Determinants of genome editing outcomes: the impact of target and donor DNA structures

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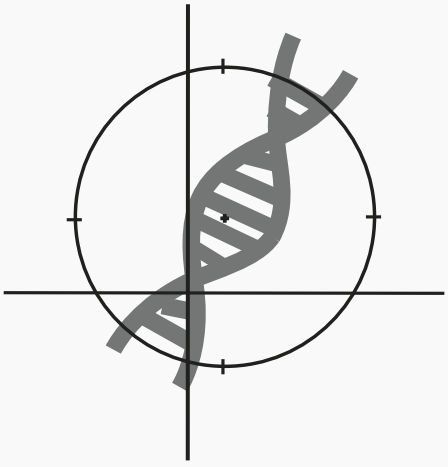
Chapter 1

CRISPR-Cas9 Genome Editing, Five Years on



Up until about five years ago, when speaking of genome editing using designer nucleases, scientists were mostly referring to transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs); now RNA-guided nucleases (RGNs) based on clustered regularly interspaced short palindromic repeats (CRISPR) systems are, undoubtedly dominating the field [1]. Native type II CRISPR-Cas9 nucleases from *S. pyogenes* were the first to be adapted into genome editing tools [2-4] and, similarly to other prokaryotic CRISPR systems, confer adaptive immunity by detecting, recording and degrading foreign DNA from invading viruses and plasmids [5, 6]. Soon after the discovery in 2012 that *S. pyogenes* CRISPR-Cas9 nucleases could cleave DNA in an RNA-guided manner [5, 6], researchers hijacked this defense system and turned it into a powerful tool to edit the genomes of eukaryotic cells, in a targeted and permanent basis [2-4]. These genome engineering technologies have wide implications for basic and applied research and bioethics, especially in the context of genetically modifying human somatic and germ cells. With the introduction and ongoing refinement of RGNs, it seems that the scientific community is on track to tackle the root cause of genetic and infectious diseases. Nevertheless, RGNs suffer from, amongst other shortcomings, off-target activities and relatively low homology-directed repair (HDR) efficiency. To overcome these hurdles, and hence unleash the full potential of RGN-based therapies, researchers are rationally engineering Cas9 variants, *de novo*-isolating Cas9 proteins, fine-tuning gRNA and donor DNA structures, and devising improved protocols for obtaining specific genome editing outcomes. This chapter reviews these efforts that, when combined, might overcome the main limitations of current genome editing technologies.

Genome editing in a nutshell

Efficient genome editing relies on designer nucleases, which, by making site-specific chromosomal double-stranded DNA breaks (DSBs), trigger cellular DNA repair pathways to establish specific genome editing outcomes., e.g., targeted gene knockouts and knock-ins [1, 7, 8]. The non-homologous end joining (NHEJ) can knockout *cis*- or *trans*-acting elements after the introduction of small insertions and deletions (indels); the homology-directed repair (HDR) can knock-in new genetic information using exogenous DNA as surrogate DSB-repairing templates (donor DNA) [1, 7]. It is noteworthy mentioning that, in mammalian cells, DNA repair often favors NHEJ over HDR, with the HDR machinery largely restricted to the G2/S phases of the cell cycle [9, 10]. Among the designer nucleases used in genome editing, ZFNs and TALENs share a similar architecture in that a sequence-specific DNA-binding protein domain is fused to the nuclease domain of the type IIS FokI restriction enzyme. Moreover, ZFNs and TALENs work in pairs, cleaving their target DNA after the *in situ* dimerization and ensuing catalytic activation of their FokI domains [1, 7]. The vertebrate Cys2-His2 zinc-finger motif is the basic unit of the DNA-binding domain of ZFNs

with each zinc-finger typically recognizing a specific DNA triplet. Thus, assembling 4-6 zinc-finger motifs per ZFN monomer generally provides enough target site specificity within complex eukaryotic genomes [11-16]. TALENs recognize DNA through an array of repeated domains of TALE proteins found in certain phytopathogenic bacteria, e.g., *Xanthomonas sp.* [17, 18]. Each TALE repeat is composed of 33-35 conserved amino acids with variable residues dubbed repeat variable di-residues at positions 12 and 13, recognizing a specific nucleotide on the DNA double-helix [17, 18]. Researchers have been exploiting ZFNs and TALENs not only for generating transgenic animal models [19], but also for treating diseases, including AIDS [20], hemophilia B [21] and certain leukemia [22]. Nevertheless, many laboratories flinch from using ZFNs and TALENs because making these protein-guided nucleases requires more expertise and higher costs than those of RGNs.

