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REVIEW

Ways of improving precise knock-in by genome-editing technologies

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Abstract
Despite the recent discover of genome-editing methods, today we can say these approaches have firmly entered our life. Two approaches—knocking out malfunctioning gene allele or correcting the mutation with precise knock-in—can be used in hereditary monogenic diseases treatment. The latter approach is relatively ineffective. Modern data about the ways of repair of double-strand DNA breaks formed by nucleases are presented in this review. The main part of the review is devoted to the ways of increasing precise and effective knock-in: inhibition of non-homologous end joining and stimulation of homology-directed repair key factors, use of small molecules with unknown mechanism of action, cell-cycle synchronization and cell-cycle-dependent activity of Cas9, donor molecule design, selection, alternative methods for insertion and other approaches.

Introduction

Genome editing is a new gene therapy approach, based on the use of so-called programmable nucleases. The first by chronology two methods, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), are based on the use of FokI nuclease, which is directed to the targeted locus using specific DNA-binding domain ZFNs (Kim et al. 1996; Bibikova et al. 2002) or TALENs (Cermak et al. 2011; Tebas et al. 2014). In both systems, DNA-binding domains consist of amino acids capable for "recognizing" groups of nucleotides (ZFNs) or individual consecutive nucleotides (TALENs). In both cases, ZFNs or TALENs pairs are used for recognizing the target DNA sequence, and FokI nuclease-binding site appears to be between them (Kim et al. 1996; Cermak et al. 2011). The use of a pair of DNA-binding domains allows to significantly increase the specificity of these editing systems (Cox et al. 2015). Method CRISPR/Cas9 [clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (Cas9)], in turn, is based on the use of an RNA-mediated recognition of target locus and nuclease Cas9. Single-guide RNA consists of specific to target DNA part (20–22 nt) and universal scaffold (about 80 nt) for Cas9 binding. PAM sequence (NGG for SpCas9 or NNGRRT for SaCas9) in target DNA locus close to sgRNA complementary sequence is necessary for Cas9 activity (Jinek et al. 2012; Zhang et al. 2014).

All of the described methods have advantages and disadvantages: ZFNs and TALENs are more specific and have small off-target effects, but CRISPR/Cas9 is simplest and easy for multiplexing. The main advantage of TALENs in comparison with ZFNs is a simple code for "recognizing" base pairs of the target locus (Cox et al. 2015). The problem of biallelic editing, in which one allele is edited correctly, whereas indel in the second is formed remains actual for all genome-editing systems (Paquet et al. 2016). The frequency of correct homozygous editing is very low and directly depends on gene activity: inactive genes are not subject to biallelic editing by any methods (Hockemeyer et al. 2009, 2011; Wang et al. 2015; Takayama et al. 2017), whereas active ones can be edited: by CRISPR/Cas9—up to 4% (Takayama et al. 2017), by ZFNs—up to 9% (Hockemeyer et al. 2009), and by TALENs—up to 16% (Hockemeyer et al. 2011).

All nucleases create double-strand DNA break (DSB), which initiates repair in one of the two ways: a non-homologous end joining (NHEJ) or homology-directed repair (HDR). In the first case, the DNA ends on the sides of the break are ligated together, while random indels (insertions or deletions of several nucleotides) are usually formed at the ligation site. It could create frame shifts and premature

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stop codon. This approach can be used to gene's knock-out. In the second case, in the presence of donor DNA [double-stranded DNA or single-stranded oligodeoxyribonucleotides (ssODN)], HDR occurs. This approach allows to insert both a small and rather large DNA fragment (Ran et al. 2013b).

Genes' knockout is widely used to develop the treatment of various diseases, including HIV infection (Tebas et al. 2014), cancer (Su et al. 2016), and autosomal dominant hereditary diseases, caused by the gain-of-function mutations (Bakondi et al. 2016). However, for other monogenic disorders, the gene knockout approach is undesirable because of necessary to correct the mutation. Therefore, it is very important to achieve a precise and effective knock-in. Many approaches to achieve this are described and will be considered in this article.

DNA repair pathways

Main types of repair processes

Double-strand break can be repaired by one of the two ways—by NHEJ or HDR.

NHEJ

NHEJ is a major repairing pathway, which is crucial for cell viability (Reid et al. 2015). About a half of DSBs in yeast and mammalian cells are repaired with precise NHEJ and its dysfunction leads to different developmental disruptions, growth retardations, and increase in level of DSB-associated chromosomal mutagenesis (Chiruvella et al. 2013). Non-homologous end joining acts during all phases of cell cycle and based on joining two DSB ends by direct ligation (Lieber 2010).

Stages of NHEJ *Break recognition and binding* First stage of NHEJ is end binding by Ku contains the Ku70 and Ku80 subunits, which formed a heterodimeric structure (Reid et al. 2015). It is supposed that each of the two DNA ends has it is own Ku–DNA complex that allows them modify separately from each other (Lieber 2010). After binding, Ku–DNA complex becomes a point of recruiting for such enzymes as polymerases, nucleases, and ligases (Lieber 2008). Another function of Ku is supporting end stability and preventing its degradation (Fell and Schild-Poulter 2015). The next events linked with DNA-PKcs, a catalytic subunit of DNA-dependent protein kinase (DNA-PK). Ku is also the part of DNA-PK holoenzyme, but it cannot form stable complex with catalytic subunit in the absence of DNA (Yaneva et al. 1997). Binding to the DNA-PKcs occurs predominantly through Ku80 (Radhakrishnan and Lees-Miller 2017).

End processing DNA-PKcs can regulate activity of different enzymes by phosphorylation and remodeling. One of the main participants of NHEJ is Artemis nuclease (encoded by *DCLRE1C* gene) that exists in bond with DNA-PK. Artemis: DNA-PKcs complex has several kinds of activities: 5′- and 3′-endonuclease and 5′-exonuclease activity, and 3′-phosphoglycolate processing activities also (Ma et al. 2002). Role of artemis is in cutting of DNA overhangs for making blunt ends. It is necessary for the next ligase sealing additional nucleotides can be provided by polymerases of Pol X family: Pol m (mu) and Pol l (lambda) (Lieber 2010).

Ligation of blunt ends The specific enzyme for NHEJ is Ligase IV (LigIV), which is the most suitable for this process due to its wide flexibility, allowing for sealing incompatible ends as well as gaps in DNA (Cottarel et al. 2013). Besides, only LigIV has ability to ligate DNA strands separately and independently of each other. Usually, LigIV acts in complex with X-ray Repair Cross Complementing 4 (XRCC4) and XRCC4-like factor (XLF) (Chen et al. 2000).

These stages are related to the so-called canonical NHEJ or c-NHEJ, which is dominant in G1 and late G2 phases (Lieber 2010).

MMEJ

To speak about S and late G2 phases, there is another end-joining pathway called alternative-NHEJ (a-NHEJ). Among subgroups of a-NHEJ, there is microhomology-mediated end joining or MMEJ, which has a feature to use microhomological sequences with length from 5 to 25 base pairs (for c-NHEJ it is 0–3 bp) (Truong et al. 2013). MMEJ is classified as high error-prone pathway (McVey and Lee 2008).

Despite the same process of joining the cut ends, a-NHEJ has differences from canonical mechanism (Figs. 1, 2). First of all, it starts from end resection, which is made with MRN/BRCA1/CtlP complex (Truong et al. 2013). Then, free 3′ ends anneal in microhomology locus. Bulge ends are cut with XRCC1, which is similar to XRCC4 in c-NHEJ (Seol et al. 2018). In addition, another type of ligase, Ligase III, works in MMEJ (Liang et al. 2008). MMEJ usually results in small insertions and deletions which depends on various cases of annealing (Sinha et al. 2016).

