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Advances in understanding the DNA-repair mechanism activated by CRISPR/Cas9

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Abstract
In recent years, clustered regularly interspaced short Palindromic repeats, clustered regularly interspaced short Palindromic repeats, CRISPR and CRISPR-associated protein 9 (Cas9) mediated gene-editing technology has been widely used in biology, essential medicine, and other research fields, and animal disease models constructed by this technology have promoted the exploration of pathogenesis and the research progress in gene therapy and other aspects. Cas9 nuclease cleavage performs gene editing in conjunction with subsequent homologous recombination (HDR) or non-homologous terminal linking (NHEJ), but the low efficiency of HDR generation limits its application. Therefore, it is crucial to study the mechanism of NHEJ and HDR repair modes to improve gene editing accuracy and efficiency. In this paper, the mechanism of NHEJ and HDR repair system in the application of CRISPR/Cas9 system is summarized, and the prospect is made based on the relevant research progress, which provides ideas for the research of efficient use of CRISPR/Cas9 technology.

Keywords: CRISPR/Cas9, DSB, homologous recombination, non-homologous terminal junction, disease model, gene therapy

Introduction
The CRISPR/Cas9 system, found in bacteria, archaea, and phagosomes, is a gene-editing tool that modifies DNA mediated by Single Guide RNA(sgRNA). Double-strand breaks (DSB) occur when Cas9 proteins are targeted to cut double-stranded DNA, and the classic pathway for DSB repair is non-homologous end-joining. NHEJ) and homology-directed repair (HDR) [1]. In addition, alternative non-homologous terminal connection (Alt-NHEJ, Alt-nhej) and single-strand annealing (SSA) [2]. NHEJ is active throughout the cell cycle, but its repair is often inaccurate and error-prone. HDR is a high-fidelity repair pathway with a low repair error rate, but it is limited to the S phase and G2 phase of mammalian cells. At present, CRISPR/Cas9 technology has been widely used in animal model construction and gene therapy, but the lack of accuracy of NHEJ and low efficiency of HDR limit the development of the CRISPR/Cas9 system [1]. Therefore, the study of molecular mechanisms of DSB repair pathways such as HDR and NHEJ is conducive to the efficient use of CRISPR/Cas9 technology. This paper mainly reviews and prospects the mechanism and selection of intracellular NHEJ and HDR repair systems during the CRISPR/Cas9 system application, providing ideas for better use of CRISPR/Cas9 technology research.

Discovery and main application progress of CRISPR/Cas9 system
Discovery and mechanism of CRISPR/Cas9
Derived from the immune mechanisms of bacteria and archaea, the CRISPR/Cas system targets DNA and RNA destruction in a sequence-specific manner, providing bacteria with immunity to invading plasmids and viruses [3-4]. CRISPR was first discovered in the genome of E. coli in 1987 [5]. Studies have shown that giant phages also have CRISPR/Cas systems [6]. Currently, various types of CRISPR/Cas systems have been discovered [7], among which type II CRISPR/Cas9 system is the most common and widely used gene-editing tool in various fields due to its simple structure, reasonable specificity, and high cutting efficiency of effecting complex [8]. It comprises Cas9 protein, crRNA(CRISPR RNA) and tracrRNA(transactivating crRNA), and the protospacer adjacent motif.

In the presence of the PAM sequence, sgRNA guides Cas9 protein to cut complementary and non-complementary chains, forming a notch, referred to as cas9-DSB notch.
Finally, cells repair broken DNA via NHEJ or HDR, resulting in base insertion, deletion (Indels), or replacement [1].

