be considered that these chimeric proteins would not be effective for increasing HDR ratio because none of these engineered proteins are involved in regulating long range 5' to 3' end resection.[106] To address these limitations, Hackley et al fused Cas9 to human Exo1 and reported that this chimeric protein compared to wild-type, Cas9 is able to increase the rate of HDR by 2.5-fold and reduce cellular toxicity about 4-fold (Fig. 4C).[107] Furthermore, miCas9 is the recently developed small-size HDR-fusion variant that increases HDR capacity. Fusion motif, Brex27 of the engineered variant interacts with RAD51 and enhances the chance of RAD51 presence at the target locus. MiCas9 increases the knock-in rates of large size genes which are mediated by dsDNA, consistently lessens insertion occurrences and off-target deletion, and keeps or enhances single-stranded oligodeoxynucleotide (ssODN)-mediated precise gene editing rates (Fig. 4D).[108] Anti-CRISPR proteins, which are naturally derived from bacteriophages, are promising inhibitory proteins for controlling the activity of CRISPR-Cas systems (Fig. 4E). Recently, Matsumoto et al observed that during phase S and phase G2 the fused human Cdt1 is degraded where HDR is dominant. When AcrIIA4, the natural anti-CRISPR protein, was fused to the N-terminus region of Cdt1, engineered CRISPR-Cas system was activated at the intended phases, S/G2, and DNA repair was boosted through HDR.[109]

Modified sgRNA, Cas9 and/or donor template

For simplifying CRISPR approaches, enhancing the efficiency of HDR seems laborious. Another helpful application is employing the assembly of ssDNA template, sgRNA, and Cas9 along with donor DNA at the targeted site to a homologous sequence. These strategies are implemented to increase the ratio of HDR through bringing donor template close to the introduced DSB site by CRISPR-Cas9 system. Several reports have corroborated the simplicity of increasing the HDR efficiency by advancing the assembly of these components individually or in a complex.

In agrobacteria, Vir proteins are able to transfer T-DNA construct into plant cells. VirD2 relaxase is a component of Vir proteins that covalently binds to the 5'-site of single-stranded T-DNA and by using its nuclear localization signal, directs attached T-DNA into nucleus and integrates it in the plant genome. When VirD2 was fused to Cas9, this chimeric protein was able to recruit repair template in close proximity to the introduced DSB by Cas9 and increased HDR rate up to 6-fold more than repair with Cas9 alone (Fig. 5A). This finding opens up novel promising opportunities for precise plant genome editing.[110] Several attempts have been taken to recruit donor oligonucleotides to introduce DSB by Cas9. It is indicated that donor template could be fused into gRNA and by using this strategy HDR efficiency was increased three times.[56] Several studies also used the advantage of biotin-streptavidin interaction for directing donor template close proximity to Cas9- mediated DSB.[111,112] Moreover, the high-affinity biotin-streptavidin interaction was also used for increasing HDR rate at DSB induced by CRISPR-Cas9 ribonucleoproteins (RNPs). RNPs have shorter half-life compared to their plasmid counterparts, so by using these components the off-target effects would
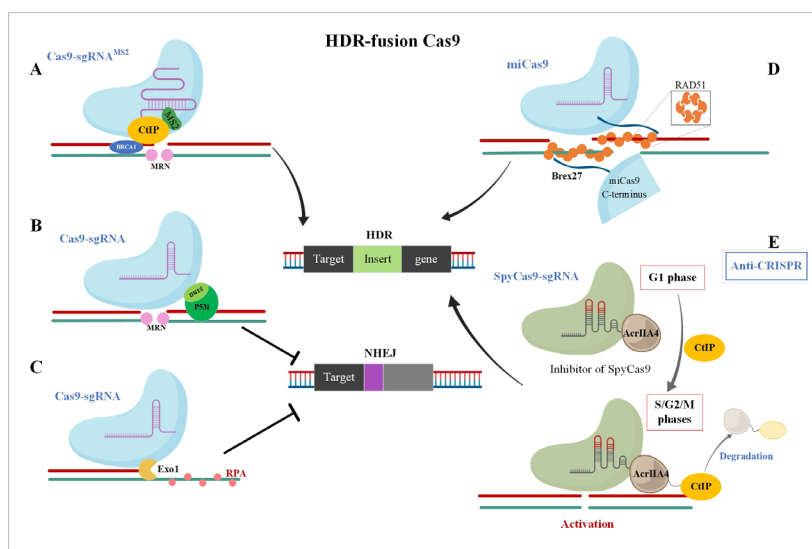
Fig. 4 Accumulation of fusion Cas9 to improve the efficiency of the HDR pathway. Generally, knock-in events are less frequent than template-free indels. (A) Accumulating CtIP via MS2 and association with special sgRNA loops of Cas9 increased knock-in ratio significantly. (B) Fusing Cas9 to DN1S, a truncated piece of p53 reduces NHEJ and increases the efficiency of HDR. (C) Fusing Cas9 to human Exo1 by inhibiting NHEJ increases the rate of HDR. (D) To improve the HDR capacity of Cas9, the miCas9 was created by adding thirty-six amino acids to spCas9. Adding Brex27 to miCas9 results in enrichment of edited region. (E) Activating specific Cas9 system by Anti-CRISPR AcrIIA4 by binding to Cas9-sgRNA acts as an inhibitor for SpyCas9 in G1 phase. In S/G2/M phases, degradation of Cdt1 (S phase degradation domain) leads to AcrIIA4 degradation and consequently, activates the SpyCas9-sgRNA complex. Figure was created with BiorRender (https://biorender.com).

