Disruptive Technology: CRISPR/Cas-Based Tools and Approaches

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Abstract
Designer nucleases are versatile tools for genome modification and therapy development and have gained widespread accessibility with the advent of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) technology. Prokaryotic RNA-guided nucleases of CRISPR/Cas type, since first being adopted as editing tools in eukaryotic cells, have experienced rapid uptake and development. Diverse modes of delivery by viral and non-viral vectors and ongoing discovery and engineering of new CRISPR/Cas-type tools with alternative target site requirements, cleavage patterns and DNA- or RNA-specific action continue to expand the versatility of this family of nucleases. CRISPR/Cas-based molecules may also act without double-strand breaks as DNA base editors or even without single-stranded cleavage, be it as epigenetic regulators, transcription factors or RNA base editors, with further scope for discovery and development. For many potential therapeutic applications of CRISPR/Cas-type molecules and their derivatives, efficiencies still need to be improved and safety issues addressed, including those of preexisting immunity against Cas molecules, off-target activity and recombination and sequence alterations relating to double-strand-break events. This review gives a concise overview of current CRISPR/Cas tools, applications, concerns and trends.

Key Points

CRISPR/Cas technology is widely applied for targeted genome modification, with ongoing discovery of new enzymes and improvements to specificity, delivery and efficiency.

Ingenious reengineering and reemployment of the basic ribonucleoprotein particles has already created versatile genome disruptors, transcriptional regulators, epigenetic modifiers and base editors.

Therapeutic approaches based on CRISPR/Cas technology have raised safety concerns in recent studies, indicating inherent risks, which require full characterization, and avoidable risks, which may be addressed by ongoing refinement of tools and protocols.

CRISPR/Cas has proven a disruptive innovation that has changed the conduct of functional studies, conception of disease models and creation of new therapies.

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1 The Context of Gene Therapy

Gene therapy, the introduction of genetic material for the cure or prevention of disease, has been 39 years in the making since its contentious beginnings [1]. Gene therapy by gene addition, providing an additional, functional copy of a disease-causing defective endogene, has already led to remarkable success for several genetic diseases, including gaining orphan drug approval [2]. Non-viral vectors are usually inefficient as delivery vehicles for gene addition [3], whereas integrating viral vectors impose size restrictions that are unsuitable for many potentially therapeutic genes, a challenge that can be addressed by combinatorial approaches but at the price of reduced efficiency [4–6]. Even in the case of smaller target genes, the size limit of the expression cassette typically remains an obstacle to adding intronic and regulatory sequences required for suitably regulated or high-level gene expression. For example, efficient lentiviral delivery of β-globin with an open reading frame (ORF) of only 441 base pairs is consistently based on size-reduced cassettes that only achieve subphysiological β-globin expression and frequently result in only partially therapeutic treatments for more severe forms of the disease [7], as reviewed in this issue by Ghiaccio et al. [8]. Delivery of short-hairpin RNAs as therapeutic cargo for posttranscriptional regulation overcomes the problem of size but is only of therapeutic interest in specific cases [9, 10] and, like all integrational approaches, still has the inherent risk of insertional mutagenesis [11]. A potentially safer option would be the application of synthetic nucleotides for modulation of gene expression or RNA processing [12], but despite its promise and decades of development, the approach continues to suffer problems with efficiency, systemic delivery, toxicity and the need for chronic application [13–15]. Finally, gene addition by non-integrating vectors even in its most advanced forms is still marred by low efficiency and durability or by adverse events upon repeat application [16–18]. What is more, therapeutic application of each of these approaches has taken years of insight into individual disease and expression mechanisms, and none is universally applicable to loss of function and toxic gain-of-function mutations. Such delays and restrictions in new therapy development for genetic diseases could be avoided if efficient straightforward correction of causative mutations were possible.