Advantages of CRISPR/Cas9 system and evolution of gene editing tools

Before the ADVENT of CRISPR/Cas9, zinc finger nucleases, ZFNs (transcription activator-like (TAL) effector nucleases (TALENs) are the main gene-editing tools. They all target specific SEQUENCES of DNA bases by building different combinations of amino acids. Both ZFNs and TALENs require a pair of nucleases to cleat a site with a low mutation rate. Compared with ZFNs and TALENs, CRISPR/Cas9 can recognize DNA sequences by sgRNA guiding specifically targeted sites, which can efficiently and quickly cut target gene loci. SgRNA vectors are easy to construct and edit at multiple sites, and CRISPR/Cas9 has higher recognition tolerance. On average, there is one targeted sequence for every eight bases [9].
The most widely used Cas9 is SpCas9, derived from Streptococcus pyogenes. Other Cas9 types are SaCas9 from Staphylococcus aureus [10], Streptococcus thermophilus St1Cas9 [11], Neisseria meningitidis NmCas9 [12], Francisella FnCas9 [13] and Campylobacter jejuni CjCas9 [14]. The targeting properties of SaCas9 and CjCas9 are comparable to those of SpCas9 [14-15]. In 2020, Chinese researcher Wang Yongming [16] developed a new small CRISPR/Cas9 tool derived from Staphylococcus oeari, named SauriCas9, with a large editing range and high efficiency. In the same year, Harvard Medical School and Massachusetts General Hospital jointly engineered Cas9 and obtained DNA-cutting Cas9 protein variants SPG and SpRY that were not limited by specific PAM sequences [17]. In March 2021, Wang Yongming's research group [18] developed slUGCAS9-HF, which has the advantages of high activity, high accuracy, and a wide editing range. In September of the same year, the Ji Quanjiang team [19] discovered the ultra-small CRISPR nuclease -AsCas12f1 with only 422 amino acids, which achieved efficient gene editing in bacterial and mammalian cells. On the same day, KIM's lab [20] developed Cas12f1, a mini-version of CRISPR system with a target similar to THAT of SpCas9 in mammalian cells, providing new ideas for the development of micro-precision gene editing and therapy tools. In addition to the CRISPR/Cas9 system that causes DSB, researchers also developed a base editing system that can achieve single base transformation without DSB [21-22]. Ddda-derived cytosine base Editor (DdCBE) for mtDNA [23]. In 2019, the LIU team [107] developed a new precision gene-editing tool PE(Prime Editor), which can achieve arbitrary insertion, deletion, and conversion of a single base without DSB and donor DNA. The proliferation of cas-based gene-editing tools has continuously injected vitality into the construction of animal disease models and gene therapy, and DSB mediated homologous recombination generated by Cas9 cutting has also provided an opportunity for the production of humanized animal models.

Application of CRISPR/Cas9 system in animal model construction and gene therapy

Gene Knockout In 2013, LE *et al.* [1] used CRISPR/Cas9 system for the first time to cut mouse genes and generate frame-shift mutation during cas9-DSB repair and obtained gene knockout mice. The application of CRISPR/Cas9 gene-editing technology in animal disease models was started. At present, CRISPR/Cas9 gene-editing technology is widely used to establish knockout animal disease models of monkeys, pigs, dogs, rabbits, and sheep [25-29]. In addition, gene knockout is also used in gene therapy research, researchers through the *in vivo* knockout of different genes, to achieve the blood low-density lipoprotein cholesterol, The expression level of LDL-C) was reduced [15] and the aim of treatment for tyrosinemia type I mice [30]. 1.3.2 Gene Knock-in DSB-mediated gene knock-in can be used to obtain ideal animal disease models and also to correct related diseases. In 2013, RUDOLF's research group [31] used the Cas9 system for the first time to successfully knock labels or fluorescent proteins into Nanog, Sox2, and Oct4 genes. In 2016, Japanese scientists successfully realized the green Fluorescent protein (GFP) gene knockin at Thy1 in rats [32]. In 2018, Chinese scientists successively obtained cyclamophagy monkeys with OCT4-GFP gene knockin, rabbit model with Wilson's disease gene knockin, and pig model with Huntington's disease gene knockin [33-35]. In the field of gene therapy, WILSON lab [36] achieved 10% ornithine carbamoyltransferase (OTC) repair in neonatal rat liver cells using SaCas9 and donor DNA template in 2016. In 2019, the substitution of exon 8 on the ATPase copper receptor beta (Atp7b) gene was successfully implemented in newborn mice [37]. In general, CRISPR/Cas9 system-mediated gene knockin is simple to operate. Only Cas9 nuclease, sgRNA, and donor DNA fragments are needed to knock in foreign gene fragments at a specific point, without any restriction of species. However, the efficiency of gene knockin is still low, especially for needle knocking and the replacement of large fragments. This limits its application in humanized animal model making and gene therapy.
The emergence of a single base editing BE system has brought new strategies for the establishment of animal disease models, as well as new treatment ideas for genetic diseases with single base mutation. Using CBE technology, KIM *et al.* [38] established mouse models of albino and muscular dystrophy (DMD) in 2017, followed by albino rabbits, single-base mutant DMD pigs, and progeria monkeys [39-41]. In addition, the mitochondrial gene MT-ND5 targeted by DdCBE was used to establish a mitochondrial disease model in mice [42]. In 2018, ROSSIDIS *et al.* [43] successfully achieved prenatal base editing gene therapy by targeting liver cells with the BE system. In the same year, Seoul National University researchers used the ABE system to correct a nonsense mutation in exon 20 in DMD mouse muscle, restoring 17% dystrophin protein expression and effectively improving mouse muscle function [44]. In May 2021, MUSUNURU *et al.* [45] used ABE8.8 to effectively reduce proprotein Convertase Subtilisin/Kexin Type 9 in blood in a non-human primate model. Pcsk9) and LDL-C. At present, the BE system has been relatively mature for editing a single base, but the operation of long fragments still needs homologous recombination-mediated by Cas9 cleavage.