HDR

Homology-directed repair (HDR, HR) is a second major mechanism in correcting DNA double-strand breaks. It becomes active only after DNA replication, thus, in S/G2 phases (Aparicio et al. 2014). In contrast to NHEJ, this pathway has no errors, because in the case of replication, there are two identical chromosomes and one of them is used as a matrix for repair (Fig. 3). Interestingly, that initial steps of HDR are similar to one in a-NHEJ, though it is not fully

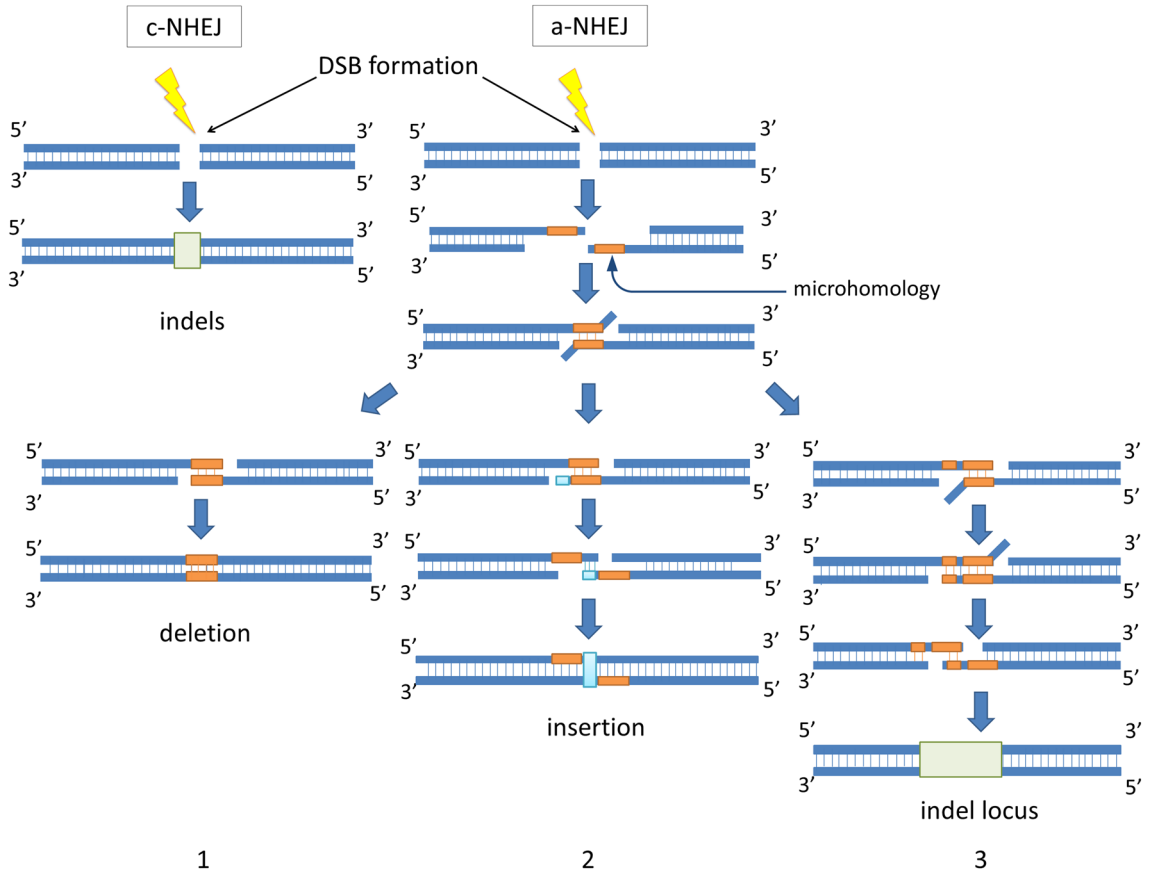


Fig. 1 Comparison of c-NHEJ and a-NHEJ mechanisms. The c-NHEJ results in indel formation during end processing and gap filling. The a-NHEJ can act through several ways that lead to different results: deletions (1), insertions (2), or mixed modifications (3). (1) The classical mechanism of a-NHEJ occurs when bulge ends are cut and deletion are formed; (2) when gaps filled with untemplated mechanism, a small new DNA region is synthetized and insertions can be formed; (3) in some cases, there is another small microhomology locus on DNA that can result in both insertions and deletions, thus, in the formation of indel locus

known, how these pathways divide later (Verma and Greenberg 2016).

1. The beginning of HDR is a two-stage end processing. The very first step is resection with MRN (Mre11–Rad50–Nbs1) complex. As HDR requires longer 3′-ends, there is the second step—deep resection with other nucleases, Exo1 and Dna2 (Symington and Gautier 2011).
2. As free single-stranded DNA end is very unstable, several proteins for maintenance and protection cover it. Heterotrimeric RPA first binds DNA, after that it is replaced with Rad-51 and forms a filament for intrusion (Pellegrini et al. 2002).
3. 3′ DNA filament intrudes in the identical chromatid and creates special structure called D-loop for synthesis of identical DNA sequence (Kakarougkas and Jeggo 2014).
4. Created structure is called as Holliday structure. It results in synthesis of lacking DNA sequence and crossover/non-crossover strands.

Thus, on the final step, cell has two identical chromatids, and HDR can occur with or without crossing over between them, what depends on subtype of homology recombination (Ceccaldi et al. 2016).

Single-strand annealing

Among subtypes of HDR, a pathway called single-strand annealing (SSA) can be highlighted because of its very specific mechanism. SSA is considered as a type of HDR when DSBs occur between two repeat sequences. In contrast to homological repair, it does not require homological chromosome, and function of matrix is performed by complementary strand (Sung 1997). In some sense, SSA reminds MMEJ, because both depend on homological loci, though they are longer in the case of single-strand annealing (Ceccaldi et al. 2016).

The mechanism of SSA is similar to microhomological end joining, but involved proteins are similar to ones in HDR.

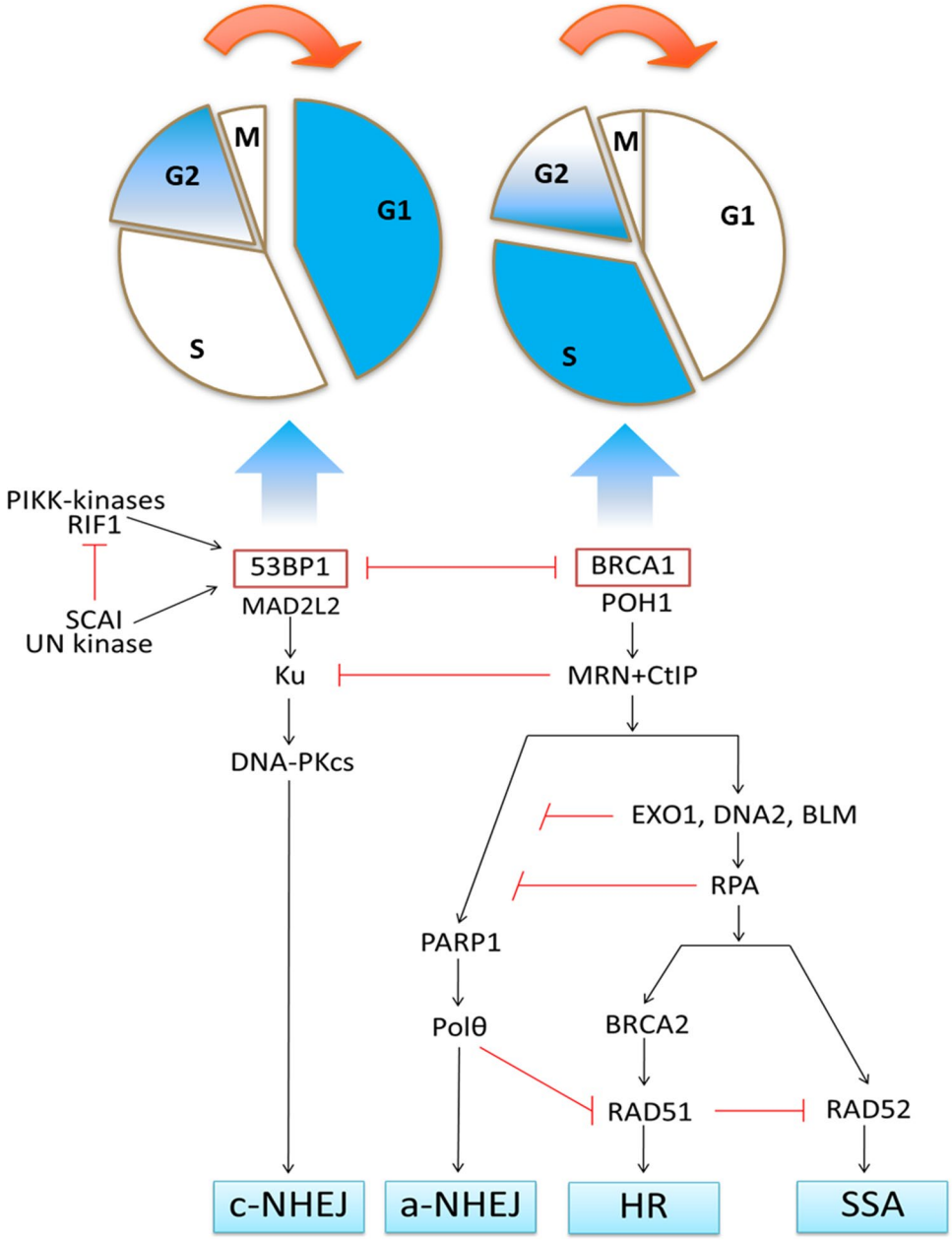


Fig. 2 DNA repair pathway regulatory network

First step begins with 5′–3′ end resection with MRN/BRCA1/CtIP complex (Garcia et al. 2011). During formation, new 3′-ends are covered by RPA protein, which supports them in single-strand condition. The next step—annealing—includes RAD-52 binding. This protein controls DNA–DNA interaction between complementary parts to join them (Verma and Greenberg 2016). Then, complex of nucleases Rad1–Rad10 removes the bulge ends (Saparbaev et al. 1996). DNA polymerase fills the gaps, and ligase sews them.