2 Gene Editing 101

Progress in gene editing technology was thus of potentially critical importance for the gene therapy field. A key factor that would encourage accelerated adoption of the technology to genetic diseases and rare diseases in particular was the establishment of more versatile editing tools, with increasing simplicity of target redesign from meganucleases [19] over zinc finger (ZF) [20] and transcription activator-like effector (TALE) nucleases [21, 22] to clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)-type RNA-guided nucleases (RGNs) [23–25] (Fig. 1). A property common to all these genome-editing technologies is the introduction of double-strand breaks (DSBs) at the site of interest as a trigger of targeted modification. ZF and TALE nucleases employ dimeric protein modules containing obligate dimeric FokI nucleases that introduce a staggered cut, whereas CRISPR/Cas9 is most frequently employed as a ribonucleoprotein complex made up of a single guide RNA (sgRNA) and a double-nickase protein component (Cas9). Most RGNs of CRISPR/Cas type introduce blunt-ended DSBs. For any nuclease platform, repair of the DSB by non-homologous end-joining (NHEJ) may achieve insertions and/or deletions (indels) or homology-independent targeted insertions (HITI) at high efficiency. Repair by homology-directed repair (HDR) in the presence of a suitable donor DNA may be used to correct or precisely insert a sequence of interest at the target site. dsDNA double-stranded DNA.
no longer suitable for recognition, which leads to highly efficient introduction of insertions and/or deletions (indels) at the DSB site as recognizable events, making NHEJ ideal for disruption of endogenous sequences. A major alternative pathway for DSB repair and the one suitable for most therapeutic applications is homology-directed repair (HDR), which can be used to introduce precise changes at the target site. However, HDR is much slower than NHEJ, normally restricted to late S and G2 phases in a cell-type- and locus-dependent fashion [28, 29] and appears to be underrepresented in long-term repopulating hematopoietic stem cells (LT-HSCs) [30–33], a cell population of particular interest for curative treatment of genetic diseases such as rare anemias, immunological disorders and lysosomal storage disorders [34]. While work continues to shift the balance toward HDR (i.e., by ongoing refinement of NHEJ inhibitors, HDR enhancers, cell-cycle regulators, design of donor DNA templates and cell cycle-specific Cas9 expression) [30, 35–40] or to find ways of enriching suitably modified cells [41] to achieve therapeutically relevant efficiencies for DSB-mediated precision editing, the CRISPR/Cas system has emerged as the editing platform of choice and already as the basis for a new generation of editing and regulatory molecules.