CRISPR/Cas9 system-induced DSB repair

CRISPR/Cas9 system induces DSB at a specific site, which is the first step in gene editing. Only when CAS9-DSB activates intracellular repair machinery can gene editing be achieved [46]. Cas9-dsb repair pathways include HDR, NHEJ, Alt-NHEJ, and SSA, as shown in Figure 1. Different repair pathways all have specific repair factors, but these

repair pathways are all driven by the same signal cascade to drive cell response to Cas9-DSB [47]. Cas9-dsb repair is accomplished in a highly complex intranuclear environment, where the cas9-DSB signaling cascade first activates intranuclear reactions in cells.

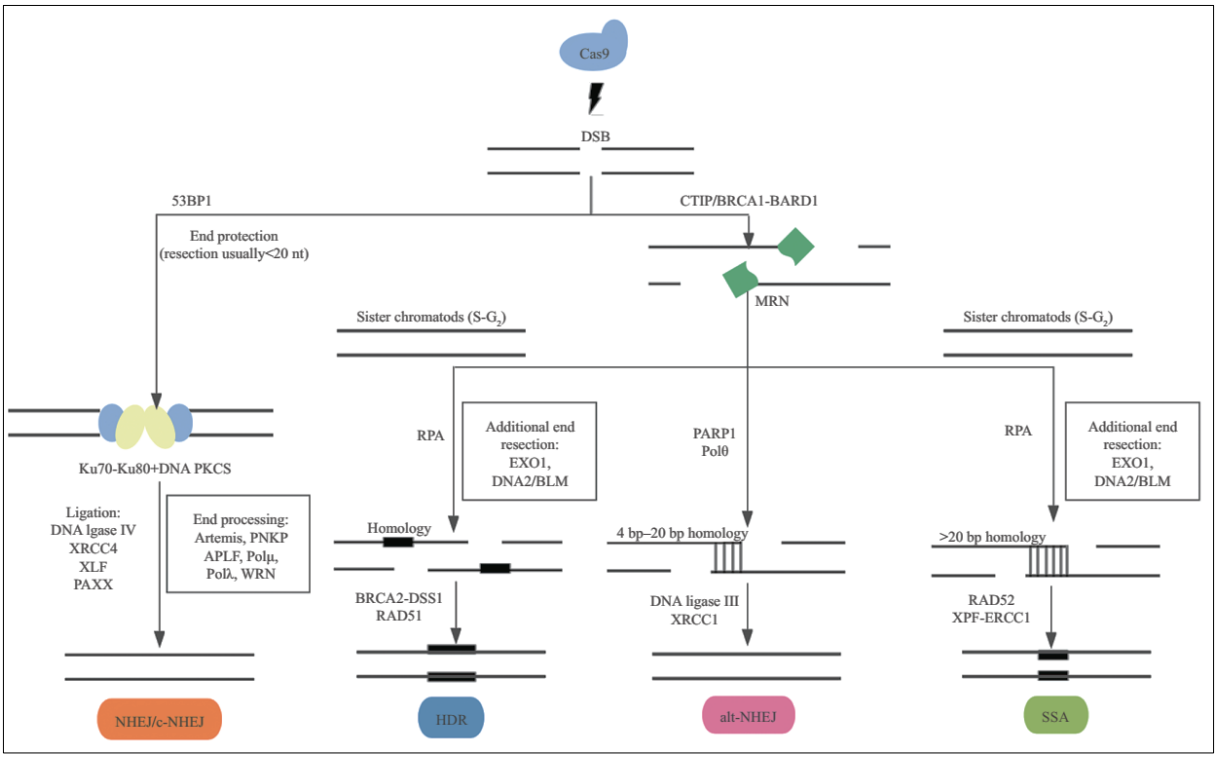

Fig 1: CRISPR/Cas9 system induced fracture repair double-stranded DNA

Intracellular reactions induced by CAS9-DSB After cas9-DSB is produced, chromatin is first initiated to undergo a highly complex ubiquitin cascade, and changes in chromatin conformation are the key to cas9-DSB modification. Changes in chromatin conformation are related to a histone modification and DNA methylation. Cas9-dsb signal first induces a series of histone modifications such as H2BK123 ubiquitination and H3K79 methylation, which can transform the inactivated chromatin template into open euchromatin. Facilitate the arrival of DNA repair proteins to damaged areas [48]. In addition, changes in histone modifications can be used as damage signals to recruit and repair proteins [49] and affect DNA methylation changes. DNA demethylation is triggered by the oxygenation of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) by TET1, TET2, and TET3, which contributes to the formation of open chromatin and promotes DNA repair [50]. The dynamic changes of chromatin conformation are also related to the selection of DSB repair pathways, p53 binding protein 1(53BP1), and breast cancer susceptibility gene 1(1). BRCA1 is a protein factor that controls the selection of the DSB repair pathway. DNA repair factors are recruited to DSB sites through specific chromatin modification and chromatin remodeling. Recruitment of 53BP1 helps DSB repair to target NHEJ, while BRCA1 leads DSB repair to target HDR [51] (Figure 1).