RGNs from the prototypic *S. pyogenes* type II CRISPR-Cas9 system and, more recently, those from other species, are offering unprecedented speed and versatility with which robust genome editing tools can be built [1, 7]. The RGNs from *S. pyogenes* consist of a fixed Cas9 nuclease (SpCas9), and two RNAs, i.e., a sequence-specific CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). Together these two RNAs form a crRNA:tracrRNA duplex that binds the SpCas9 nuclease and directs it to a target site consisting of an NGG triplet called protospacer-adjacent motif (PAM) and a 20-nt long sequence complementary to the 5′ end of the crRNA (spacer) [5, 6, 23]. The recognition of the NGG triplet by the PAM-interacting domain of SpCas9 is followed by double helix melting, local RNA-DNA hybridization, R-loop elongation and PAM-dependent allosteric activation of the HNH and RuvC-like nuclease domains [24]. This series of events lead to the generation of a blunt DSB mostly positioned 3-bp distal from the PAM [5, 6, 24]. The initial adaptation of the native *S. pyogenes* CRISPR-Cas9 nuclease system into a powerful genome editing tool involved expressing human codon-optimized *Cas9* ORF and single guide RNAs (sgRNAs) formed by the fusion of crRNA and tracrRNA moieties that mimic the original crRNA:tracrRNA structure [2, 4]. Hence, by simply swapping the spacer sequence in the sgRNA, researchers can target virtually any genomic sequence with unprecedented flexibility. Depending on the target cells —cultured *in vitro* or located in a living organism—RGNs can be delivered as ribonucleoproteins (RNPs) or encoded in mRNA, plasmid DNA or viral vector genomes; and depending on the purposes, they can be designed to induce (i) disruption of specific sequences (knockout), (ii) gene knock-ins, (iii) targeted DNA deletions, (iv) directed chromosomal translocations and (v) single nucleotide substitutions.

Notable bottlenecks of CRISPR-Cas9 technologies

Admittedly, there are no perfect designer nucleases. As for ZFNs and TALENs, RGNs can cleave at unintended off-target sites, which can only be partially identified

through *in silico*-guided and genome-wide assays [1, 25]. Given the complexity and very large size of the human genome (~3 billion base pairs per haploid genome), the generation of off-target DSBs poses a non-negligible risk, which might knockout essential genes or mutate cancer-associated genes [26-30]. These outcomes must especially be avoided when the goal is to develop genetic therapies that exploit the NHEJ or the HDR pathways. In the context of gene knockout approaches, the unpredictable indel footprints from NHEJ might produce aberrant products, e.g., truncated or misfolded proteins or proteins displaying immunogenic epitopes. In the context of genome editing strategies involving multiple DSBs at different loci, chromosomal rearrangements might arise contributing to genomic instability [31]. Regarding HDR-based genome editing, RGNs, next to faithfully integrating the exogenous DNA at one allele, often also disrupt the other allele due to the prevalence of NHEJ over HDR in mammalian cells [32]. Moreover, HDR-mediated chromosomal DNA insertion triggered by site-specific DSB formation often is not efficient enough (< 1%), especially when donor templates encompass entire transcription units stretching several kilobases. Finally, the native chromatin environment in eukaryotic cells poses yet another hurdle compromising the efficiency and predictability of genome editing efforts [33, 34].

To address the aforementioned issues, many researchers are investigating a broad range of strategies that are as diverse as engineering or mining for new nucleases, redesigning sgRNA moieties and testing new donor DNA structures aiming at achieving efficient and accurate genetic manipulations in complex (epi)genomes.

Isolating and engineering new Cas9 proteins

Although the SpCas9 PAM sequence NGG occurs in the human genome every 8 bp on average, it can nonetheless constitute a limitation, especially when the genomic sequences of interest have a high A/T content. Using a selection-based SpCas9 system in bacteria, Kleinstiver et al. isolated the VQR and VQER variants of SpCas9, each of which recognizing the PAMs NGAN and NGCG, respectively [35]. Importantly, their directed evolution system has also yielded the D1135E SpCas9 variant which displays a superior discriminating profile between canonical NGG and cryptic NGA PAMs than that of the wild-type SpCas9 protein [35].