Repair pathway choice and regulation mechanisms

Choice between 53BP1- and BRCA1-pathways

The main question, which researchers had been asking for many years, is how cell uses different types of repair in different periods of cell life and how this complex switching network is regulated.

First and global problem is choice between BRCA1-mediated and 53BP1-mediated mechanisms (Fig. 2).

It is known that NHEJ can be active also in S and G2 phases, but that time, it is suppressed by HDR (Kakarougkas and Jeggo 2014). The question is what mechanism allows 53BP1-replacing with BRCA1 and why is it active in this particular time?

The main step for all BRCA1 pathways is end resection of damaged ends. For initiating this process, the special complex MRN needs to get an access to damaged ends (Symington and Gautier 2011). However, in fact, access is blocked by 53BP1-protein, which together with Ku locates on damaged DNA ends. It was confirmed that the role of 53BP1 is permanent inhibition of HR, and making DSB not available for HR factors in G1 phase.

Many factors are involved in replacing 53BP1 with BRCA1. First of all, these are kinases that directly interact with 53BP1 and BRCA1. For NHEJ, phosphorylation of 53BP1 in S/TQ sites stimulates recruiting RIF protein to DSBs (Callen et al. 2013). Function of RIF1 protein is inhibition of end resection and this complex stay on the DNA until entering S phase (Chapman et al. 2013). Phosphorylation of 53BP1 can be stimulated by various PIKK-kinases, including DNA-PK catalytic subunit, ATM and ATR kinase. It was revealed recently that when S phase starts, phosphorylation of 53BP1 occurs again, but this phosphorylation is in other sites (S/TP) and it is promoted by other, yet unknown kinases (UN kinases, CDKs, and MAPKs are proposed to be good candidates for that). Phosphorylation in S/TP sites recruits SCAI protein, which inhibits RIF1 and stimulates its dissociation from 53BP1 (Isobe et al. 2017).

One the other hand, at the same time, cell-cycle-dependent phosphorylation of CtIP nuclease by CDK promotes binding with BRCA1 and forming CDK–BRCA1–MRN–resection complex (Ferretti et al. 2013). This complex does not only perform resection, but also deletes Ku from DNA strand (Langerak et al. 2011). Moreover, it was shown that BRCA1 removes 53BP1 through the ubiquitination-dependent manner with participation of POH1, proteasome component (Kakarougkas et al. 2013). Thus, BRCA1 complex and other necessary proteins get access to DNA. It was recently shown that TIRR1 can take part in pathway choice. It was proposed that TIRR1 binds to the 53BP1 in normal condition by masking histone-binding motif, and maintains its expression (Drané et al. 2017). However, when DSBs occurs, phosphorylation of 53BP1 stimulates dissociation of 53BP1–TIRR1 complex and recruits RIF1. Interesting that both depletion and overexpression of TIRR1 cause defective function of 53BP1 and enhance HDR (Zhang et al. 2017a, b). Another protein, MAD2L2, which is protein of mitosis checkpoint, makes complex with RIF1 and 53BP1 in G1, and also suppresses BRCA1-mechanisms in S/G2 phases, when it is present ectopically (Boersma et al. 2015). In addition, depletion of MAD2L2 can enhance HDR despite normal function of 53BP1 and RIF1 (Simonetta et al. 2018). To sum up, nowadays, we have several proteins as targets to increasing percentage of BRCA1-mediated pathways.

Another factor that influences pathway choice can be chromatin condition. 53BP1 can bind to DNA with special histone marks: H2AK15ub, which is specific for DSBs, and H4K20me2 (Fradet-Turcotte et al. 2013; Botuyan et al. 2006). It was unclear how the last can influence repair being non-linked with DSBs directly. However, it was revealed that the number of H4K20me2 is reduced in S/G2 and increases in G1 by some unclear reasons. Other words, chromatin remodeling presents us another level of pathway choice regulation (Simonetta et al. 2018).

Choice between BRCA1-mediated pathways

Another one object is choice between BRCA pathways. Note that all mechanisms act at the same time; thus, we cannot speak about cell-cycle factors.

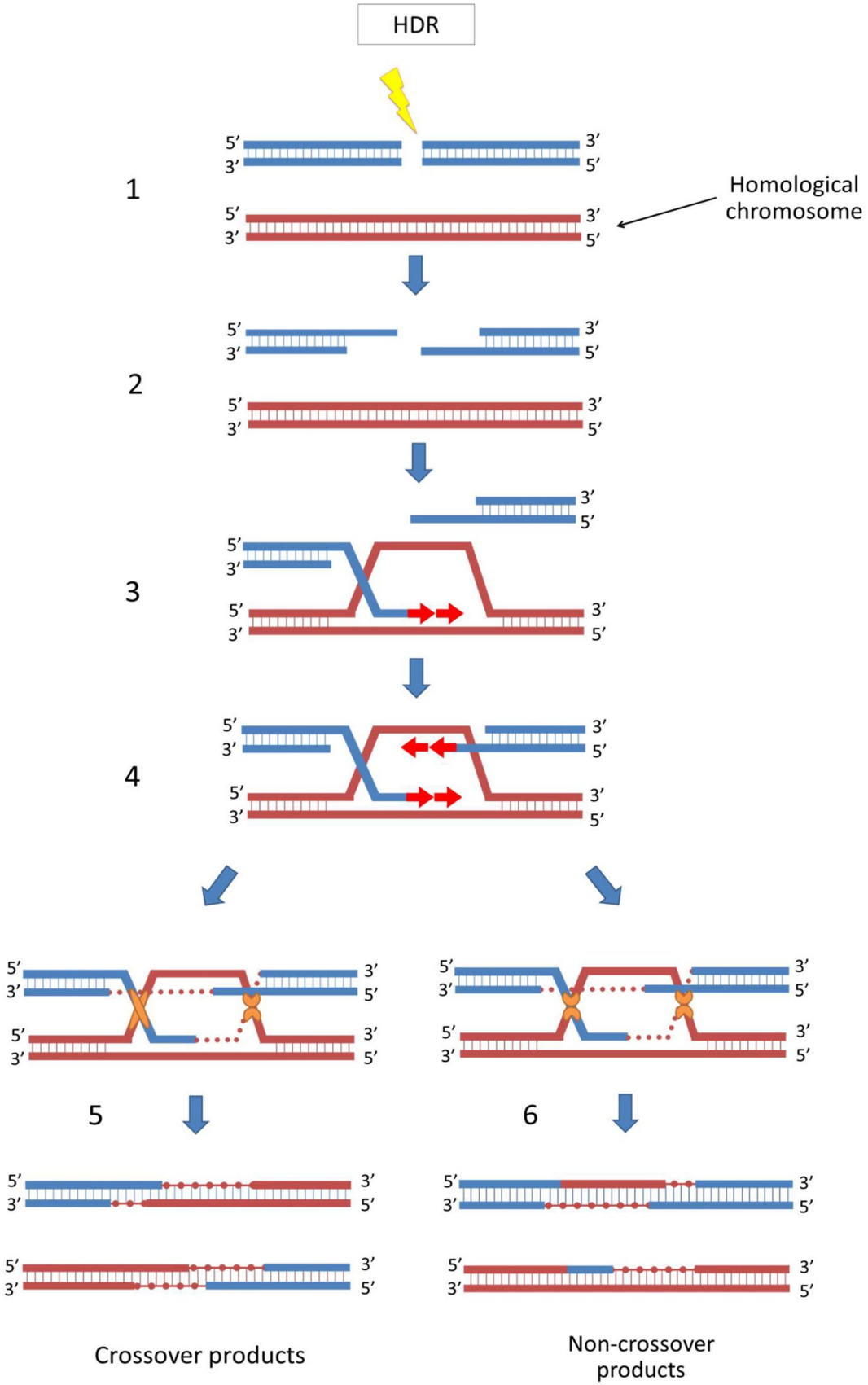
The main ways of BRCA1-mediated repair are classical HR, SSA, and a-NHEJ (some authors distinguish MMEJ as a subtype of a-NHEJ, others make these concepts synonymous, and we consider that nevertheless, these ways have the same trigger points and factors) (Verma and Greenberg 2016).

End resection is the general step for all three ways. However, the extent of resection is a factor related to balance between both HR and SSA vs. MMEJ. In fact, end resection contains two parts: “end clipping” by MRN complex together with CtIP, and “extensive resection” with contribution of EXO1, DNA2, and BLM proteins (Ceccaldi et al. 2016). If the resection stops after first stage, MMEJ occurs. Otherwise, longer resected ends are generated, and microhomology connection is no longer available (Symington and Gautier 2011).

RPA binding also was shown as process that prevents repair through MMEJ (Deng et al. 2014). This step is also required for HR and SSA, so it is another HR-SSA-factor.