Repair Mode and Mechanism of CAS9-DSB When Cas9-DSB signals drive the conformation change of chromatin in the nucleus, cells initiate signal cascades in response to CAS9-DSB, first of all, ataxia telangiectasia mutated, ATM, ATR(ATM and RAD3-related) and DNA-dependent protein kinase, DNA-pk) Moreover, phosphoinositide 3-kinase related protein kinases (PIKKs) are activated [52]. ATM activation phosphorylates the c-terminal site S139 of histone H2AX to form γH2AX, initiating staining-based signaling modifications including phosphorylation, ubiquitination, and other post-recording modifications [49]. At the same time, ATM activates CHK2 and phosphorylates mediator of DNA damage checkpoint 1 (MDC1) at S964. Phosphorylated MDC1 recruited E3 Ubiquitin ligase (RNF8) and RNF168 proteins, ubiquitinated H1 and histone H2A/H2AX, respectively. Recruitment of 53BP1 or BRCA1 for the subsequent repair of CAS9-DSB was selected [53].

Cas9-DSB homologous recombination repair The main components involved in intracellular homologous recombination repair are MRN(MRE11-RAD50-NBS1) complex and RAD51, RAD54, BRCA1, CTIP (CTBP intervening proteins), ATM and ATR factors [54]. The repair of Cas9-DSB recruits BRCA1 protein to guide cells to HDR repair. BRCA1 is the most critical mediator in 5 '-end resection in cells. Brca1-bard1 interacts with CTIP and MRN in a cytopercycle dependent manner. Brca1-bard1 or CTIP stimulates the activity of MRE11 and initiates DNA end resection. Further remote excision is performed by EXO1 (EXO1) and heterodimer DNA2/Bloom syndrome protein (DNA2/BLM) nuclease -- disultase complex, resulting in the production of large amounts of ssDNA [51]. The unstable ssDNA rapidly binds to replication protein A(RPA) to form RPA-SSDNA, which then binds to RAD51, A recombinant enzyme recruited with the help of the BRCa2-DSS1 complex. After a homologous search with the help of RAD51, homologous chromosomes were invaded, and displacement loop (D-loop) recombinant intermediates were generated based on this template. After that, DNA polymerase extended from the 3 'end to the other end of DSB using the homologous strand as a template. Effectively

seal the damaged area [55]. Therefore, an extensive 5 'terminal of DNA is the key for DSB to select the HDR repair mode. The proteins BRCA1-BarD1 and CTIP that interact with MRN are the critical factors for the initiation of DNA turbinectomy [56].

Cas9-DSB non-homologous terminal Junction repair NHEJ/ C-NHEJ pathway is the DNA direct connection repair process of Cas9-DSB with the participation of some repair elements. The main components involved in NHEJ include Ku protein, DNA-PK, DNA ligase IV, X-ray repair cross-complementary 4(XRCC4), APLF, and other molecules [57].

NHEJ is the main pathway for DSB repair in eukaryotes due to its high repair efficiency, and this pathway can be divided into several steps. The first step of NHEJ is that DNA-PK is recruited to DSB with the assistance of Ku isomer (KU70-KU80 protein) and activates DNA-PK kinase activity to perform the function of connecting broken DNA double strands [57]. DNA-PK kinase can autophosphorylate and phosphorylate chroma near DSB and many downstream NHEJ factors, promoting timely recruitment and activation of downstream NHEJ factors under the interaction of LIG4-XRCC4 complex and two other proteins, XLF and PAXX, Promoting the correct connection of the ends of parts of DNA. However, since broken DNA ends are usually not complementary and may contain modified nucleotides, they need to be processed before joining. Artemis, PNKP, APLF, Polμ and Polλ, Werner(WRN), Aprataxin and Ku can make DNA terminal splice. They act on different DNA terminal structures, such as Artemis nuclease on the hairpin structure. Aprataxin can remove AMP residues at the 5 '-end of fractured DNA. Ku processing enzyme can help to excise alkaline sites near DSB. DNA Polλ and DNA Polμ can add nucleotides to the 3 'end of the fracture [51]. Indels are easy to generate due to the end processing mentioned above, leading to frame-shifting mutation or premature termination of codon formation [58], thus achieving the effect of gene knockout.

Alt-NHEj pathway of cas9-DSB alternative terminal connection is the backup mechanism of C-NHEJ, including microhomologous terminal connection (MMEJ) [59]. Proteins in the Alt-NHEj pathway include PARP1, DNA ligase I, DNA ligase III, XRCC1, and MRN [57].