Multiple CRISPR-Cas systems isolated from different species have been adapted for genome editing in human cells, including those from *S. aureus* and *Acidaminococcus sp.*. Often, the resulting RGNs display an improved or complementary set of attributes when compared to the SpCas9. For instance, in contrast to the 4.1-kb ORF of the SpCas9 nuclease (1368 amino acids), the ORF coding for the *S. aureus* Cas9 (SaCas9) protein (1053 amino acids) spans only ~3.2 kb, making it easily packaged within the limited confinements of commonly-used viral vectors, e.g., adeno-associated viral vectors [36]. In addition, Cas9 orthologs offer alternative specificity profiles and

genomic targeting ranges owing to their different PAM sequences, e.g., SaCas9 has as PAM the NNGRRT motif instead of NGG [36]. To further increase the versatility of SaCas9-based RGNs, Kleinstiver et al. generated the KKH SaCas9 variant, which recognizes the degenerate PAM sequence NNNRRT and, as a result, has broadened the genomic coverage of these RGNs [37]. Different from the RGNs based on type II CRISPR-Cas9 systems of *S. pyogenes* and *S. aureus*, those adapted from type V CRISPR-Cpf1 systems recognize T-rich PAM sequences, generate staggered DSBs with 5′-overhangs, display enhanced specificities and do not have a tracrRNA component [38]. Similarly to the development of Cas9 proteins with altered PAMs, using structure-guided mutagenesis, Gao and colleagues engineered mutants of *Acidaminococcus sp*. Cpf1 (AsCpf1) which recognize the PAMs TYCV and TATV instead of the native PAM sequence TTTV [39].

In parallel to modifying the target range of RGNs, recent research is also leading to improvements in reducing RGN off-target activities. Previous studies on the development of ZFNs and TALENs have set the stage for optimizing the specificity of RGNs by mimicking the *modus operandi* of ZFNs and TALENs, which encompass FokI nuclease domain dimerization [13, 40]. Tsai et al. and Guilinger et al. fused the FokI nuclease domain to the N-terminus of a catalytically inactive SpCas9 protein to form FokI-dCas9 monomers (where "d" stands for "dead") [41, 42]. The binding of a pair of these monomers to their bipartite target sequences forms a dimeric RNA-guided FokI nuclease (RFN), offering a higher specificity profile when compared to conventional RGNs as the target sequences of RFN span up to 44 bp. Despite of its improved specificity, the RFN approach has limited genomic DNA coverage due to their strict gRNA design and spacing requirements. In particular, RFNs require a so-called PAM-out gRNA design with their activities peaking within a 14-17 bp spacing between the two hemi-nucleases [41, 42]. These limitations, together with the fact that RFNs are larger than conventional RGNs, are keeping this approach at the proof-of-principle stage. The nuclease domains of SpCas9, HNH and RuvC, cut the target and non-target strands, respectively. Mutating catalytic residues in each of these nuclease domains yields the RuvC mutant D10A and the HNH mutant H840A "nickases", which cleave the target and non-target strands, respectively [6]. Delivering into cells a Cas9 "nickase" together with two different gRNAs targeting opposite DNA strands of a bipartite recognition sequence induces a DSB owing to the local coordinated action of both RGN "nickases". Should either of the nicking complexes cleave at off-target sites, the resulting SSBs are, for the most part, faithfully repaired. Thus, this confers an overall high specificity to the dual nicking approach. When compared to conventional RGNs, this dual nicking strategy can reduce off-target activities (up to 1,500-fold) [43, 44]. Similar to RFNs, the relative positions and spacing of the two gRNAs are important for efficient target DNA cleavage. Usually, dual RGN "nickases" yielding 5′-overhangs and a spacing between the 5′ ends of the two gRNAs

between -4 and 20 nt result in the highest DSB formation activities [43, 44]. Of note, SaCas9 "nickases" bearing a D10A or an N580A mutation were also compatible with the dual nicking strategy, although in this case, the optimal distance between the gRNAs seems to lie between 0 and 125 bp [45]. One drawback of the dual nicking strategy is that, often, there is a trade-off in which the gains in specificity are accompanied by a decrease in efficiency [43-45].