Speaking about positive a-NHEJ regulators, PARP1 has been described as modulator of this pathway (Audebert et al. 2008). It acts as a platform for recruiting of a-NHEJ repair factors, such as TLS polymerases (for example, Polθ) (Ceccaldi et al. 2016). Polymerase θ itself also serves as factor providing of a-NHEJ (Chan et al. 2010). On one hand, it binds to Rad-51 and thus suppresses formation of Rad-51–DNA filament; on the other hand, through binding to resected ends, Polymerase θ can extend each strand using other overhang as template, thus providing microhomology pairing (Ceccaldi et al. 2015; Kent et al. 2015).

Therefore, the key choice between HR and SSA occurs after process of RPA covering of resected ends. Each way has several choice factors, and they interrelationships determine the method of repair.


HDR
1
Homological chromosome
2
3
4
5
6
Crossover products
Non-crossover products

◂**Fig. 3** Most common mechanism of HDR. After double-strand break induction (1) the repair starts with resection of 5′-ends of the lesion (2). Then, stabilization of single-strand ends and DNA filament invasion in the homological chromosome occurs (3). The filament search for homology locus and complementary binds with it and then promotes synthesis of the lack bases (4). After this stage, strands become able to form a Holiday junctions—a key intermediates of HR mechanism that can be resolved with crossover (5) or non-crossover (6) products' generation

For homological repair, the crucial factor is the presence of certain template (Karanam et al. 2012). In the case of lack of preferred template (sister chromatide), repair switches on SSA. The same result will be expected when strand invasion fails (Bhargava et al. 2016). Striking into, one of the main positive regulator of HR is BRCA2 that mediates loading of Rad-51 to DNA (Ceccaldi et al. 2016). There is some evidence that RAD51 can prevent SSA through RAD52 (Wu et al. 2008).

Knock-in enhancement

Inhibition of NHEJ key factors

To increase HDR rate, the proteins participating in NHEJ can be inhibited or their expression reduced. Using ZFN injected into embryos, Beumer et al. showed that in *Drosophila melanogaster* lacking DNA ligase IV (LigIV), HDR rate was significantly greater than in wild-type (WT) flies (up to 99% flies carried corrected allele). This observation was valid for both types of donor DNA templates—plasmid and ssODN (Beumer et al. 2008, 2013). Raghavan and colleagues (Srivastava et al. 2012) synthesized LigIV inhibitor Scr7 as DNA Ligase I/III/IV inhibitor L189 (Chen et al. 2008) derivate. In series of experiments, authors demonstrated that significant dose-dependent decrease of NHEJ in different models in vitro and in vivo. The main mechanism of Scr7 action is inhibition of LigIV interaction with DNA (Srivastava et al. 2012).

Subsequently, the addition of Scr7 in culture media during transfection was often used to enhance HDR in genome editing with varying success (Table 1). It was shown that Scr7 in CRISPR/Cas9 experiments could increase HDR rate in porcine fetal fibroblasts (twofold, up to 47% with G418 selection) (Li et al. 2017), HEK293 (fivefold) (Chu et al. 2015), HEK293T (twofold) (Robert et al. 2015), A549 (threefold enhanced HDR), and MelJuSo (19-fold) cell cultures (Maruyama et al. 2015), perhaps due to decreased expression of some NHEJ factors (*LIG4, MRE11, DCLRE1C*, and *XRCC4*) (Li et al. 2017). In experiments in murine zygotes, Scr7 increased HDR rate of tenfold (Singh et al. 2015) and twofold (Maruyama et al. 2015) and according to Maruyama et al. (2015) contributed correct homozygous editing. However, according to other publications Scr7 either does not demonstrate any effects [in rabbit embryos (Song et al. 2016), iPSCs (Zhang et al. 2017), human embryonic stem cells (Yang et al. 2016) and porcine fetal fibroblasts (Gerlach et al. 2018)], or only slightly increases the HDR rate [CHO (Lee et al. 2016) and HEK293A cells (Pinder et al. 2015)]. In addition, it is shown that Scr7 inhibited cell growth and decreased transfection efficiency of CHO cells in dose-dependent manner (Lee et al. 2016). Possible explanation of such discrepant effects of Scr7 in various cell cultures is the difference in LigIV expression—cell cultures with high LigIV expression are more sensitive to Scr7 (Srivastava et al. 2012). Recently, Greco et al. noticed that Scr7 inhibits DNA Ligase I and Ligase IIIα more than LigIV and suggested that effect of increasing HDR is determined by other mechanisms, rather than by inhibition of LigIV, especially when using low (less than 200 uM) doses of Scr7 (Greco et al. 2016).

Adenovirus 4 (Ad4) E1B55K and E4orf6 proteins which mediate the ubiquitination and proteasomal degradation of LigIV can also be used for HDR enhance. Chu et al. demonstrated that added to culture media Ad4 decreased NHEJ eightfold (to 0.7%) and increased HDR sevenfold (up to 36%) in HEK293 cells (Chu et al. 2015). In HEK293T, E1B55K + E4orf6 enhanced HDR by 3.5-fold (Robert et al. 2015). In contrast, it has been shown that in iPSCs, E1B55K + E4orf6 decreased HDR rate by 2.5–4-fold depending on genome locus (Zhang et al. 2017).

Another approach to improving HDR rate is to inhibit expression of key genes involved in NHEJ by small interfering RNA (siRNA) or short hairpin RNA (shRNA). Knocking down of *XRCC6* (coding KU70), *XRCC5* (coding KU80) or *LIG4* (coding LigIV) genes can reduce NHEJ rate by twofold to threefold (Chu et al. 2015; Robert et al. 2015). In the paper of Ye et al., it was shown that the repression of such key NHEJ factors as KU70, KU80, and LigIV by CRISPRi increases the level of HDR by 2–3 times in HEK293T cell culture (Ye et al. 2018).

Inhibition of DNA-dependent protein kinase (DNA-PKcs) by siRNA or pharmacological inhibitors, such as NU7441 and KU-0060648, could increase HDR by twofold to fourfold (Robert et al. 2015). Despite the existence of multiple DNA-PKcs inhibitors (Hollick et al. 2003; Neal et al. 2011; Durisova et al. 2016), they are rarely used to improve HDR rate in genome editing, possibly because of their low efficiency, for example, in iPSCs (Zhang et al. 2017).

Ad4 with NU7441 or KU-0060648 could induce HDR by fivefold in HEK293T cells (Robert et al. 2015). Finally, combination of Scr7 and inhibitions of other NHEJ factors is used. In some cases, Scr7 with *XRCC6* and *LIG4* knock down demonstrated fivefold increase (up to 25%) of HDR (Chu et al. 2015), but in others, no effects were obtained (Shy et al. 2016). Combination of Scr7 with NU7441 or

Table 1 Scr7 treatment

Object	Scr7 dose	Donor DNA	Knock-in improving	References
A549 cells	0.01 uM	Plasmid	3.1-fold (3.1% vs. 1% in control)	Maruyama et al. (2015)
CHO cells	0.1–20 uM	Plasmid	None	Lee et al. (2016)
MelJuSo cells	1 uM	Plasmid	19.1-fold (19.1% vs. 1% in control)	Maruyama et al. (2015)
HEK293 cells	1 uM	Plasmid	Fivefold (normalized data)	Chu et al. (2015)
HEK293A cells	1 uM	Plasmid	None (11.7% vs. 9.9% in control)	Pinder et al. (2015)
HEK293T cells	1 uM	Plasmid	1.8-fold (normalized data)	Robert et al. (2015)
iPSCs	1 uM	Double-cut plasmid donor	None	Zhang et al. (2017a, b)
Porcine fetal fibroblasts	1 uM	ssODN	None (32.0% vs. 30.7% in control)	Gerlach et al. (2018)
Rabbit embryos	40 uM	Plasmid	None (9.4% vs. 7.1% in control)	Song et al. (2016)
Murine zygotes	50 uM	ssODN	9.7-fold (56.2% vs. 5.8% in control)	Singh et al. (2015a, b)
Porcine fetal fibroblasts	100 uM	Plasmid	1.9-fold with neomycin selection (49.7% vs. 26.2% in control)	Li et al. (2017)
Porcine fetal fibroblasts	200 uM	Plasmid	Twofold (11.2% vs. 5.6% in control)	Li et al. (2017)
Murine zygotes	1 mM	ssODN	2.2-fold (59.3% vs. 26.8% in control)	Maruyama et al. (2015)
Human embryonic stem cell lines H1-WA01	NA	Plasmid	None	Yang et al. (2016)

KU-0060648 stimulated HDR by threefold (Robert et al. 2015).

Summarizing, indeed, enhancing of HDR is shown by inhibiting key NHEJ factors for some cell lines. The danger of NHEJ inhibition is that double-strand DNA breaks can accumulate in the cell, leading to increased apoptosis and cell death (Vartak and Raghavan 2015; Chen et al. 2008), so this approach may cause increased cytotoxicity and should be used with caution.