be reduced significantly. Recently, it is elucidated that by combining two cells homologous recombination CRISPR genome editing with a modified biotin–streptavidin approach, in a site-specific manner, the knock-in efficiency of any large fragment could increase up to 95% compared to standard methods in mouse embryos. Although the function of sgRNA may be interrupted by linking an aptamer, engineered Cas9 could be recruited to the biotinylated DNA template. Ma et al fused avidin to Cas9 in mouse embryos for enhancing the efficiency of accurate knock-in from 0 to 15-22%. On the other hand, it was indicated that when wild-type Cas9 is used all mutations contained indels, generated through NHEJ (Fig. 5B).[111] Furthermore, Gu et al reported the use of mSA fused to Cas9 for localizing donor DNAs to target sites. Fusing mSA, because of its monomer structure, to Cas9 demonstrates a biotin-binding domain to recruit repair templates without accumulating the nuclease (Fig. 5C). By incorporating reporter genes at 20 different loci in various cell types, this design was evaluated.[113] Several studies were developed that consistent with other observations have revealed the crucial role of donor localization to increase HDR pathway.[114-116]

Another strategy for directing donor template to close proximity of Cas9-mediated DSB is to bind donor template to Cas9 through covalent linkage. In this line, covalent tethering was reported between RNP complex form of CRISPR-Cas9 and ssODN via a fusion of porcine circovirus 2 (PCV) target sequence with HUH endonuclease. The need to alter donor ssDNA or the sgRNA is resolved via HUH-tagged recombinant protein (Fig. 5D). It is estimated that this strategy augmented

HDR efficiency up to 30-fold.[116] In addition, Savic et al reported that donor template can physically link to Cas9 enzyme through a SNAP-tag (Fig. 5E). They indicated that O6-benzylguanine (BG)-labeled DNA oligos are able to covalently bind to Cas9-SNAP chimeric proteins and the efficiency of HDR increased up to 24-fold.[115] There are several limitations for these introduced methods, such as i) there are technical and commercial difficulties for obtaining terminally modified long ssOND; and ii) the expression level of Cas9 or its delivery efficiency might be changed by fusing a functional domain or linker and also maybe a susceptible chimeric protein to protease cleavage.

In order to overcome these limitations, recently a novel innovative strategy known as genetic code–expansion technology was developed. This technology is established by using chemically modified Cas9 mutant including an azide-containing noncanonical amino acid which is able to recruit modified or unmodified repair template to the DSB site (Fig. 5F). Such modifications enable conjugation of alkyne-azide cycloaddition by dibenzylcyclooctyne (DBCO)-DNA adaptor or dibenzylcyclooctyne modified donor ssODN, which in turn facilitates recruitment of the repair template to the cleavage complex for the HDR pathway. A universal platform is developed by these Cas9 conjugates for using unmodified ssODNs which are commercially available at high purity and a low cost to the RNP complex by base pairing. This strategy increased the HDR efficiency up to 10-fold in both mouse zygotes and human cell culture.[117]