AltNHEJ is activated to repair DSB when the critical protein of C-NHEJ is defective [60]. PARP1 is the critical protein of Alt-NHEj. When INTRACellular DSB occurs, the receptor PARP1 recruits the alt-NHEJ repair factor to the DNA fracture point, and Mre11 and CTIP and other factors participate in the terminal base resection. DNA ligase III interacts with XRCC1 as a ligase to participate in terminal ligating, and finally, DSB ligating is mediated by factors such as DNA ligase I and DNA ligase III [57]. Alt-nhej usually excised the 5 'end of DNA at 15-100 nucleotides of the DSB site, and the exposed 4-20 bp microhomologous sequence contributed to the reconnection of the broken end [2]. PARP1 can also recruit Polθ, which can replace the annealing of RPA to stabilize the 3 '-SSDNA long tail and use the annealing part as a template to extend the 3' end of the DNA chain to strengthen the binding between the broken ends. When annealed microhomologous fragments are embedded into the long tail of 3 '-SSDNA, the non-homologous 3' -SSDNA tail needs to be excised before Polθ binding. Therefore, nucleases such as XPF-ERCC1 nuclease, Artemis, and APLF are utilized in the alt-NHEJ process. These nucleases remove non-homologous 3 '-single-stranded DNA(3' -SSDNA) tails, setting the stage for Polθ binding. Recent studies have reported the relatively high frequency of alt-NHEJ based target integration using CRISPR/Cas9 [61-62]. However, alt-NHEJ has some technical disadvantages related to NHEJ: Indels problems at the 5 'and 3' junctions of DNA insertion sites [63].

Cas9-DSB single-chain annealing repair of SSA and AlT-NHEJ also requires the removal of the 5 'end of DNA. However, the scope of removal is more prominent because the initiation of SSA requires the 3' -SSDNA tail with 25 to hundreds of nucleoside long homologous sequences. Such homologous sequences are usually found in double-stranded repeats of DNA molecules [2]. MRN and CTIP initiate terminal resection of SSA to produce a 3 '-ssDNA tail. EXO1 and DNA2/BLM perform extensive terminal resection to produce a 3' -SSDNA tail with a longer homologous sequence. SsDNA and RPA then form the RPA-SSDNA complex, but unlike HDR, the final step of SSA relies on RAD52-mediated annealing to connect two 3 '-SSDNA ends with long homologous sequences. If there are non-homologous sequences at the 3' -SSDNA ends, They will be excised by xPF-ERCC1 endonuclease [64]. As the SSA pathway often involves extensive terminal resection before defining pairing, SSA leads to large DNA fragment deletion, partial loss of genetic information, and an error-prone repair process [65]. Some studies believe that most mammalian gene sets are composed of repeated sequences, so SSA may be an essential alternative pathway for HDR [66] and may also be one of the mechanisms for missing large fragments in the preparation of animal models.

Selection of CRISPR/ Cas9-induced DSB repair mechanism

Cas9-DSB repair mechanism selection and cell cycle

Cas9-dsb repair to achieve specific gene editing purposes is one of the essential means of gene editing. Research on the DSB repair pathway selection mechanism is of great significance for improving gene editing efficiency [67]. The selection of the DSB repair pathway is related to the cell cycle, and the selection of DSB repair mode is shown in Figure 2. NHEJ does not require sequence homology and is active at all stages of the mitotic cell cycle. In contrast, HDR requires extensive sequence homology and acts in meiosis, anaphase S, and G2 phases [68]. After DNA replication, an identical DNA sequence can be used as a template for error-free DSB repair in the nucleus. Both HDR and NHEJ pathways can repair DSB in the late S and G2 phases of mitosis, but cells will preferentially choose HDR repair with high repair accuracy. The mechanism is that CYREN is a cell cycle-specific inhibitor of NHEJ, and CYREN interacts with Ku dimer to inhibit NHEJ repair and promote HDR repair [69-71]. When two significant pathways, NHEJ and HDR, are damaged, cells initiate SSA and alt-nhej repair pathways, and both SSA and alt-nhej require DNA terminal resection, so they are active in cell S G2 phases [2, 72].

Selection of Cas9-DSB repair mode in somatic cells

The selection of DSB repair mode is determined by the complex molecular interaction network caused by activation of crucial NHEJ factor (53BP1) or key HDR factor (BRCA1). 53BP1 and BRCA1 are mutually antagonistic. Both recruit many additional factors to reach the mutation point [73]. Mitosis is the primary way of somatic cell

proliferation in mammals, and the repair of DSB in somatic cells mainly depends on the NHEJ pathway. Only some proliferating cells in S and G2 phases tend to choose the HDR pathway accurately. However, the DSB repair pathway selection in the S and G2 phases also depends on DNA terminal excision. 53BP1 is a crucial regulator of the selection of repair mode, which induces Ku dimer to bind to DNA end to inhibit the initiation of NHEJ repair after DNA end removal, and promotes HDR repair after DNA end removal [74]. The structure of THE DNA terminus is also an essential factor affecting the removal of ssDNA. For example, the long tail of ssDNA is weakly bound to Ku dimer, while the flat or concave DNA terminus is better bound to Ku dimer. SsDNA or interspace DNA ends directly activate PARP1 and may evade end recognition through Ku dimer, supporting Ku dimer replacement and promoting the removal of long fragments of DNA ends. Finally, cells will choose a more precise HDR repair. At the same time, alt-NHEJ and SSA, the two pathways, can also act opportunistically on the ssDNA terminal, leading to chromosome rearrangement for DSB repair [72]. Therefore, the CRISPR/Cas9 incision generates a flat-end DSB incision to guide the repair to NHEJ, while the sticky end DSB incision tends to HDR repair.