3 CRISPR/Cas Versatility

RGNs (Fig. 2a), initially identified as a prokaryotic adaptive immune system, were the first programmable nuclease system to act as ribonucleoprotein particles (RNPs) and to employ simple Watson–Crick base pairing for target recognition. The corresponding ease of redesigning target specificity at long last allowed speedy and widespread adoption of genome-editing technology for new targets. Demonstration of effective redesign, characterization of two independent strand-specific nickase domains and modification for easier exploitation as genome-editing tools [42, 43] then allowed successful application of CRISPR/Cas9 to mammalian cells [24, 25]. Its versatility and ease of target design, construction and delivery suggested early on that the CRISPR/Cas system may bring about a landmark shift in gene therapy development [44], despite apparent limitations. For instance, the originally employed Streptococcus pyogenes Cas9 (SpCas9) restricted the choice of target sequence by sequence requirement of a DSB-proximal NGG protospacer adjacent motif (PAM) trinucleotide. However, this could be overcome by turning to naturally occurring CRISPR-associated enzymes from other species or by reengineering or evolving Cas9 to achieve alternative or relaxed RGN specificities [45–49]. Of note here are Staphylococcus aureus Cas9, which is particularly small and therefore most suited for adeno-associated virus (AAV)-mediated delivery [50, 51], and the Cas12a (aka Cpf1, CRISPR from Prevotella and Francisella) RGN system. Cpf1 nuclease uses a shorter RNA component more suitable for direct synthesis than that of other CRISPR/Cas systems and creates staggered DSBs 18 base pairs from the TTTV PAM tetranucleotide, with 5’ overhangs of 4–5 nucleotides [52–54]. These overhangs may favor directional and HDR-mediated events compared with Cas9-induced DSBs, which typically have blunt ends or single-nucleotide 5’ overhangs [55]. Incidentally, the presence of blunt- and sticky-end cutting enzymes in the adaptive CRISPR/Cas system parallels the presence of both types of enzymes in the “innate” prokaryotic defense system of restriction enzymes, which likewise uses DSB induction to fend off intruders and which provides valuable lessons in the dynamics of related repair events [56]. As data accumulate for large-scale application of RGNs across cell types, on-target events surrounding the DSB will become more predictable [57], facilitating designs for particular outcomes, such as frame-shift mutations, at the design stage [58]. As a problem common to all RGNs and designer nucleases, the relatively short recognition sequence, which for RGNs are encoded by the single guide RNA (sgRNA) component of the RNP, mediates on-target cleavage but also off-target cleavage of sequence-similar sites [59]. In this context, employment of mutated Cas9 nickases or heterologous nickases for dimeric target recognition [60, 61] of truncated sgRNAs [62] and of modified high-fidelity Cas9 molecules with minimal unspecific protein-DNA interactions [63, 64] all led to decreasing off-target activity and maintenance of on-target activity. In this vein, programmable Cas9 molecules, such as the recently engineered single-molecule protease-sensing Cas9s [65], may not only expand the possible uses of RGNs but may help reduce any residual off-target activity and its impact. Generally, off-target cleavage is exacerbated by prolonged expression of the designer nuclease and can thus be addressed by highly transient expression of RGNs [66], which will also depend on the delivery method. For instance, RNP-based delivery of CRISPR/Cas allows RNP detection for up to 48 h [66], where delivery as RNAs allows RNA detection for up to 24 h [67] and ongoing RNP action for up to 96 h [68], although this will vary with each specific application. By contrast, plasmid DNA in cells and tissue is highly stable, persists in vivo for several months [69–71] and, moreover, poses the risk of illegitimate integration in the genome [72–74]. The same is true for AAV vectors, which are highly efficient for in vivo delivery but persist as episomes for months [17]. For clinical application, delivery of the RGN components themselves (rather than of possible donor templates) as RNPs or RNAs is therefore highly preferable to delivery as naked DNA or as persistent viral vectors.
CRISPR/Cas9-based tools for therapy development. CRISPR/Cas ribonucleoprotein complexes already offer up an array of molecular tools, which all exploit sequence-specific recognition of DNA or RNA molecules by the sgRNA component of the complex. The following are important categories of CRISPR/Cas-based tools. a RNA-guided nucleases introduce a DSB to trigger repair by NHEJ or HDR. NHEJ introduces indels or can be exploited for homology-independent targeted integration of donor sequences, whereas HDR allows precise repair and homology-based insertion of donor sequences. b Designer base editors (BEs) exploit chemical conversion of DNA bases to introduce permanent sequence changes without DSB. Shown is the conversion of C to U, the correction of the complementary base from G to A triggered by the Cas9 nickase activity, and the eventual establishment of a T-A base pair after DNA replication. c Epigenome regulators incorporate or recruit DNA or histone-modifying enzymes, such as the DNMT3 DNA methyltransferase or the TET1 DNA demethylase, which add or remove a methyl group (Me) from proximal CpG sequences. d Transcriptional regulators incorporate or recruit transcriptional activators or repressors for nonpermanent modulation of target gene expression. e Posttranscriptional regulators, such as Cas13, CasRx and derivatives, are mostly based on the Cas13 family of RNA-guided RNases and perform targeted RNA cleavage without sequence restriction to cognate PAM sites. f Deactivated CasRx may bind to cis-regulatory elements of pre-messenger RNA (pre-mRNA) and effect alternative splicing. g RNA BEs, in analogy to DNA BEs, contain a Cas13 fusion with an ADAR2 domain, which performs adenosine-to-inosine conversion. This A > I conversion is read as an A > G base change during translation and may be exploited to achieve codon changes or the removal of premature stop codons in the open reading frame of mRNAs. In contrast to DNA-targeting Cas molecules, RNA-targeting Cas molecules are not restricted to target sequences with a corresponding PAM sequence. For clarity, Cpf1, with its staggered DSB, RNA BEs and a plethora of additional tools, such as many additional transcriptional regulators and epigenome regulators [45] and different flavors of paired nickases (two-component RGNs), are not shown.

Exclusion mark activation of expression; Red cross deactivation of expression; STOP sign translation termination (nonsense) codon, dCas9 deactivated Cas9 without endonuclease activity, dsDNA double-stranded DNA, catalytic domains for functional expansion of the RGN complex: CyD cytidine deaminase domain for C > U conversion in the ssDNA loopout, currently with precision of ≤ 2 bp, UGI uracil DNA glycosylase inhibitor domain to prevent base excision repair and removal of base edit, DNMT3a catalytic domain of DNA methyltransferase 3 alpha for DNA methylation and potentially persistent repression of gene expression for affected promoters [82], TET1 catalytic domain of Ten-Eleven Translocation dioxygenase 1 (TET) for DNA demethylation and potentially persistent transcriptional activation of affected promoters [84], VPR VP64 (four tandem repeats of herpes simplex virus VP16) linked to p65 (the transactivation domain of nuclear factor [NF]-κB) and Rta (the Epstein-Barr virus transcriptional activation domain) for broad and potent transcriptional activation of affected promoters [174], KRAB catalytic domain of Krüppel-associated box epigenetic repressor [175], ADAR2 adenosine deaminase acting on RNA 2 [101]