As mentioned about the advantage of biotin-streptavidin interaction, this approach was only successful for knocking-in large fragments into a limited number of
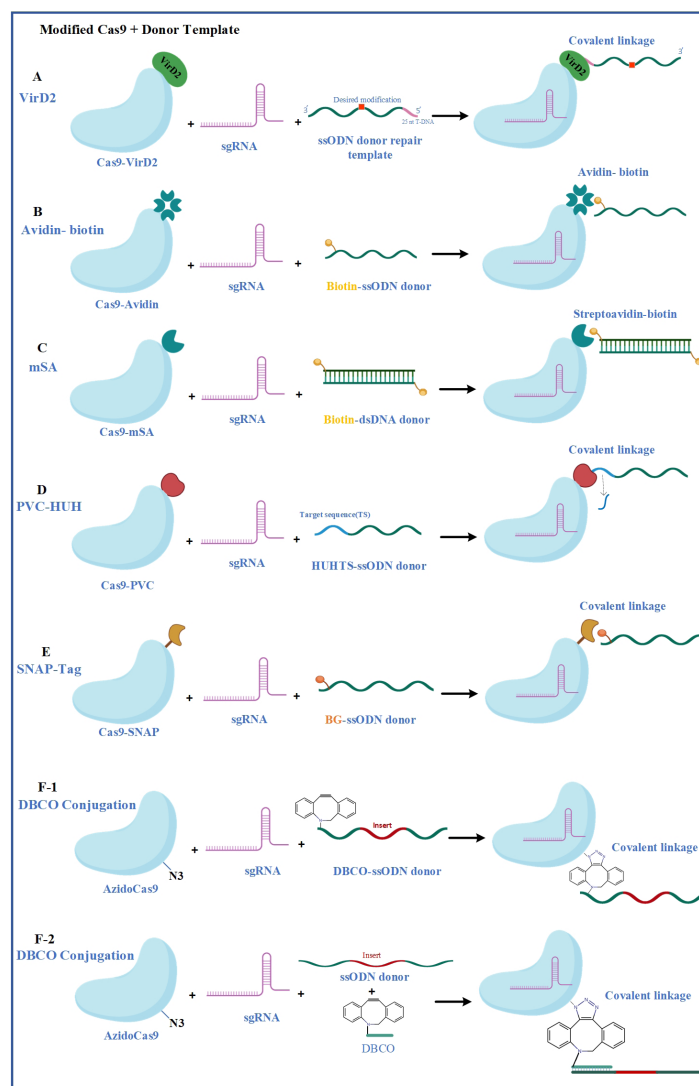
Fig. 5 Variation methods for tethering the ssODN donor template to modified Cas9 for enhancing gene editing efficiency. Modified Cas9 along with sgRNA were joined to ssODN donor template to enhance the rate of precise repair via the targeted HDR pathway (A-F). Figure was created with BiorRender (https://biorender.com).

targeted loci. Two-cell stage has an exceptional long G2 phase in embryos. In addition, the chromatin state in this extended phase is open and provides a golden opportunity for editing enzymes and repair template to easily reach targeted sites. Recently, S1mplex, a modular RNA aptamer-streptavidin strategy (Fig. 6), was developed for delivering RNP form of purified CRISPR-Cas9 system together with an engineered sgRNA containing a streptavidin-binding aptamer into target cell for *in vitro* or *ex vivo* genome editing. It is reported that this system increased precise editing up to 18-fold and augmented pool cells including multiplexed precise edits by 42-fold.[112]

Furthermore, new genetic information can help to increase HDR efficiency by utilizing timed delivery of CRISPR-RNP complexes along with various drugs which are arresting the cell cycle.[118] In addition, recently inhibiting main factors in NHEJ and stimulating proteins participating in HDR by using small molecules are highly regarded.[40]

Small molecules impact on improving CRISPR-Cas-mediated gene or transcript editing