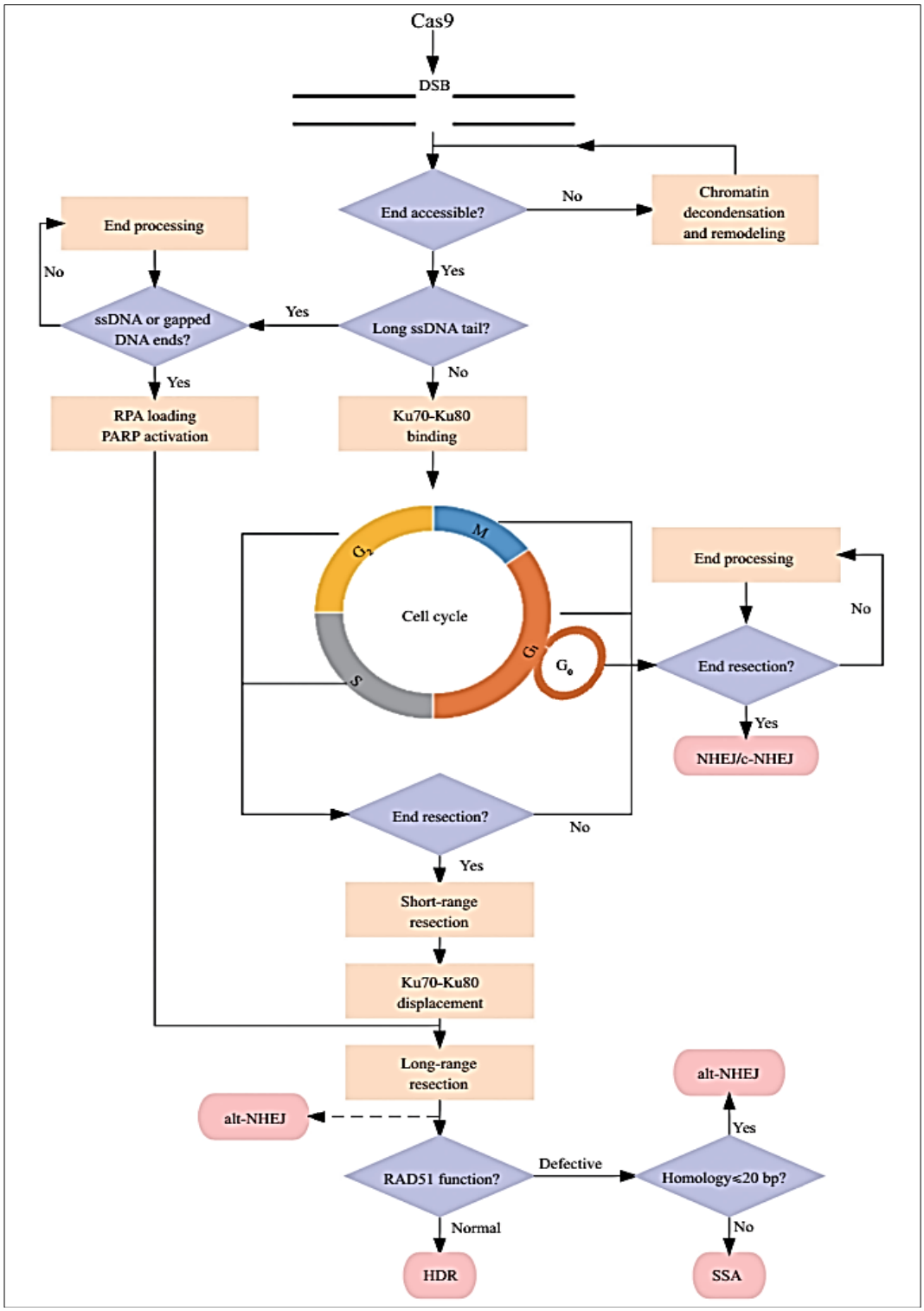

Fig 2: CRISPR/Cas9 mediated the choice of the ways of double-stranded DNA fracture repair