To obviate the need for a pair of suitable gRNAs for dual nicking to increase the specificity of RGNs, researchers have generated high-specificity SpCas9 variants through structure-guided protein engineering involving mutations-to-alanine of specific DNA-interacting residues [46, 47]. They reasoned that the resulting reduction in the RGN-DNA binding energies would be adequate to cleave the target site but insufficient to cleave at off-target sites [46, 47]. Slaymaker et al. reported enhanced specificity of the SpCas9 variants eSpCas9(1.0) and eSpCas9(1.1) containing triple mutations (i.e., K810A/K1003A/R1060A and K848A/K1003A/R1060A, respectively) at the positively charged nt-groove of SpCas9, which plays a role in stabilizing the non-target DNA strand [47]. Kleinstiver et al. generated in turn quadruple mutant SpCas9-HF1 (i.e., N497A/R661A/Q695A/Q926A). These mutations-to-alanine are thought to attenuate hydrogen bonds between SpCas9 and the phosphate backbone of the target DNA strand [46]. Both strategies significantly reduce off-target chromosomal DNA cleavage while retaining on-target activities similar to those of wild-type SpCas9 for the majority of target sequences tested [46, 47]. However, it is noteworthy mentioning that the eSpCas9(1.1) and SpCas9-HF1 variants are less efficient than wild-type SpCas9, when the gRNAs contain a 5'-mismatched nucleotide (e.g. 21-mer spacer with a 5' end "G" extension) or are truncated (i.e. < 19- to 20-mer spacers) [33, 48]. Interestingly, the SpCas9 variant HeFm2SpCas9, harboring combinatorial mutations from eCas9(1.1) and SpCas9-HF1, albeit more specific than their parental proteins, suffers from low activity at most target sites tested [49]. More recently, Chen et al. showed that the specificity gains of eSpCas9(1.1) and SpCas9-HF1 were likely due to a failure in the conformational change of the REC3 domain necessary for HNH catalytic activation at off-target sites [50]. Based on this information, they engineered a SpCas9 protein containing the REC3 mutations N692A/M694A/Q695A/H698A, and showed that this so-called HypaCas9 has comparable or higher specificity than SpCas9-HF1 and eSpCas9(1.1) [50].

To circumvent DSB formation and the undesirable NHEJ-derived mutations, researchers are developing strategies that resort to the induction of SSBs instead of DSBs to facilitate targeted genome editing [32, 51, 52]. Komor et al. and Nishida et al. introduced the base editing concept, which ultimately replaces one base pair for another [51, 52]. The rationale behind base editors is that the cytidine in a C:G pair can be converted to a U:G pair by cytidine deaminases, e.g., APOBEC1 [51] and AID [52]. After mismatch repair the U:G becomes a U:A pair, which finally turns into a

T:A pair after DNA replication. The first generation of base editors made by Komor et al. has the cytidine deaminase APOBEC1 fused to the dSpCas9 (BE1). To avoid removal of uracil by uracil glycosylases, a uracil glycosylase inhibitor (UGI) was added to their second generation base editor (BE2). To increase the base editing efficiency, the dSpCas9 was replaced by a SpCas9 "nickase", yielding a third generation base editor (BE3) consisting of a fusion between APOBEC1, a UGI and the SpCas9 D10A "nickase" scaffold. BE3 generates an SSB in the G-containing strand converting any C:G base pair to a T:A (C to T) within a 5-nt window about 12 bp away from the PAM [51]. More recently, a BE3 mutant named YEE-BE3 with a weaker cytidine deaminase activity has narrowed the peak of C-to-T transitions from a 5-nt to a 2-nt window [53]. In addition, base editors containing as scaffolds SpCas9 variants or SaCas9 have either expanded the PAM requirements of base editors [54] or improved their specificity [55]. With a fine-tuned design, the fourth generation base editor, BE4, has larger linkers between the SpCas9 "nickase", APOBEC1 and UGI, and incorporates a second copy of UGI [54]. This construction enriches for the intended C-to-T transitions and reduces unwanted conversions likely mediated by uracil glycosylases. Other BE3 and BE4 base editors (e.g. BE3-Gam, SaBE3-Gam, BE4-Gam, and SaBE4-Gam) incorporate the bacteriophage Mu Gam protein which purportedly, by binding to DSBs, reduces the residual levels of nick-derived chromosomal breaks [54]. Finally, The recent development of adenine base editors (ABEs) nicely complements their BE counterparts as these new based editors can convert A:T to G:C pairs (A to G) in a highly efficient manner [56, 57]. Theoretically, with current base editors, many pathogenic single base mutations can be corrected and, in addition, ATG start codons can be disabled for targeted and precise gene knockouts.