Over the past decade, several studies have been done on the effect of small molecules on improving the genome editing systems. Pruett-Miller et al used small molecules to enhance the rate of gene targeting by ZFN and reduced its toxicity. Indeed, they could increase transiently expression level of the modified ZFNs, by adding a manipulated destabilizing FKBP12 domain to the N-terminus, fusing a ubiquitin moiety to the N-terminus, linking a modified destabilizing FKBP12 domain to the N-terminus, adding MG132 proteasome inhibitor and Shield1 synthetic ligand.[119] Intuition of the CRISPR-cas9 system has revolutionized genomic editing approaches. Using small molecules for regulating Cas9 activity to improve the efficiency of this system have been recently investigated in both direct and indirect approaches. In the direct approach, Davis et al generated a Cas9 endonuclease with impaired cleavage activity by incorporating a 4- hydroxytamoxifen
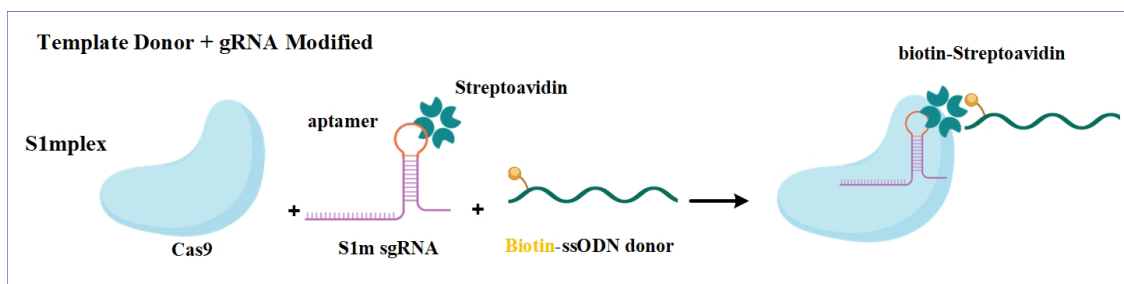
Fig. 6 Engineered sgRNA which is harboring a streptavidin aptamer that contains streptavidin-binding aptamer was joined to a biotinylated ssODNA donor providing an efficient method of recruiting biotinylated DNA. Figure was created with BioRender (https://biorender.com).

(4-HT)- responsive intein sequence at specific positions. In the presence of 4-HT, this small molecule binds to intein, enforces conformational changes, provokes protein splicing, and restores the DNA cleavage activity of inactive Cas9. This approach showed that conditionally activated Cas9 corrects target genomic sites with higher specificity (up to 25-fold) than wild-type Cas9 in human cells.[120] The indirect approach has relied on the regulation of Cas9 activity by using small molecules that target endogenous cellular processes.[91] By analyzing 4000 small molecules with known function, Brefeldin A, a small molecule inhibiting protein transportation between endoplasmic reticulum to Golgi apparatus and L755507, an β3-adrenergic receptor agonist, increased targeted reporter gene integration by 2-fold and 3-fold, respectively.[7] It is indicated that SCR7 is able to enhance the proficiency of the genome editing which is performed by CRISPR-Cas9 technology.[77] Maruyama et al demonstrated that by using CRISPR-Cas9 technology, SCR7 is able to increase the HDR efficiency approximately to 19-fold in mammalian cells and mouse embryos. Indeed, this increased efficiency has a positive dual function on the HDR pathway and CRISPR-Cas9 system.[8] Moreover, the small molecules involved in the stimulation of the HDR pathway, such as RS-1, are able to increase the CRISPR-Cas9 efficiency. Pinder et al showed that RS-1 increases the performance of both Cas9 and HDR up to 3-fold.[45] Moreover, the combination of small molecules known as "CRISPY mix", such as NU7026, MLN4924, inhibiting neddylation of CtIP, Trichostatin A, and NSC15520, in collaboration with Cas9 nickase, enhanced the efficiency of knock-in experiments.[46] Besides all successful achievements in increasing HDR efficiency through stimulation of HDR or inhibition of NHEJ, it should be considered that these strategies are harmful to genome integrity.[121] On one hand, inhibition of NHEJ would increase premature aging and the incidence of cancers. On the other hand, stimulation of RAD51 would lead to an augmentation in spontaneous recombination especially across widespread repetitive sequences which in turn, could result in loss of key genetic information.[122,123] In this line, discovering small molecules with minimal off-target effects on global genome stability sounds extremely crucial. Recently, Zhang et al established a novel and easy-to-score screening system