Selection of cas9-DSB repair mode in early embryos

In order to maintain the genome integrity of the reproductive system, different DNA damage repair systems are used for gametes and fertilized eggs. In general, using NHEJ or HDR to repair DSB depends on the cell cycle. When DNA replication is complete and sister chromatin can be used as a repair template, DSB repair tends to HDR, and HDR machinery is down-regulated in M and early G1 phases, thus favoring NHEJ-induced genome editing. However, when CRISPR/Cas9 was introduced into MII oocytes during ICSI, no reduction in HDR efficiency was observed, which may be due to the difference in DNA repair response between the M phase of cleavage and M phase of mitosis, or the fact that DSB occurred in M phase or G1 phase of the cell cycle. HDR repair subsequently occurs in the S or G2 phase of the cell cycle [75]. In 2013, Li Jinsong [76] found that mouse embryos could use endogenous wild-type alleles as templates for repair, but adding exogenous DNA templates could not significantly improve the repair efficiency of HDR. We demonstrate that HDR using endogenous wild-type is epitome as template plays a dominant role in CRISPR/Cas9 mediated repair of mouse embryos. In 2019, Professor WOLF *et al*. [77] discussed the repair of DSB in early embryos. The repair efficiency of HDR using exogenous ssODN template only accounted for 1%-5%, while the repair efficiency of ENDOGENOUS wild-type allele template reached 64%. In 2021, Professor Feng Guoping *et al*. [78] obtained efficient homozygous gene knock-in mice using CRISPR/Cas9 for gene knock-in mouse embryos, which further confirmed that HDR uses endogenous wild-type DNA alleles as templates play a leading role in DSB repair in mouse embryos. RAD51 is one of the essential factors in this process.

Research using the CRISPR/Cas9 system to inject fertilized eggs is mainly used to construct animal disease models and gene therapy. NHEJ repair mainly produces the result of gene knockout, while HDR repair can carry out gene knockout. The upstream and downstream of the commonly used HDR repair template contain more than 500 bp homologous sequences, and the middle contains the sequence to be replaced. However, HDR is usually relatively inefficient, limiting its use. In order to address the deficiency of HDR, many studies have used a variety of donors as templates for repair. The simplest of these templates is single-stranded oligodeoxynucleotides (ssODNs) with homologous arms less than 100 bp. The repair pathway is often called single-stranded template repair (SSTR). This process includes HDR(precise integration of templates) and NHEJ(formation of INDELs) repair results, which generally achieve higher integration rates than traditional HDR [79]. In terms of gene therapy, CRISPR-Cas9 technology can repair harmful gene mutations, but its main limitation is that the NHEJ pathway will introduce INDELs, and HDR repair efficiency is low. In treating heterozygous mutation, gene transfer has also attracted much attention. Gene transfer is the process of one-way conversion of donor sequence to a highly homologous receptor sequence without an external template. Typically, gene transfer occurs in the period of meiosis non-crossover recombination and is activated by the DSB repair mechanism. In turn, the complete homologous sequence is copied to the DSB region. It has been proved that the gene conversion and repair efficiency of CRISPR/ Cas9-induced mutant gene MYBPC3 in early embryos reaches 64% [75].

Study on improving the efficiency of CAS9-DSB mediated HDR in gene editing

The efficiency of CRISPR/ Cas9-mediated gene editing depends on the repair pathway of Cas9-DSB. Due to the randomness of the NHEJ repair mechanism and the inefficiency of HDR repair, it is not easy to obtain correct animal models for editing target gene sites. The key to improving the accuracy and efficiency of DSB repair lies in the regulatory selection of HDR and NHEJ repair pathways [80]. Methods to enhance the efficiency of HDR include altering homologous donor sequences, such as changing different homologous arm lengths in double-stranded plasmid donors, using long oligonucleotide form of single-stranded DNA donor molecules, using rAAV generation of single-stranded DNA donor molecules, and linearization of donor molecules *in vivo* by designed cleavage sites. HDR-mediated gene insertion efficiency can also be improved [80], and the RNP-SSDNA complex can be constructed by combining Cas9 protein, sgRNA, and ssDNA to improve HDR efficiency [81]. In addition, HDR activity can be enhanced in the following ways.

Key factors that inhibit the NHEJ pathway enhance HDR efficiency

When CRISPR/Cas9 induces DSB signal, cells in the S and G2 phases will first try to use HDR for DSB repair. When NHEJ and HDR co-occur, key factors that inhibit the NHEJ pathway can lead to DSB repair to the HDR pathway [82]. DNA ligase IV is a crucial protein in the last step of the NHEJ connection and a potential target for NHEJ inhibition [83]. SCR7 is a small molecule inhibitor of DNA ligase IV. In 2015, SCR7 was successfully used to inhibit NHEJ, increasing the frequency of CRISPR/ Cas9-mediated HDR. MARUYAMA *et al*. [84] found that SCR7 treatment increased the gene knocking efficiency of mammalian cells by 2-19 times, and co-injection of CRISPR/Cas9 and SCR7 into the zygotes could improve the HDR-mediated knocking efficiency. SCR7 has also been used to increase the frequency of precise genetic modifications [85-86]. In addition to small molecule inhibitors, DNA ligase IV activity can be blocked by other methods, such as shRNA-mediated gene silencing and proteome degradation of DNA ligase IV can also improve the efficiency of HDR in mammalian cells [86]. In addition, the efficiency of the HDR pathway can be improved by inhibiting key factors upstream of the NHEJ pathway. Ku-specific siRNA has been used to down-regulate the expression of Ku70 and Ku80 and significantly increase the frequency of CRISPR/ Cas9-mediated HDR in porcine fibroblasts [87]. Inhibition of DNA-PK by chemical inhibitors NU7441 and KU-0060648 resulted in a two-fold increase in the efficiency of the HDR repair pathway of Cas9-DSB in HEK-293T cells [88]. Considering the importance of NHEJ in maintaining genomic stability, inhibition of this repair pathway may lead to timely repair of DSB in cells, resulting in cell death [82] or late embryonic death [89]. Therefore, the safety of these inhibitors *in vivo* needs to be carefully evaluated.