4 CRISPR/Cas Beyond the Double-Strand Break

CRISPR/Cas molecules form the basis for new classes of sequence-targeted DNA modifiers by modular combination of their RNP components with additional functional domains. These molecules currently include designer base editors (BEs) [75–81], epigenome modifiers [82–86] and transcriptional regulators [87]. BEs (Fig. 2b) exploit CRISPR/Cas9-mediated target recognition and Cas9 fusion with nucleotide-specific chemical modifiers plus auxiliary components to achieve chemical conversion of DNA bases. To date, conversion of cytosine to uracil (read as thymine; by cytidine deaminase) [76, 78–80] and of adenine to inosine (read as guanine; by artificially evolved adenine deaminase) [81] has been demonstrated. Triggered by nickase activity of a mutated Cas9 and made more efficient by inhibiting base excision repair of the altered base, the conversion is then copied over to the opposite strand to create a double-stranded sequence change without DSB and thereby with
mutations. Epigenome modifiers (Fig. 2c) can modify the use to only a fraction of all pathogenic single-nucleotide and a limited editing window, therefore constrains their midines and vice versa, combined with PAM restrictions base transversions, i.e., substitutions of purines with pyrimidine to another pyrimidine. This inability to catalyze However, BEs are restricted to catalyzing base transitions, i.e., substitutions of adenine-deaminase BEs that have the capacity to correct G > A mutations, which account for approximately half of all known pathogenic single nucleotide polymorphisms [81]. However, BEs are restricted to catalyzing base transitions, i.e., substitutions of purines with pyrimidines and vice versa, combined with PAM restrictions and a limited editing window, therefore constrains their use to only a fraction of all pathogenic single-nucleotide mutations. Epigenome modifiers (Fig. 2c) can modify the accessibility of target DNA and contain fusions of nuclease-deactivated Cas9 (dCas9) with functional enzyme domains or with a binding domain recruiting such enzymes, including histone methylases [82, 83], histone acetyltransferases [82, 83, 86], DNA methylases [82] and DNA demethylases [84]. In transcriptional regulators (Fig. 2d), the dCas9 or sgRNA component of RGNs is fused to transcriptional activator or repressor domains [45] and can be used to regulate gene expression of specific target genes or even gene networks [90, 91]. Application of CRISPR/dCas9 alone can also lead to repression of gene transcription by interfering with the successful formation of the RNA polymerase complex machinery at the initiation step or by disrupting the elongation stage, both via steric hindrance [85, 92]. Modification of protein or RNA components of the RGN allows regulated modulation of transcription [93, 94] and even differential ligand-induced regulation of multiple genes [94]. Sufficiently small artificial transcriptional regulators of this type can be delivered by AAV vectors, which has achieved activity in target cells for over 5 months [95]. However, action of CRISPR/Cas RNPs is not restricted to DNA. Analyses of CRISPR/Cas molecules as a prokaryotic defense system had already indicated targeted RNA cleavage by certain Cas molecules [96] 5 years before CRISPR/Cas9 was also harnessed for targeted cleavage of eukaryotic messenger RNA (mRNA) in 2014 by provision of mRNA-complementary PAM-containing oligonucleotides [97]. Discovery and application of new RNA-targeting CRISPR/Cas molecules continues [98–100] and has recently led to the discovery of the RNA-editing Cas13 family [99–101] and the engineering of CasRx, which shows potent and specific RNA cleavage in human cells and, at a small size of 930 amino acids, is suitable for delivery by AAV vectors [102] to primary cells and for in vivo delivery (Fig. 2e). In addition to the knockdown activity of RNA-binding Cas complex, utilization of a catalytically inactivated CasRx guided by single or multiple guide RNA (gRNA) has the capacity to regulate pre-mRNA splicing (i.e., exon excision) and act as splice effectors (Fig. 2f) [102]. Moreover, and in analogy to designer DNA BEs, modification of Cas13-type molecules can be exploited to edit RNA and convert adenosine to inosine (read as guanosine), for instance to repair premature stop codons, without PAM sequence constraints on the target sequence [101] (Fig. 2g).