by doing a mechanistic study and analyzing 722 natural small molecules which are commonly used in traditional Chinese medicine. It is elucidated that farrerol, isolated from *Rhododendron dauricum*, with anti-inflammatory and anti-bacterial properties, scaled up homologous recombination without any effect on NHEJ. Furthermore, this natural small molecule enhanced CRISPR-Cas9-mediated genome editing in different *in vitro* and *in vivo* models.[124] Precise control over exposure time and expression level of CRISPR-Cas9 system during gene editing approaches is extremely important for enhancing its application. To address this serious challenge, Wu et al generated a chimeric endonuclease by fusing small molecule-assisted shut-off (SMASh) to Cas9 protein. It was well-demonstrated that in the presence of hepatitis C virus (HCV) protease inhibitor asunaprevir (ASV), a clinically approved small molecule for HCV, this chimeric endonuclease degraded but upon ASV removal, this phenomenon reversed. Generating chimeric endonuclease such as Cas9-SMASH fusion could increase the accuracy and versatility of genome editing approach based on CRISPR-Cas9 technology.[125] Although CRISPR-Cas9 technology has presented high efficiency in various *in vitro* and *in vivo* models, knock-in efficiency by this technology has not been successful in hESC. Cas12a (also known as Cpf1) is a CRISPR effector which is categorized in class II- type V. CRISPR-Cas12a has shown promising potential for expanding genome editing toolbox. One special feature of this system is introducing a staggered DSB in targeted regions which seems to have increased the HDR rate.[126] A high-throughput chemical screening for identifying the candidate small molecules elucidated that AZD-7762 and VE-822 are able to improve CRISPR-Cas12a -mediated precise genome engineering.[54] Furthermore, the design of a new paradigm for stimulation of the structure of Cas protein and/or small molecules interaction with Cas-DNA seems a logical approach for the enhancement of the efficiency of the CRISPR-Cas mediated gene editing. In this line, Li et al provided a new paradigm for modulating the efficiency of CRISPR-Cas12a-mediated gene editing by small molecules. According to their results, a rational small molecule, quinazoline-2,4(1H,3H)-dione, is able to work like a molecular glue between Acidaminococcus Cas12a (AsCas12a) and crRNA and stabilize endonuclease-crRNA

complex which in turn, would improve the efficiency of gene editing which is mediated by AsCas12a mammalian cells.[127] Manipulation of genetic information flow at the RNA level provides the opportunity to change the expression level of genes without long-term modification to the host genome. Cas13 proteins are CRISPR effectors with RNA targeting activity and are classified into class II- type VI. CRISPR-Cas13 has shown promising results in RNA targeting strategies and RNA-based diagnostic tests.[128] The large size (~100-130 kDa) and bacterial origin restrain the applications of CRISPR-Cas13 technology in both research and therapeutic developments. Recently, a novel CRISPR-Cas-inspired RNA targeting system (CIRTS) was generated to overcome these limitations. CIRTS is an engineered RNP that is able to recruit protein cargo site-selectively to target the transcript through using Watson-Crick-Franklin base-pair interactions with a gRNA.[129] In order to temporally control CIRTS dynamics, a small molecule-inducible RNA-targeting platform was established based on this effector by coupling heterodimerization domains of the abscisic acid small molecule system with CIRTS. This system provides an opportunity to easily target any desired transcript under a small molecule inducible-CIRTS platform.[130]

CRISPR-Cas applications in in vivo preclinical and clinical models

Currently, most of clinical applications which are mediated by CRISPR-Cas technology for monogenic disorders are *ex vivo* approaches. During last years, several *in vivo* gene editing studies have been represented that target both HDR and NHEJ pathways. Since the efficiency of *in vivo* editing is lower compared to *in vitro*, a screening approach for detecting the modified cells through accurate editing boosts the feasibility of HDR approach in clinical models.[131] Although CRISPR-Cas9 has been used in *ex vivo* clinical trials related to human immunodeficiency virus-1 (HIV) infection, cancers, b-thalassemia (ClinicalTrials.gov: NCT03655678), and sickle cell disease(ClinicalTrials.gov: NCT03745287), mentioned strategies such as synchronization, modified CRISPR-Cas Systems, and small molecules in the clinical models could be promising in future. Some of the recent applications based on HDR gene editing in preclinical and clinical models are presented in Table 3. Hereon, we emphasize several HDR-based accurate *in vivo* gene editing experiments utilizing CRISPR-Cas that can be potentially applied to human in future.

Table 3. List of the recent therapeutic gene editing studies based on HDR in preclinical models