Stimulation of HDR key factors

Another way to switch DNA repair from NHEJ to HDR is to stimulate key HDR factors. Bozas A. et al. demonstrated necessity of RAD51 in HDR in *Drosophila*. *SpnA* (*Rad51*) deficient flies demonstrated significantly lower HDR rate when using ZFN, than the WT flies (Bozas et al. 2009). Jayathilaka et al. in pre-genome-editing era based on screening of 10,000 small molecules discovered 3-[(benzylamino)sulfonyl]-4-bromo-*N*-(4-bromophenyl) benzamide, which was called RAD51-stimulatory compound 1 (RS-1). RS-1 demonstrated twofold increase of key HDR factor RAD51 activity due to its stabilization, enhancing DNA binding with this protein, and stimulation homologous strand assimilation activity (Jayathilaka et al. 2008). Later, usefulness of added to culture media RS-1 was shown in genome editing using different methods (Pinder et al. 2015; Song et al. 2016; Pan et al. 2016). In HEK293A, RS-1 enhanced HDR rate by sixfold (up to 21%) using Cas9 and by fourfold (up to 4%) using $Cas9^{D10A}$ nickase (Pinder et al. 2015); in COS-7 cells using FokI-dCas9—by 1.3–1.4-fold (up to 26.7%) (Pan et al. 2016). In rabbit embryos, RS-1 also increased HDR rate by sixfold for ROSA26-like locus (*RLL*) (up to 26.1%) and 2.4-fold for *CFTR* gene in vitro. *EGFP* knock-in was greater in RS-1 treatment (26.3% vs. 7% without RS-1) in vivo (Song et al. 2016). RS-1 may enhance HDR whatever HDR template is used—dsDNA (Pinder et al. 2015) or ssODN (Pan et al. 2016). It is also shown that RS-1 did not affect NHEJ (Pinder et al. 2015) and did not alter reproduction in rabbits (Song et al. 2016). However, in iPSCs, RS-1 did not increase HDR rate (Zhang et al. 2017).

It is also possible to perform that overexpression of the genes involved in HDR. It has been shown that the addition of mRNA of *BRCA1* slightly increased HDR rate in HEK293A cells (Pinder et al. 2015); meanwhile, *RAD51* overexpression led to sixfold increase of knock-in (25% vs. 4.4% in control) in rabbit embryos (Song et al. 2016). However, in study performed by Zhang et al. in iPSCs, *RAD51* overexpression leads to decrease of HDR (Zhang et al. 2017).

Ye et al. showed that the activation of such key HDR factors as CtIP and CDK1 by CRISPRi increases the level of HDR by 2–4 times in HEK293T cell culture (Ye et al. 2018). Besides, it is possible to design a vector carrying the fusion gene SpCas9 and CtIP, a protein participating in HDR. It is shown that this approach increases HDR level by twofold in HEK293T cells and by 1.5-fold in iPSCs. It is not necessary to use the whole CtIP gene, a fragment from 1st to 296th nucleotides is sufficient (Charpentier et al. 2018).

Based on published studies, it seems that HDR stimulation is more effective way of precise knock-in than NHEJ inhibition.

Use of small molecules with unknown mechanism of action

Different small molecules with unknown mechanism of action in DNA repair, added to culture media, can also be used for improving HDR (Table 2). Discovered in 1998 (Parmee et al. 1998) selective agonists of the human β_3 adrenergic receptor, L755507, in high-throughput chemical screening assay showed twofold (33.3% vs. 17.7% in control) HDR increase in mouse ES cells with CRISPR/Cas9. In other cell cultures, L755507 demonstrated improving HDR by twofold with plasmid DNA donor, and ninefold enhancing of HDR using ssODN (Yu et al. 2015). In porcine fetal fibroblast, L755507 increased HDR rate up to twofold with and without cell selection, probably due to enhanced HDR key factors expression, or arrest cells at the S phase (Li et al. 2017). However, in study published by Pinder et al., L755507 did not demonstrate any HDR improvement (Pinder et al. 2015). Indel formation was decreased by 14% in human iPS cells using L755507 (Yu et al. 2015).

Discovered in 1968 (Handschin et al. 1968) as antiviral agent in *Penicillium brefeldianum*, Brefeldin A prevents protein transport from endoplasmic reticulum to the Golgi apparatus (Ktistakis et al. 1992; Helms and Rothman 1992). As L755507 Brefeldin A was estimated to increase HDR in CRISPR/Cas9 using high-throughput chemical screening assay, performed by Yu et al. and demonstrated improvement of HDR by twofold in mouse ES cells (Yu et al. 2015).

Resveratrol is necessary dietary supplement found in some plants (Vang et al. 2011). In a single study, based on the hypothesis that resveratrol potentially reduces the expression of NHEJ key factors, it has been shown that this molecule in small concentrations reduces expression of *LIG4, MRE11*, and *XRCC4* in porcine fetal fibroblasts. Resveratrol demonstrated increased HDR rate by threefold without selection (up to 15%) and twofold with G418 cell selection (up to 46%). Authors suggested that mechanism of Resveratrol effect on HDR enhancing is its possibility to arrest cells at the S phase (Li et al. 2017).

Cell cycle synchronization

G2/M phase

Nocodazole is widely used to arrest cells in G2/M phases due to disturbance of microtubules formation (Luduena and Roach 1991; Lu et al. 2012), which can be used for HDR improvement in genome-editing technologies. Nocodazole treatment leads to reversible cell-cycle synchronization in various cell cultures, including human pluripotent stem cells (hPSCs) and iPSCs (Lin et al. 2014; Yang et al. 2016; Zhang et al. 2017), but not in primary neonatal fibroblasts and embryonic stem cells (Lin et al. 2014). In HEK293T, Nocodazole improved HDR rate with CRISPR/Cas9 and ssODN as template by 1.4–6-fold, depending on-target locus (Lin et al. 2014). In iPSCs—by 1.7-fold (from 13 to 22%) (Zhang et al. 2017). Yang et al. demonstrated that up to 80% of hPSCs were in G2/M phase after Nocodazole treatment. HDR rate increased by 3.5-fold (up to 1.5%) without cell selection and up to 78% using Cas9 nickase and antibiotic selection. Knock-in was also improved by threefold to six-fold using other genome-editing approaches—ZFNs and

Table 2 Small molecules

Small molecule	Cells	Donor DNA	Knock-in improving	References
L755507	Mouse ES cells	Plasmid	1.9-fold (33.3% vs. 17.7% in control)	Yu et al. (2015)
	HUVEC	Plasmid	Twofold (1.8% vs. 0.9% in control)	Yu et al. (2015)
	K562	Plasmid	1.6-fold (1.3% vs. 0.8% in control)	Yu et al. (2015)
	HeLa	Plasmid	1.5-fold (5.1% vs. 3.5% in control)	Yu et al. (2015)
	Fibroblast CRL-2097	Plasmid	1.7-fold (6.0% vs. 3.5% in control)	Yu et al. (2015)
	Human ES cell-derived cells (neural stem cells)	Plasmid	1.3-fold (1.0% vs. 0.8% in control)	Yu et al. (2015)
	Human iPS cells	ssODN (200 nt)	8.9-fold (3.13% vs. 0.35% in control)	Yu et al. (2015)
	Porcine fetal fibroblasts	Plasmid	1.9-fold (10.9% vs. 5.6% in control)	Li et al. (2017)
	Porcine fetal fibroblasts	Plasmid	Twofold with neomycin selection (51.6% vs. 26.2% in control)	Li et al. (2017)
	HEK293A	Plasmid	None (10.1% vs. 9.9% in control)	Pinder et al. (2015)
	iPSCs	Double-cut plasmid donor	None	Zhang et al. (2017a, b)
Brefeldin A	Mouse ES cells	Plasmid	Twofold (27.2% vs. 17.7% in control)	Yu et al. (2015)
	iPSCs	Double-cut plasmid donor	1.3-fold (170% vs. 13% in control)	Zhang et al. (2017a, b)
Resveratrol	Porcine fetal fibroblasts	Plasmid	2.7-fold (15% vs. 5.6% in control)	Li et al. (2017)
	Porcine fetal fibroblasts	Plasmid	1.8-fold with neomycin selection (46% vs. 26.2% in control)	Li et al. (2017)

TALENs. Besides reversibility of cell synchronization, it has also been demonstrated that Nocodazole did not affect the pluripotency of stem cells (Yang et al. 2016).

Like Nocodazole ABT-751 disrupts microtubules formation, so synchronizes cells in G2/M phases (Hande et al. 2006). In work, published by Yang et al., authors demonstrated 3.1-fold improving of HDR rate in different hPSC lines (up to 1.35%) using CRISPR/Cas9 without selection. Cell synchronization was reversible and did not alter pluripotency (Yang et al. 2016).

Vinblastine binds to tubulin and blocks microtubule dynamics (Fernandez-Garcia et al. 2010), so may cause cell synchronization in G2/M phases. Rahman and colleagues demonstrated that Vinblastine induced G2 cell arrest in immortalized cell cultures HeLa, HT-1080, and U-2 OS. However, HDR rate was increased in some of these cell cultures, but not increased in umbilical cord-derived mesenchymal stem cells using meganuclease I-*Sce*I and ZFNs (Rahman et al. 2013).