Redesigning and modifying gRNAs

sgRNAs have secondary structures forming hairpins and stem loops which mimic the architecture of native gRNAs consisting of the sequence-specific crRNA (42 nt) and the scaffolding tracrRNA (89 nt) [2, 6]. The 5'-terminal 20 nts of the sgRNA (spacer) and the Cas9-interacting scaffold function as essential elements in sgRNA molecules [2]. Truncated gRNAs (tru-gRNAs) displaying 17-19 mer spacers instead of the typical 20 mer spacers, have a weaker DNA-binding potential which, presumably, biases the cleaving activity to fully complementary target sites in detriment of mismatched (off-target) sequences. Indeed, Fu et al. showed that RGNs bearing tru-gRNAs and wild-type SpCas9 can display greatly decreased off-target effects (up to 5,000-fold) while maintaining their on-target activity [58].

The most commonly used sgRNA structure has (i) a shorter crRNA:tracrRNA duplex region next to the spacer sequence when compared to that of the native crRNA:tracrRNA duplex, potentially impairing stable Cas9:gRNA complex assembly, and (ii) a continuous stretch of four uridines, which might serve as a premature

RNA polymerase III termination signal. On the basis of this information, Chen et al. and Dang et al. optimized the gRNA scaffold by extending the duplex region next to the 5′ spacer region by 5 bps, and showed significantly enhanced dSpCas9-based chromosomal imaging and SpCas9-mediated gene knockout, respectively [59, 60]. By combining the sgRNA duplex extension with the disruption of the uridine stretch, Dang and coworkers demonstrated higher *CCR5* and *CD4* gene knockout frequencies when compared to those achieved by the original sgRNA scaffold [60]. Considering that these optimized scaffolds seem to yield more stable and full-length sgRNAs, they might also be compatible with different (epi)genome editing tools, e.g., Cas9 variants, dCas9-based transcriptional regulators and base editors.

Adding functional RNA elements into sgRNA secondary structures can repurpose RGN-based tools, without diminishing their DNA targeting proficiency [43, 61]. For instance, hairpin aptamers can be inserted into the sgRNA scaffold at the tetraloop and lower stems, so that sgRNAs bind the MS2 bacteriophage coat protein. When such aptamer-containing sgRNAs are delivered together with Cas9 and MS2 fused to effector domains (e.g. VP64 transcription activator or Tet1-CD demethylation domains), potent transcription activation and epigenetic remodeling of target loci can be achieved [62, 63].

Currently, RNA polymerase III (Pol III) promoters are the most widely used elements for sgRNA synthesis as they evolved for expressing short, unprocessed, transcripts in eukaryotic cells. Although highly active, Pol III promoters require a G (e.g., U6 and 7SK promoters) or an A or a G (e.g., H1 promoter) to efficiently initiate transcription, therefore the most suited sgRNAs have spacers starting with a G or an A at their 5′ terminus [64, 65]. Of note, there is an impairment on the activity of the eSpCas9(1.1) and SpCas9-HF1 variants when the 5′ terminal G does not hybridize to the target site [33, 48]. Although RNA polymerase II (Pol II)-driven transcripts are subjected to complex downstream processes and nucleus-to-cytoplasm translocation, they offer the possibility for conditional or tissue-specific expression or for the expression of multiple sgRNAs from a single template. In this regard, Nissim and coworkers designed Pol II expression units containing the sgRNA coding sequence flanked by recognition sites for the RNA endonuclease Csy4 (Csy4-gRNA-Csy4), so that single or multiple sgRNAs can be released after Csy4 cutting [66]. The Csy4-gRNA-Csy4 unit was also inserted into an artificial intron construct with splicing sites and branching point. Hence, after splicing, the Csy4-gRNA-Csy4 module can be set free as an intron [66]. However, inserting the sgRNA sequence alone (i.e., without the flanking Csy4 sites and Csy4 expression) into the artificial intron construct did not yield stable sgRNA due to rapid degradation [66]. To avoid the concomitant delivery of the RNA endonuclease Csy4, the sgRNA sequence can be put after a 5′ hammerhead ribozyme (HH) or in-between a 5′ HH and a 3′ HDV ribozymes (HH-gRNA-HDV), which generate mature sgRNAs through self-cleaving [66-68]. Of note,