5 Brief Instructions for the Toolkit

The current momentum of novel discovery and developments of and around CRISPR/Cas suggests that the tools we have in hand after barely 6 years of research into tailored RGNs are all but the tip of the iceberg. However, these tools already provide a veritable arsenal of possible treatment strategies for genetic diseases. Based on HDR with short donor templates or by base editing, we can fix causative point mutations, activate positive disease modifiers of disease severity and introduce stop codons into gain-of-function toxic ORFs or into negative modifiers. Based on HDR in combination with gene addition, we can minimize insertional mutagenesis by targeting large expression cassettes into inert genomic loci, such as the paradigmatic safe-harbor AAVS1 locus inside the PPP1R12C gene in the case of hematopoietic stem and progenitor cells. Alternatively, we can target gene fragments or complementary DNA (cDNA) into endogenous loci to replace defective sequences and achieve physiological expression from the resulting chimeric expression cassette. Based on NHEJ, we can disrupt toxic mutated genes or regulatory sequences of disease modifiers or, using pairs of RGNs, excise and rearrange parts of the genome. Based on epigenetic and transcriptional regulators, we can boost or dampen expression, respectively, of positive and negative disease modifiers or directly counteract the effect of causative mutations in regulatory regions. For transcriptional regulators, this can already be done in a regulated and multiplexed fashion for several regulatory targets [94]. Finally, the recent addition to the CRISPR/Cas-based toolkit of posttranscriptional regulators that perform targeted RNA cleavage allows the removal of toxic RNAs or accelerated turnover of normal RNAs to alter the transcriptome and cell state [83].

Approaches acting at the level of genomic DNA introduce permanent changes and are best performed using one-off transient RGN applications to limit off-target effects.
Conversely, transcriptional and posttranscriptional modifiers need to act continuously and thus require one-off permanent RGN delivery or repeated transient application over time. For epigenetic modifiers, long-term maintenance and thus the need for reapplication of epigenetic reprogramming depend on the nature and locus of the modification [85]. Delivery of CRISPR/Cas components may be performed by a plethora of methods [68, 103], viral delivery (as AAV or lentivirus [LV]) and electroporation (as RNP or RNAs) being amongst the most widely applicable. Transient delivery may be achieved by electroporation of RNPs or RNAs or by transduction with integration-defective viral vectors, whereas permanent delivery would rely on integrating LVs or, at lower efficiency but also lower risk of insertional mutagenesis, by employing an additional designer nuclease for integration into AAVS1 or other suitable loci of choice. Either methodology may be employed for ex vivo and in vivo delivery of the CRISPR/Cas complex, with a preference of DNA-free delivery by electroporation for ex vivo approaches for reasons of biosafety, and of AAV-mediated delivery for in vivo approaches for reasons of efficiency in a range of tissues [18]. Where donor templates or tags for integration at DSBs are required, those may be delivered as either AAV vectors or as naked nucleic acids or analogs, depending on insert size and type of application [18, 103–105].

6 A Lever to Move the Medical World

Based on these CRISPR/Cas-based technologies, therapy development for many human diseases, including infectious diseases, cancers and monogenically inherited diseases, is underway. As regards human infectious diseases, one of the most intensely researched viral diseases is HIV, for which several CRISPR/Cas-based therapeutic approaches are being evaluated. These include antiviral action by elimination of the C-C chemokine receptor type 5 (CCR5) HIV co-receptor [106] and excision of the HIV provirus from latently infected cluster of differentiation (CD)-4+ T cells for complete elimination of the virus [107]. Besides disruption of integrated proviruses as antiviral therapy, CRISPR/Cas can also eliminate episomal viral DNA species, such as the covalently closed circular DNA responsible for relapse after withdrawal of antiviral treatments in hepatitis B and C infections [108, 109]. In cancers, CRISPR/Cas therapeutic application has two main angles. On the one hand, CRISPR/Cas has been used to accelerate development of chimeric antigen receptor (CAR) T cells [110], enhance their action or enhance the action of naturally occurring T cells [111] as an auxiliary factor to therapy. On the other hand, CRISPR/Cas may be used as direct and possibly the primary therapeutic agents, as proposed for noncoding RNAs [112] or as shown with pro-apoptotic effect for cancer cells by knockout of the programmed cell death protein 1 (PD-1) [113]. The latter approach is at the basis of at least seven current clinical trials (ClinicalTrials.gov ID NCT02793856, NCT02863913, NCT02867332, NCT02867345, NCT03044743, NCT03081715, NCT03342547) for various types of cancer and of an additional trial based on PD-1 knockout in combination with gene-edited CAR T-cells (NCT03545815). As regards monogenically inherited disease and reviewed elsewhere in this issue [114], ease of CRISPR/Cas design favors therapy development for an abundance of rare diseases, most of which are currently without a cure. The CRISPR/Cas strategies employed for rare diseases are highly varied and, depending on the disease mechanism and type of causative mutation, may include disruption, precision editing, targeted integration of expression cassettes or of gene fragments, and transcriptional and posttranscriptional regulation. Despite the experimental nature of therapies based on the nascent CRISPR/Cas technology, three clinical trials aiming to treat patients with rare diseases are already registered, all three with a focus on β-hemoglobinopathies (NCT03655678, NCT03728322 and NCT03745287).