It has been shown that indirubins can cause cell-cycle arrest in G1/S or G2/M in various cell cultures due to inhibition of several cyclin-dependent kinases (Hoessel et al. 1999; Eisenbrand et al. 2004). In published by Rahman et al. study indirubin-3′-monoxime was shown to induce G2/M arrest in cell lines HeLa, HT-1080, and U-2 OS, which leads to increase of HDR rate by 2–5-fold using meganuclease I-*Sce*I and ZFNs. In addition, in mesenchymal stem cells, indirubin-3′-monoxime also leads to HDR increase by tenfold (Rahman et al. 2013). However, it has been shown that indirubins (indirubin and its derives) may lead to increased apoptosis due to pro-apoptotic Bcl-2 family members stimulation (Shi and Shen 2008).

A number of studies have shown that lithium chloride (LiCl), a specific inhibitor of glycogen synthase kinase-3β (Stambolic et al. 1996), can also be used to synchronize different cell cultures (Ha et al. 2014; Mao et al. 2001; Lee et al. 2016). In one of the works, it has been demonstrated that after 10 mM LiCl addition, 54.7% of CHO cells were in G2/M phase on day 3, but without treatment, only 39% of cells were in such a phase ($p < 0.05$) (Ha et al. 2014). In another study, it was also shown that 10 mM LiCl increased bovine aortic endothelial cells in G2/M phase up to 40.4% (Mao et al. 2001). However, in human prostate carcinoma LNCap cells, LiCl along did not significantly increase number of cells in G2/M phase (Azimian-Zavareh et al. 2012). Despite the fact that LiCl leads to G2/M cell-cycle arrest in CHO cells, this does not result in an increase in HDR rate with CRISPR/Cas9 (Lee et al. 2016).

S phase

Aphidicolin blocks DNA synthesis due to inhibition of DNA polymerase-α and δ and leads to S phase cell arrest (Pérez-Benavente and Farràs 2016). Synchronization in S phase of primary neonatal fibroblasts, embryonic stem cells (ESCs) (Lin et al. 2014), and HCT116 cells (Rivera-Torres et al. 2014) increased HDR rate by 1.3-fold, 1.6-fold, and 2–3-fold, respectively. As in Nocodazole treatment, ESCs after Aphidicolin synchronization kept their pluripotency (Lin et al. 2014).

It was shown that in bacteria hydroxyurea, inhibitor of ribonucleotide reductase, caused S-phase arrest in all tested strains and increased HDR by twofold to eightfold in different strains and loci (Tsakraklides et al. 2015). Mammalian cells are also successfully synchronized in the S phase when hydroxyurea is added (Borel et al. 2002), but to date, there are no data about its influence on HDR.

2′,3′-dideoxycytidine (ddC) can slow down replication fork movement, which leads to extension of S phase—up to 70% of DLD-1 cells were in S phase after 24 h post treatment. Brachman and Kmiec (2005) demonstrated significant increase of HDR rate by threefold (3% vs. 1% in untreated control) with the addition of ddC.

Cyclin D1 (CCND1) induces cell-cycle transition from G0/G1 to S phase (Baldin et al. 1993). Nevertheless, the addition of CCND1 slightly increased HDR in iPSCs and worked only in combination with Nocodazole (Zhang et al. 2017).

Cell synchronization, especially in the G2/M phase, significantly increases HDR rate by 1.4–6-fold in different cell cultures using various methods of genome editing. Synchronization of cells is reversible and does not affect the pluripotency of stem cells, allowing to be widely used. However different chemical agents have different effects on various cells, so it is necessary to perform preliminary experiments to identify the most optimal substance.

Cell-cycle-dependent activity of Cas9

As described above, NHEJ and HDR dominate at different cell-cycle phases—G1 and S/G2, respectively. Thus, making Cas9 active exactly in S and G2 phases, we can increase homology-directed repair rate. In addition to chemical methods of cell-cycle synchronization (nocodazole, aphidicolin, etc.), recent studies use modifications to Cas9 itself—fusion of protein with molecules that active in certain cell-cycle phase.

Gutschner et al. made a fusion of Cas9 with geminin—the replication licensing factor which contains a destruction box motif in its N-terminal region mediating ubiquitinligase recognition. Cas9 was fused to the N-terminal region of human Geminin, and thus converted into a substrate for E3 ubiquitinligase complex APC/Cdh1 (Gutschner et al. 2016). This manipulation resulted in lower expression of Cas9 in G1 and higher expression in S/G2/M phases (Howden et al. 2016). Geminin-Cas9 fusion increased HDR efficiency at the

MALAT1 locus from ~9.7% to ~13.8% in HEK293T cells. Combination of this approach with nocozadole treatment increased HDR rate to 16.2% (Gutschner et al. 2016).

Later, Howden et al. showed reduced NHEJ rate, but the same HDR rate in iPSCs in *DNMT3B* and *SCN2A* loci using SpCas9-Geminin compared with unmodified SpCas9 (Howden et al. 2016). The last for nowadays study with SpCas9-Geminin demonstrated increased knock-in in porcine fibroblast cultures by 1.9 ± 0.1-fold in *MYH7* locus also (Gerlach et al. 2018).

Thus, the usage of a fused Cas9-Geminine in all published studies shows an absolute or relative increase of HDR level in different cell cultures, making it the most promising tool for enhancing HDR.

Donor molecule design

It has been shown that HDR rate depends on the matrix used for the DSB repair. In genome-editing studies, a double-stranded DNA (dsDNA) molecule (Chu et al. 2015; Shy et al. 2016; Beumer et al. 2013; Byrne et al. 2015; Zhang et al. 2017; Auer et al. 2014; Pinder et al. 2015; Yang et al. 2014) or short single-stranded oligonucleotide (ssODN) (Richardson et al. 2016; Yang et al. 2013; Beumer et al. 2013; Lin et al. 2014; Rivera-Torres et al. 2014) can serve as a donor molecule. In a study performed by Davis and Maizels (2014), it was shown that donor molecule determines how the DNA will be repaired—dsDNA initiates the canonical HDR, whereas ssODN—alternative HDR.

Double-strand DNA

In the classical HDR, occurring in the cell, the sister chromatid is used as the donor molecule for DNA repair. This molecule is double-strand and contains long homology arms (Waldman 2008). By analogy with it, in the case of genome editing, the plasmid with long fragment of DNA with homology arms more than 1 kb is most often used (Chu et al. 2015). In addition to the high specificity of insertion to target gene this approach allows to insert a large fragment (reporter or antibiotic resistance gene) into the target locus also, which may be used further for selection of cells with successful editing.

There are a lot of published works demonstrating rather high efficiency of HDR using dsDNA (Chu et al. 2015; Shy et al. 2016; Beumer et al. 2013; Byrne et al. 2015; Zhang et al. 2017; Auer et al. 2014; Pinder et al. 2015; Yang et al. 2014).

It is shown that lengths of insert and homology arms are very important for HDR efficacy (Shy et al. 2016). In an effort to improve correct knock-in efficacy, researchers modify the donor dsDNA molecule by increasing or decreasing the length of insertion and/or homology arms. Chu VT et al. showed the best efficacy of HDR using donor DNA with long homology arms (≥ 1 kb)—5.4–5.8% in HEK293 cells. Short homology arms (350 bp) decrease HDR rate by 2.5-fold (5% vs. 2%) (Chu et al. 2015). Shy et al. demonstrated that short insertion (less than 200 bps) and long arms (more than 1 kb) are better and increase on-target insertion threefold for insert length and threefold for arms lengths in mouse embryonic stem cells (Shy et al. 2016). In *Drosophila melanogaster* embryos, increased HDR efficacy with increasing of length of homology arms (HA) was shown using ZFN. HR was absent in cases of 200 bp HAs and increased up to 60% in LigIV lack embryos with HAs ≥ 2.1 kb (Beumer et al. 2013). Another study performed by Byrne SM et al. confirms the absence of HDR using dsDNA short HAs (100 bp) and very low HDR frequency in case of one short and second long HAs (3–4%) in human iPCS cells. The maximum HDR rate was achieved using dsDNA with long HAs (> 0.8 kb)—up to 17% (Byrne et al. 2015). However, it is also shown that very long homology arms (3.16 kb at 5′ and 1.0 kb at 3′ or > 4 kb both) do not increase, but even decrease the frequency of HDR in Drosophila embryos using ZFN (Beumer et al. 2008) and human iPSCs (Byrne et al. 2015), respectively.