however, Yoshioka et al. showed that ribozyme-flanked sgRNAs expressed from the RNA Pol II CAG promoter (CAG-HH-gRNA-HDV) induced less genome editing events than those from the commonly used U6-driven sgRNA [68]. Alternatively, Xie et al. hijacked the endogenous tRNA maturation mechanism, and constructed a Pol III promoter-driven tRNA-sgRNA tandem array, which, after cleavage by endogenous RNAses, yielded functional sgRNAs in rice [69]. Follow-up studies confirmed that sgRNAs produced from the tRNA-gRNA module could achieve efficient genome editing in *Y. Lipolytica*, maize and, more recently, in *Drosophila* [70-72].

Pre-assembling Cas9 and gRNA ribonucleoprotein complexes *in vitro* before their delivery into target cells bypasses chromosomal integration of foreign DNA encoding these RGN components, and ensures a more transient activity window of the nucleases [73, 74]. The gRNAs are usually made from *in vitro* transcription or by solid-phase chemical synthesis [73, 74]. Unlike *in vitro* transcribed gRNAs, chemically synthesized gRNAs bypass the difficult-to-upscale enzymatic reactions. Lately, several groups extensively investigated the chemical modification of the gRNA at different positions of the nucleotide (base, ribose sugar and phosphate group), to increase their stability and genome editing efficiency [75-78]. Hendal et al. showed that sgRNAs modified with 2′-O-methyl, 2′-O-methyl-3′-phosphorothioate or with 2′-O-methyl-3′-thioPACE at both ends of the sgRNA induced robust genome editing at the *CCR5* and *HBB* loci in the CD34+ hematopoietic stem and progenitor cells [75]. In addition, Rahdar and co-workers systematically screened crRNAs modified at different positions with chemical substitutions in their phosphate and/or sugar groups. They identified a shortened, yet equivalently functional, 29 nt-long crRNA containing 2′-fluoro (2′-F) and 2′-S-constrained ethyl (2′-cEt) at their 5′ and 3′ ends, respectively [76]. Interestingly, in addition to improving the stability and reducing the size of gRNA components, chemical modifications are also paying off in terms of generating reagents with enhanced specificity. A recent study demonstrated that 2′-O-methyl-3′-phosphonoacetate modifications at positions 5 or 11 of sgRNAs can remarkably reduce off-target activity while maintaining on-target cleavage [77]. Instead of substituting chemical groups in the ribose-phosphate backbone, Lee and co-workers added large groups at the termini of crRNAs (e.g. 5′-Rhodamine and 5′-Amine) to endow them with new functions, i.e., fluorescence for enriching edited cells [78].

Manipulating donor DNA templates

As aforementioned, when partnered with designer nucleases, donor DNA molecules can act as DSB-repairing templates to precisely incorporate customized DNA changes into specific genomic positions through the HDR pathway [1]. Regardless of their foreign nucleic acid composition, conventional donor DNA templates have sequences identical to those framing a designer nuclease target site (“homology

arms"). These "homology arms" serve as strand invasion regions that allow for homology-directed insertion of foreign DNA, whose size can vary from single base pairs to whole transgenes [1]. For the targeted insertion of large transgenes, the donor DNA is often provided in recombinant plasmids or viral vector genomes. Yet, regardless of the delivery method, HDR occurs mainly during the late G2 and S phases of the cell cycle, and is particularly inefficient in human cells, such as hematopoietic stem/progenitor cells and iPSCs [1, 9, 10]. Moreover, the competing and constantly available NHEJ pathway leads to a large fraction of heterozygous edited alleles due to the concurrent action of HDR and NHEJ, or NHEJ alone.