Enhancing the activity of vital HDR factors to improve HDR efficiency

HDR agonists are considered to be a safer choice in clinical practice. RAD51 is a core protein in the HDR pathway, mainly involved in chain exchange and homology search. Rs-1 is a chemical agonist of RAD51, which can enhance

the binding activity of RAD51. Researcher [90] showed for the first time that RS-1 treatment could increase THE HDR stimulated by Cas9 in HEK-293A cells by 3-6 times and was successfully applied to CRISPR/ Cas9-mediated knocking in human pluripotent cells [91]. As a RAD51 homolog, RecA can effectively improve HDR efficiency by increasing the copy number of DNA plates through directional enrichment. Researcher [92] showed that the HDR efficiency of the Cas9/RecA system was higher *in vivo* and *in vitro*. Researcher [93] showed that the overexpression of RAD51 can increase the knocking efficiency of neural precursor cells by about 2 times. In addition to HDR agonists, Researcher [94] showed that overexpression of yRAD52 or Y R D 52-C A S 9 fusion protein increased the HDR frequency of HEK-293T and porcine PK15 cells. HDR in chicken cells using single-chain donor oligonucleotide (SSODN) as template increased by 3 times [95]. At the same time, Researcher [96] found that CTIP, RAD52, and MRE11 could improve the precision editing efficiency by 2 times when fused with Cas9. In addition, Researcher [97] also found that small molecule L755507 and Brefeldin A could increase the efficiency of large-fragment knocking by 2-3 times and the point mutation efficiency by 9 times.

Enhance HDR efficiency by regulating 53BP1 and BRCA1 proteins

53BP1 is a crucial regulator of repair selection between NHEJ and HDR. Researcher [98] found that 53BP1 inhibitor (I53) works by blocking the interaction between 53BP1 and H4K20ME2 and inhibiting the accumulation of 53BP1 at DSB. It increased CAS9-mediated HDR by a factor of 5.6 in different human and mouse cells. Mdn53BP1 enhances the activity of CAS9-mediated HDR in HEK-293T cells by competitively inhibiting the recruitment of 53BP1 to DSB [99]. Recently, Researcher [100] found that DNA damage response (DDR) factor RAD18 is an effective enhancer of CAS9-induced HDR, and RAD18 binds H2AK15Ub with a higher affinity than 53BP1. Thus, the recruitment of 53BP1 to DSB is suppressed to enhance the efficiency of HDR. As for the vital protein OF HDR pathway BRCA1, Researcher [90] stated that cells overexpressing wild-type BRCA1 or its recombinant variant (BRCA1K1702M, BRCA1M1775R) also showed a cas9-induced increase of HDR.

Inducing Cas9 activity to synchronize with cell cycle to increase HDR efficiency

Since the HDR pathway occurs in the S and G2 phases, the formation of Cas9-DSB is restricted to the S/G2 phase and synchronizes the formation of Cas9-DSB with the cell cycle to improve the efficiency of HDR. Cas9-dsb formation can be synchronized with the cell cycle by adding blockers such as Nocodazole, ABT751, or RO-3306. When Nocodazole was combined with direct delivery of the pre-loaded Cas9 RNP complex, cas9-mediated HDR increased 1.38 times in heK-293T cells treated with Nocodazole compared to non-synchronous cells. Cas9-mediated HDR increase in human primary fibroblasts and embryonic stem cells is effective [101]. ABT751 showed the same biological function as Nocodazole, with a six-fold increase in targeting efficiency in IPSCs [102]. Researcher [103] reported an increased ratio of HDR in human hematopoietic stem cells treated with RO-3306(CDK1 inhibitor). Researcher [104-106] found a new small molecule, XL413, targeting CDC7, which can temporarily stop the cell cycle in the S phase and increase the efficiency of HDR by 3.5 times. Because of their potential toxicity, their use *in vivo* is limited.

Summary and prospect

Using CRISPR technology, large animal models of disease could advance research into pathogenesis and gene therapy. HDR-mediated precision gene editing provides scientists with a tool that can precisely modify genomic DNA. Improving the efficiency of this tool will contribute to applying gene-editing technology in various fields, reduce non-specific gene editing events, improve the safety of gene targeting, and ultimately promote the development of human disease modeling and gene therapy research.

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