Some studies show that more efficient recombination is observed with using linearized vectors compared to circular donors (Bibikova et al. 2003). Studies of ZFN activity performed on *Drosopila melanogaster* revealed that the expression of ZFN in the presence of a linear donor yielded in 15-fold higher HDR efficiency in the female germline and 60-fold in the male germline (Beumer et al. 2006). The ZFN study performed in 2013 year demonstrated that donor integration required ZFN-mediated linearization. First, the authors showed that a donor without a ZFN site was not integrated despite efficient cleavage of the target locus, and second, transfection of a donor without co-transfection of the corresponding ZFN also gave not targeted integration (Cristea et al. 2013). Conversely, another experiment demonstrated that co-injection of a circular plasmid is less toxic and more efficient in triggering plasmid integration at the desired locus. Auer et al. tested the efficiency of insertion linearized plasmids vs. circular in locus after CRISPR/Cas9 cleavage. Linearized donor DNA co-injected with sgRNA *eGFP* 1 and *Cas9* mRNA into one-cell stage zebrafish embryos increased death rate compared to circular plasmid (35% vs. 15%, respectively). Frequency of in-frame integration events was reduced compared to circular donor integration (11% vs. 76% with circular plasmid) (Auer et al. 2014).

Use of plasmid with insertion flanked by sequencing for sgRNA, which will cut and create a linear insert (double-cut donor) instead of the usual circular plasmid may be another approach to increasing HDR. A study by Zhang et al. showed that HDR frequency is increased by fourfold in the HEK293T cells culture with the mCherry insert (21.2% vs.

5.2%). At the same time in iPSCs cells, such a significant difference was not obtained: 12% vs. 10.4% when trying to insert mNeonGreen into *CTNNB1* locus. The main problem with double-cut donor—incorrect insertions, which include reverse insertions, insertions with HA and insertions of plasmid backbone. In all analyzed cases authors obtained incorrect insertions, in up to 50% (Zhang et al. 2017).

There is also evidence that certain ratio of Cas9 + gRNA/donor DNA plasmid ratio may influence HDR rate using CRISPR/Cas9. The best HDR efficacy in HEK293A to insert monomeric green fluorescent protein Clover was observed with 5:1 ratio (HDR rate 10% vs. 6% in the case of 1:1) (Pinder et al. 2015). It has also been shown that large deletions (> 1 kb) in donor dsDNA at both ends of the ZFN site increase HDR frequency by twofold in *Drosophila melanogaster* embryos with lack of DNA LigIV (20% vs. 44–48%) (Beumer et al. 2013). After successful editing of target locus, it can be repeatedly recognized by sgRNA and Cas9 will produce a DNA cut in this place. To avoid this, it is necessary to modify the donor DNA by inserting silent mutations with the aim to disturb homology with sgRNA and/or change the PAM (Yang et al. 2014).

ssODN

Single-stranded oligodeoxynucleotide (ssODN) is a short matrix for HDR. The main advantage of ssODN is its length (up to 200 bp), which allows to transfect large quantities into cell, so theoretically increase the effectiveness of HDR. However, length is also a disadvantage, because it does not allow the insertion of a large fragment of gene or gene for selection. There are many examples of successful application of ssODNs in genome editing (Chen et al. 2011; Ran et al. 2013b).

Many published works demonstrated different HDR efficacy using differently designed ssODNs (Richardson et al. 2016; Yang et al. 2013; Beumer et al. 2013; Lin et al. 2014; Rivera-Torres et al. 2014). Richardson et al. showed that the highest HDR frequency (up to 60%) is achieved when using ssODN, homologous to target strand (the strand to which sgRNA anneals), with total length of 127 nt and asymmetrical homology arms: 91 nt upstream and 36 nt downstream PAM (Richardson et al. 2016). Yang et al. received the highest HDR rate in iPSCs using ssODN 90 nt with symmetric homology arms (Yang et al. 2013), Yumlu et al. in similar cell culture obtained the best HDR efficacy using ssODN of 120 nt with symmetric homology arms (Yumlu et al. 2017). It was shown that 111 nt ssODN with 1 nt deletion around ZFN site to the sense DNA strand increased HR up to 30% in Drosophila melanogaster embryos with lack of LigIV (Beumer et al. 2013). In synchronized by nocodazole HEK293T cells, best HDR rate (21–28%) was received using 90 + 90 arms of ssODN with no influence of DNA strand for homology (Lin et al. 2014). In HCT116 cells, ssODN 72 and 100 nt demonstrated best HDR efficacy (Rivera-Torres et al. 2014). Robert et al. noted that antisense donor ssODN increased HDR level by threefold (normalized data) (Robert et al. 2015).

Notably, there is a serious problem that could appear after successful editing—locus can be detected and cut again, often repairing with NHEJ; therefore, HDR summary frequency will be lower. To solve this problem, ssODN with substitutions in locus of sgRNA junction or PAM locus was synthesized. This allowed increasing HDR efficiency in 2–10 times (Paquet et al. 2016).

Selection

Selection of edited cells may help to obtain higher proportion of HDR-corrected cells.

A donor HDR plasmid with the fluorescent reporter gene (Tálas et al. 2017) or antibiotic resistance (Firth et al. 2015) can be used. It allows inserting selective marker for FACS or antibiotic selection into the desired locus. However, this approach has several drawbacks: an additional insert increases the size of the plasmid, which can reduce transfection efficiency; selective marker gene will be inserted into the target locus and disrupt it, so it is necessary to cut it out after selection, which significantly increases the labor costs for genome editing. Therefore, researchers are developing new approaches for cell selection.

A plasmid containing the fluorescent reporter gene in addition to Cas9 and sgRNA can be used. In this case, successfully transfected cells can be selected with FACS. This approach allows increasing HDR frequency to 17% in iPSCs (Yumlu et al. 2017). One of the approaches based on cell sorting is fusion of Cas9 (Gopalappa et al. 2018) or other nucleases (Lonowski et al. 2017) with a fluorescent protein via a 2A peptide with further selection by FACS. This approach allows increasing the HDR rate up to 40 times in HEK293T cell culture (Gopalappa et al. 2018). Fluorescence enrichment also enhanced HDR by threefold in CHO cells (Lee et al. 2016).

Another approach is to use the temporary resistance of the edited cells to some antibiotic. So-called surrogate target plasmid (pBSR) with out-frame resistance gene against Blasticidin S (*bsr*) can be used. sgRNA targets locus upstream *bsr* and allows Cas9 to form DSB and indel resulting in reading frame restoration. Therefore, cell begins to produce resistance protein to Blasticidin S. After selection of resistant cells, it is possible to select the potentially edited cells. Subsequent discontinuation of antibiotic allows to get rid of the pBSR plasmid. Thus, it is possible to increase the HDR frequency up to 37% (in control 2%) in CH12F3 cells (Niccheri et al. 2017).

Editing cells can also be sorted by cell cycle. Yang et al. tested Nocodazole and ABT to arrest cells in the G2/M phase in three different hPSC lines, H1, HUES8 and Fucci-H9 cells, two human diabetic iPSCs (DiPSCs), and hPSC-derived neural progenitor cells (NPCs). Using Nocodazole treatment in H1 cells, they received 3.5-fold increase of HDR in GFP fluorescent system (up to 1.5%), and 3.1-fold (up to 1.35%) using ABT. Simple cell sorting by cell cycle may increase HDR by tenfold. Using Nocodazole or ABT treatment with antibiotic selection (Neo resistance insert) may improve HDR up to 78% using Cas9 nickase (Yang et al. 2016).

Finally, there is another approach—the use of the so-called phenomenon coincidental insertion (COIN). It is shown that editing each allele is an independent event. Authors inserted *GFP* gene into one locus of the genome, and at the same time, Neo resistance gene into other locus and after G418 selection the number of GFP-positive cells was increased by 21-fold. The main explanation of this discovery is the enrichment of cells in the G2/M phase when HDR is active (Shy et al. 2016). Reporter gene or resistance to some antibiotic gene can be used as a selection marker in this case. In a paper published by Mitzelfelt et al., the HDR rate in the target locus (*CRYAB, BAG3, LMNA* or *MTERF4* genes) was enhanced by an average of 50 times, and the HDR/NHEJ ratio was increased by 18–27-fold, depending on the iPSCs culture, using the simultaneous insertion of the puromycin resistance gene into the locus *AAVS1* (Mitzelfelt et al. 2017).

Wu et al. proposed a slightly more complex approach. The authors offered the use of two selective plasmids carrying disrupted puro-mycin-resistant and zeocin-resistant genes with targets for sgRNA inside these genes, as well as subsequent out-frame fluorescent reporter genes. When CRISPR/Cas9 system is "working", SSA restores the resistance genes and reporters. The latter allows to visually evaluate CRISPR/Cas9 work, and the first—to perform an antibiotic double selection. It is possible to substantially increase HDR rate to 34.1% in HEK293T and 18.2% in PK15 cells while using the same target for sgRNA in the gene of interest (Wu et al. 2017).

Despite its efficacy, cell selection has a number of limitations, including high cost, labor costs, and the inability to use in vivo. This significantly limits the use of this approach and makes it necessary to look for more accessible ways to increase precise knock-in.

Alternative methods for insertion

Targeted genome knock-in can be performed not only via HDR pathway. Recent studies discovered variety of methods based on alternative mechanisms of DNA repair.