Studies on the design and testing of different types of donor DNA molecules unveiled attractive research avenues to increase the efficiency, specificity and/or accuracy of HDR-based genome editing [1]. Holkers and coworkers demonstrated that the donor templates delivered in protein-capped adenoviral vector genomes greatly enhance the specificity and fidelity of HDR-based genome editing [79]. By using a "double-cut" donor DNA design in which the "homology arms" are flanked by sgRNA target sites, Zhang et al. achieved 8 % and 12 % transgene knock-ins in human iPSCs at the *CTNNB1* locus with 300 bp and 600 bp "homology arms", respectively [80]. These targeted DNA insertion frequencies were 2- to 5-fold higher than those achieved by the standard covalently-closed plasmid templates [80]. Chen et al. repoted a similar effect after comparing "double-cut" with standard donor plasmids at the *DMD* and *AAVS1* loci in human cells [32]. To bypass the HDR pathway, researchers are also looking into "double-cut" donors containing no or short "homology arms" (e.g., 10-40 bps) for DNA insertions, which are achieved by NHEJ and microhomology-mediated end joining (MMEJ) pathways instead [81-83]. By using RGNs together with homology-free "double-cut" donors, He et al. showed up to 20 % and 1.7 % of a 4.6-kb transgene insertion at the *GAPDH* locus in LO2 cells and ESCs, respectively [83]. Suzuki et al. also applied the homology-free "double cut" donors and inserted different expression units in post-mitotic mouse neurons, both *in vitro* and *in vivo* [84]. Alternatively, "double-cut" donors with short "homology arms" were efficient substrates for MMEJ-mediated transgene insertion in zebrafish [85] and, more recently, in mice [82]. However, it is of note that free DNA termini generated from "double-cut" donors increase the risk of random and/or foreign DNA insertions resulting from imprecise recombination processes [32, 79, 80].

Albeit at low levels, SSB-induced HDR using designer "nickases" can yield targeted and accurate chromosomal insertion of foreign DNA in mammalian cells without the attendant catalytic induction of DSBs [32, 86, 87]. Recently, Chen and co-workers have developed an efficient SSB-induced HDR approach, which can place large DNA segments into specific genomic target sites without provoking the mutagenic NHEJ [32]. The key aspect to this approach, named in trans paired nicking, consists

of combining a Cas9 "nickase" with a modified donor DNA in which the "homology arms" are framed by the sgRNA recognition site [32]. This arrangement assures concomitant SSB formation at target and donor sequences, generating homologous recombination DNA substrates as postulated by Holliday in 1964 [88]. The in trans paired nicking not only increased HDR at the human *AAVS1*, *CCR5* and *DMD* loci in different cell types, including iPSCs, but also facilitated multiplexing HDR [32].

Single-stranded oligodeoxyribonucleotides (ssODNs) are commonly used donors for small genomic modifications, e.g., point mutations, polymorphisms or short protein-tagging motifs. Typical ssODN donors are symmetrical in that they harbor similarly sized "homology arms" at both ends. Recent studies have shown that, when compared to symmetric ssODNs, asymmetric ssODNs can induce more efficient RGN-assisted genome editing [89]. Specifically, optimal ssODN donors should be complementary to the sgRNA non-target strand and encompass shorter homology at the PAM distal end [89]. Biophysical data indicate that this configuration favors donor-target hybridization owing to the generation of a 3′-ended flap on the non-target strand after DNA cutting [89]. Liang et al. confirmed the superiority of asymmetric ssODNs and, in addition, tested short double-stranded ODNs (dsODNs) consisting of annealed ssODNs displaying either 3′ or 5′ 30-nt overhangs [90]. They showed that dsODN donors with 3′ overhangs lead to higher frequencies of RGN-induced genome editing than those achieved with asymmetric ssODNs or with dsODNs containing 5′ overhangs [90].

Similarly to sgRNA components, donor DNA templates are also substrates for chemical modifications to improve the performance of genome editing. For instance, chemically modifying the terminal nucleotides of ssODNs with phosphorothioate increased the efficiency of genome editing by 2-3 fold [91]. In another study, Mang et al. introduced the CAB system, short for Cas9-Avidin-Biotin ssDNA, in which Avidin is fused to Cas9 and binds Biotin-conjugated ssODNs inside cells [92]. Equally relying on high-affinity Biotin-Avidin interactions, Carlson-Stevermer et al. developed the S1mplex system, where Biotin-conjugated ssODNs are tethered *in vitro* to RGNs containing sgRNAs displaying a Streptavidin-binding S1m aptamer [93]. Both the CAB and S1mplex systems increased the frequencies of HDR and the ratio of HDR to NHEJ in mammalian cells [92, 93].