Human disease	Organ	Editing strategy	Delivery system	Gene editing tool	Ref.
Hemophilia A and B	Mouse liver	HDR-dependent gene insertion	Systemic injection of AAV8	ZFN	[132]
Hemophilia A and B	Mouse liver	HDR-based corrective gene editing	Systemic injection of AAV8	ZFN	[133]
Hemophilia B	Mouse liver	HDR-based point mutation correction	Injection of AAV8	CRISPR-Cas9	[134]
Hemophilia B	Mouse liver	HDR-mediated insert into mFIX into murine ROSA26 safe harbor	Intravenous injection of AAV8	CRISPR-Cas9	[135]
Hemophilia B	Mouse liver	HDR-based point mutation correction in *F9* locus	Intravenous injection of adenovirus	CRISPR-Cas9	[136]
Hunter's syndrome	Mouse liver	HDR-mediated integration into albumin locus	Systemic injection of AAV8	ZFN	[137]
HTI	Mouse liver	HDR-based point mutation correction	Intravenous injection of AAV2/8 and LNP	CRISPR-Cas9	[138]
HTI	Rat liver	HDR-based point mutation correction	Intravenous injection of adenovirus	CRISPR-Cas9	[139]
AATD	Mouse liver	HDR-based point mutation correction	Intravenous and intraperitoneal injection of AAV	CRISPR-Cas9	[140]
DMD	Mouse muscle	HDR-mediated dystrophin gene correction	Intramuscular and retro-orbital injection of dual AAV6	CRISPR-Cas9	[141]
DMD	Mouse muscle	HDR-based point mutation correction	Intramuscular injection of Gold nanoparticle Cas9 RNP and donor DNA	CRISPR-Cas9	[142]
Retinitis pigmentosa	Mouse Eye	HDR-based point mutation correction	Subretinal and electroporation of RecA-MS2 Plasmid and ssDNA donor	CRISPR-Cas9/ RecA	[143]
OTC deficiency	Mouse liver	HDR-based point mutation correction	Intravenous injection of AAV8	CRISPR-Cas9	[144]
OTC deficiency	Mouse liver	HDR- mediated insert codon optimized human *OTC* into intron 4 of mouse *OTC* locus	Intravenous injection of AAV8	CRISPR-Cas9	[145]
MPS type I	Multiorgan	HDR- mediated insert Idua into murine ROSA26 safe harbor	Intravenous injection of cationic liposomes	CRISPR-Cas9	[146]

AAV, adeno-associated virus; mFIX, factor IX; HTI, hereditary tyrosinemia; LNP, lipid nanoparticle; AATD, alpha-1 antitrypsin deficiency; DMD, Duchenne muscular dystrophy; OTC, ornithine transcarbamylase; MPS, mucopolysaccharidosis.

Review Highlights

What is the current knowledge?
√ HDR is attractive for its high fidelity; the choice of repair pathway is biased in a biological context.
√ Mammalian cells preferentially employ NHEJ over and HDR is restricted.

What is new here?
√ Facilitating the development of a modified CRISPR-Cas9 system to achieve more precise gene editing.
√ Suggesting the remarkable advantage of small molecules for establishing drug inducible genome editing platforms.
√ Drug inducible genome editing platform would help to control editing approaches dose- and exposure time-dependently.

Conclusion

The stimulation of HDR pathway can increase the accuracy of CRISPR-Cas-mediated engineering systems. Generating chimeric programmable endonucleases provides this opportunity to direct DNA template close proximity of CRISPR-Cas-mediated DSB. However, it should be considered that by fusing a functional domain or linker, the expression level of Cas9 or its delivery efficiency might be changed and also the generated fusion protein may be prone to protease cleavage. Small molecules and their derivatives are able to proficiently block or activate certain DNA repair pathways. Most small molecules increase precise genome editing through HDR. The results of most studies suggest that for increasing HDR-driven knock-in events, blockade of NHEJ is more efficient than improving HDR. Furthermore, the targeting upstream protein in NHEJ provides a greater potential for increasing the HDR-mediated gene insertion. Nonetheless, small molecules have unequal and even diverse influences on the efficiency of precise genome editing in various cell models. Hence, it is necessary to survey the efficacy of this approach in certain cells, different DNA loci, and *in vivo* studies. In summary, inhibitors of DNA-PK and ligase complex enhance the efficiency of precise genome editing efficiency in various cell types. There is serious consideration about genome integrity by inhibiting NHEJ or stimulating HDR components. In this line, discovering safe natural products such as farrerol, which is able to increase HDR with no effect on NHEJ, brings up a novel perspective for increasing the efficiency of genome editing approach. Ultimately, high throughput screening of small molecule libraries could result in more discoveries of promising chemicals that improve HDR efficiency and CRISPR-Cas9 systems. Another remarkable advantage of small molecules is their usefulness for establishing drug-inducible genome editing platforms. This finding would help researchers temporally control the dose and exposure time of genome or transcriptome editing tools.

Ethical statement
Not applicable.

Competing interests
Authors declare they do not have any conflict of interests.

Authors' contribution
FSh: conceptualization and draft preparation, writing and reviewing. HD: study consultation, writing and reviewing. OM: conceptualization and draft preparation. SM, HV, MS: study consultation and validation. AR: supervision, writing and reviewing, and project administration.

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