One of this methods, named the Precise Integration into Target Chromosome (PITCh) system, assisted by microhomology-mediated end joining (MMEJ). PITCh uses very short (5–25 bp) microhomologous sequences as homology arms, which can be easily added to the PITCh vector by PCR or by the insertion of annealed oligonucleotides. It was developed in 2014 by Sakuma et al. Authors developed two types of PITCh system: TAL-PITCh and CRIS-PITCh. In TAL-PITCh, a special TAL-PITCh vector containing a TALEN target site was co-introduced with the pair of TALENs. The vector contained different spacer sequence compared with the original genomic sequence with switched anterior and posterior halfs. Pair of TALENs cut both genomic and vector sequences, and the linearized TAL-PITCh vector contains microhomologous DNA ends corresponding to the genomic cleavage site. MMEJ-dependent mechanism allows the whole vector to be precisely integrated into the genome. CRIS-PITCh system is more complex than based on TALENs. Actually, TAL-PITCh strategies can be applicable to CRIS-PITCh, but it was improved to remove unnecessary vector backbone and to abolish restriction of the gRNA target sequence. There are three guide RNAs (gRNAs) and Cas9 nuclease which should be co-expressed, and CRIS-PITCh vector contains two different gRNA target sites. Using this system, authors could produce knocked-in HEK293T cells without any additional sequence. Nucleolar localization of fluorescence was observed, similar to the TAL-PITCh experiment. Thus, TAL-PITCh and CRIS-PITCh have on average comparable efficiency (Nakade et al. 2014). Next, CRIS-PITCh system was applied for targeted knock-in of an scFv-Fc antibody gene into the *hprt* locus of Chinese hamster ovary cells. However, there it is shown less efficiency than traditional HR method (50% vs. 100%) (Kawabe et al. 2018).

To explore the possibility of CRISPR/Cas9-induced NHEJ in promoting of DNA integration [non-homology (NH)-targeting], He et al. constructed two donor plasmids that carry promoterless IRES-eGFP, which has no homology to the *GAPDH* locus. In these NH donors, a single sgRNA (sg-A) target site at 5′ of IRES-eGFP (single-cut donor) was inserted, or two sg-A sites at both sides of IRES-eGFP (double-cut donor). This allows introducing cleavage for desired integration and to generate IRES-eGFP fragments of different lengths. The IRES element was used to bypass any frameshift caused by NHEJ and to confirm GFP expression after reporter integration. With this strategy, it became possible to integrate a 4.6 kb promoterless IRES-eGFP fragment into the *GAPDH* locus with efficiency up to 20% in somatic LO2 cells and 1.70% in human embryonic stem cells (He et al. 2016).

In the article of 2016 Suzuki et al. called that mechanism as homology-independent targeted integration (HITI). Authors compared knock-in efficiencies in transfected

neurons with an HDR (Tubb3-HDR), a PITCh (Tubb3-MH), and four different HITI donor plasmids. It was observed little to no knock-in effectiveness with HDR (Tubb3-HDR) and PITCh (Tubb3-MH) vectors, but observed higher knock-in efficiency with HITI donors (IRESmCherry-). Usage of the NHEJ inhibitor NU7026 made HITI efficiency significantly lower, confirming the dependence of HITI on the NHEJ repair machinery. HITI vectors allowed efficient GFP knock-in in non-dividing primary neurons with rate up to 50% (Suzuki et al. 2016).

NHEJ-based strategy for DNA integration was developed for TALENs and ZFNs named Obligate Ligation-Gated Recombination (ObLiGaRe). This method allows inserting a 15-kb inducible gene expression cassette at a defined locus in human cell lines. For direct ligation of exogenous DNA fragment into the genome, initially, ZFN-binding sites are introduced into a donor plasmid. Here, two halfs *AAVS1* ZFN-binding sites were inserted in the vector without changing the orientation of the linker region. The ZFNs cut both *AAVS1* sites in the genome and in the donor plasmid to produce complementary overhangs, which then were ligated. The newly formed junctions are resistant to further digestion by *AAVS1* ZFNs (Maresca et al. 2013).

Alternative methods of DNA inserting are quite effective, but often involve a large number of errors.

Other approaches and combinations

There is variety of nucleases of CRISPR/Cas systems; those are also used in HDR experiments. Feature of Cas9 nickase mutants [for example, Cas9 (D10A) and Cas9 (H840A)] is their ability to make a nick in one DNA strand. In addition, despite the levels of HDR repair obtained with the nickases were lower than obtained with wtCas9 (Robert et al. 2015; Richardson et al. 2016), it was proved that double nicking may be required to promote 200 to over 1500-fold greater specificity than the wild-type Cas9 (Ran et al. 2013a) and high-efficiency homology-directed repair, comparable with that with SpCas9.

Cpf1 is a novel class of CRISPR-Cas DNA endonucleases. It was demonstrated that Cpf1 from Lachnospiraceae bacterium (LbCpf1) can significantly increase homology-directed repair in zebrafish (up to ~ fourfold in two out of four tested loci) compared to SpCas9 (Moreno-Mateos et al. 2017). Another study performed on N2a mouse neuroblastoma cells reports about 24% HDR efficiency with using LbCpf and 15% with AsCpf1. Lb- and AsCpf1 were equally or more efficient in inducing SSA than three tested Cas9 counterparts (Tóth et al. 2016).

Cas9 ribonucleoprotein (RNP) together with AAV donor can be used, it increases HDR rate by tenfold (from 1 to 10%). However, RNP with a plasmid donor or AAV donor together with the plasmid SpCas9 reduces the HDR rate (Gaj et al. 2017).

To increase HDR, it is possible to link SpCas9 and donor molecule (ssODN) covalently through the SNAP-tag fusion protein, which allows to increase HDR 3–24 times depending on the length of ssODN, cell culture, and the target locus. This approach is effective even when using the combination of SpCas9 and inactive SaCas9 (SadCas9) connected to ssODN (Savic et al. 2018).

An approach based on the use of the PiggyBac transposon in combination with CRISPR/Cas9 is increasingly used (Woodard and Wilson 2015). In this case, expression cassette with one or several genes for selection, for example, reporter gene or/and antibiotic resistance gene, is inserted by CRISPR/Cas9 into the target locus using a plasmid with long homology arms. This fragment is flanked by the PiggyBac sites, which further are recognized by PiggyBac transposase, transfected additionally in expression plasmid, what provides scarless removal of the donor cassette without disturbing the nucleotide sequence of the targeted gene (Woodard and Wilson 2015; Singh et al. 2015a, b). Editing is achieved by changing the nucleotide sequence of one of the homology arms (Firth et al. 2015).

The most used and effective combination is the suppression of NHEJ and the activation of key HDR factors. Simultaneous using of several approaches, including NHEJ inhibition, HDR stimulation, and others, can facilitate homology-directed repair. Shao et al. made an experiment with co-expression of Rad52 with Cas9 and Rad52–Cas9 fusion and revealed that both manipulations yielded approximately threefold increase in HDR (from 2.3 to 8.4%) during the reporter assays in human HEK293T cells, and also in the genome-editing assays. Combination with Scr7 for NHEJ inhibition resulted in significant HDR improvement to about 40% (Shao et al. 2017). Ye et al. demonstrated that the repression of KU80 and the activation of CDK1 by CRISPRi increase HDR level in HEK293T and HeLa cell cultures by fivefold to sixfold depending on the targeted locus (Ye et al. 2018).

HDR efficiency can also be increased with combination of NHEJ inhibition and using Cas9 protein (RNP) instead of plasmid. In rat experiments at the presence of Scr7 (1 μM), 140 injected zygotes were transferred to 5 recipients and 17 pups were born. 11 rats contained the $CreER^{TA}$ insertion with the efficiency up to 64%. Using Cas9 RNP yielded in 51% (15/29 rats) of HDR rate. Then, authors used both approaches simultaneously. A total of 100 injected zygotes were transferred to four recipients and 13 pups were born. Ten rats with the efficiency up to 76% (10/13) contained the $CreER^{TA}$ insertion (Ma et al. 2016).

Conclusion

Precise knock-in efficacy is not very high, but there are a large number of approaches aimed to increase it. It seems that cell selection and cell-cycle synchronization, which increase knock-in up to almost 100%, are the most effective, but these approaches can only be used in in vitro studies. However, the use of Cas9 fused with Geminin can solve the problem of Cas9 activity in certain phases of the cell cycle in vivo. In vivo, most likely, methods that enhance HDR factors or inhibit NHEJ (or a combination thereof), although not as effective, will be more applicable. The type (ssODN or dsDNA) and design of the donor molecule for HDR are very important and allow to significantly increase knock-in efficiency. Mentioned in this review approaches show that there is no unambiguous method for achieving high knock-in efficacy. In each case, preliminary work on the search of the optimal method or their combination, which give maximal efficacy in certain cells and DNA locus, is necessary to perform.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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