Tweaking experimental conditions for improving genome editing outcomes

Besides engineering novel genome editing components, e.g., the above-described Cas9 proteins, sgRNA structures and donor DNA templates, researchers are also optimizing experimental protocols to improve the efficiency and predictability of genome editing procedures. A simple methodology involves modulating the temper-

ature at which the designer nucleases operate. When compared to the normal 37°C mammalian cell culture condition, ZFN and TALEN induced higher gene knockout frequencies under a transient, 30°C hypothermic period [94, 95]. In contrast, at least in certain mammalian cell lines, transfection of RGN-encoding plasmids at 39°C triggered significantly higher targeted gene knockout frequencies, presumably owing to increased sgRNA expression in these lines [96]. Other protocols entail enriching for cells that have undergone genome editing - by co-delivering reporter units that reflect the expression of designer nucleases [97], or by co-targeting an endogenous gene (i.e., *ATP1A1*) whose loss-of-function is selectable [98].

Steering DNA repair machineries in favor of HDR over NHEJ is also being exploited when targeted gene knock-ins are the desired genome editing outcomes. One can distinguish two main strategies: genetic manipulations or pharmacological interventions. Regarding the former, Chu and colleagues expressed short hairpin RNAs to silence the genes encoding the NHEJ factors KU70, KU80 or DNA ligase IV or co-expressed adenovirus serotype-4 E1B55K and E4orf6 proteins to induce proteasomal degradation of DNA ligase IV [99]. These and other authors have also applied the small-molecule DNA ligase IV inhibitor Scr7 to bias the repair of site-specific DSBs towards the HDR pathway in mammalian cell lines [99, 100], as well as in fertilized mouse and rat zygotes [100, 101]. Robert and co-workers have equally applied a pharmacological approach to dampen the rate of NHEJ and increase the frequency of HDR, but used the DNA-PKcs inhibitors NU7441 and KU-0060648 instead [102]. Next to testing NHEJ inhibitors, researchers are also evaluating HDR-enhancing agents as well. For instance, RS-1, a molecule previously shown to activate RAD51, which play a role in homology search and strand exchange, increased knock-ins by 2- to 5-fold in cell lines and pronuclear stage rabbit embryos [103]. This enhancement on HDR was observed after DSB formation by RGNs and TALENs [104, 105]. By screening a library of roughly 4,000 small molecules, Yu and coworkers found that the β3-adrenergic receptor agonist L755507 stimulates HDR-mediated *Nanog* targeting by 3-fold in murine PSCs [106]. Alternatively, timed delivery of *in vitro* assembled ribonucleoprotein RGNs together with ssODN donors to cells that are synchronized at the cell cycle G1/S phase border by aphidicolin or at the G2/M phase by nocodazole, enhanced HDR-mediated genome editing up to 6-fold when compared with unsynchronized cells [107]. Of note, whether the use of specific genetic and/or pharmacological agents for modulating HDR/NHEJ ratios have long-term pleiotropic effects on target cells or organisms will require further investigations.

Concluding remarks

The ongoing discovery of Cas9 orthologs combined with structure-guided and directed evolution-based protein screens are yielding an ever-increasing number of Cas9 variants with new properties, e.g., strand-specific cleaving activities, alternative

PAM requirements and higher target DNA specificities. In addition, albeit not suited for large genomic DNA changes, base editors promise precise, DSB-free, genome editing. Next to isolating and engineering new Cas9 proteins, reshaping the native gRNA architecture and chemically modifying the gRNA composition are improving the activity and specificity of RGNs. By the same token, manipulating the structure and composition of donor DNA molecules is paying off in terms of enhancing the efficiency and specificity of HDR-based genome editing. To expand and complement hypothesis-driven studies for identifying HDR-enhancing agents, high-throughput screenings of small-molecule drug libraries should yield new compounds for modulating DNA repair pathways. Finally, sensitive and unbiased genome-wide assays will become ever-more important to thoroughly access the global mutation levels upon the application of the constantly improved genome editing tools.

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