Towards future therapies, through the ease of creating large sgRNA libraries and corresponding viral vectors or RNP complexes, CRISPR/Cas technology also greatly facilitates functional genetic screens for forward or reverse genetics [115]. Compared with screens based on knockdown by RNA interference (RNAi), CRISPR/Cas screens have much greater versatility, in that CRISPR/Cas-based transcriptional inactivation screens mimic RNAi-based screens [116], whereas knockout screens allow more reproducible, complete inactivation of targets in coding as well as non-coding parts of the genome [117, 118], and whereas gain-of-function screens additionally allow assaying for the effects of transcriptional activation [118]. In the context of viral infections, genome-wide screens using CRISPR/Cas have thus already achieved identification of additional host-dispensable critical host factor for HIV for future therapy [119], of antiviral host defense mechanisms against flaviviruses [120] and of critical host factors for additional viruses, such as Dengue, hepatitis C, West Nile and Zika viruses [121], with a clinical trial for the identification of norovirus-critical host factors underway (NCT03342547). As examples for cancer research, CRISPR/Cas is employed to validate shortlisted drug-resistance candidates [122, 123], knockout screens have already identified therapeutic targets for BRCA-, KRAS- and MYCN-linked cancers and large B-cell lymphomas [124–128], and a transcriptional activation screen has revealed genes conferring resistance to cancer treatment by BRAF inhibitors [129]. Because high-throughput screens depend on readily scorable phenotypes, such as viral infection, cell death or cell proliferation, screening for nonmalignant genetic diseases with phenotypes that mostly only appear at the organismal level is more difficult. Nevertheless,
here CRISPR/Cas screens have already revealed novel genes with therapeutic relevance in β-hemoglobinopathies [128], Parkinson’s disease [130], thromboembolisms [131] and inflammatory responses [132] and have shortlisted genes required for processes as diverse as neuronal fate [133], energy metabolism [134] and ferritinophagy [135], giving pointers to novel disease-related pathways and candidate genes.

CRISPR/Cas-type molecules thus have great potential both as therapeutic tools and for the identification of future therapeutic targets. Combined with the wider importance of CRISPR/Cas technology for therapy development, where it also contributes to several-fold accelerated development of disease models by facilitating the creation of isogenic cell-line [136–138] and animal [138–146] models, CRISPR/Cas technology has already become an essential and ubiquitous component of biomedical research for infectious, malignant and nonmalignant diseases.

7 Room for Improvement

As versatile and powerful as CRISPR/Cas technology may already be, many aspects of the technology still need to be improved, understood, or both, for therapeutic application. Whereas for research applications, suboptimal aspects of CRISPR/Cas technology will become an inconvenience or a significant cost factor, for therapy these same aspects will prevent regulatory approval for clinical application or may turn a trial into a tragedy. The issues that remain to be addressed may be divided into aspects of safety, efficiency and utility, with some overlap between the categories.

7.1 Safety

A key safety concern is that of off-target activity, which is still hard to predict, despite large-scale analysis efforts, and is in part brought about by issues of cell-type- and development-specific genome accessibility, which cannot be fully controlled [147]. The problem of potential off-target activity also affects BEs, which moreover have an editing window of 2–5 nucleotides that limits their use for precision editing of certain target sequences [78, 148]. While off-target activity at least may be reduced by high-fidelity variants or, for DSB-based approaches, by paired CRISPR/Cas nickases or CRISPR/FokI dimeric nucleases, approval for clinical trials will be difficult to obtain without comprehensive experimental analysis of potential off-target effects, so as to minimize the residual risk to patients [149]. Another safety concern is that of pre-existing immunity against Cas molecules in humans [39, 150, 151]. While this issue may be of minor importance for most ex vivo approaches and may be overcome for in vivo approaches by alternative enzymes or transient immunosuppression [39], it nevertheless casts a shadow of doubt over in vivo applications of CRISPR/Cas, which are already at risk of adverse reactions or lowered efficiency by pre-existing immunity to AAV and other delivery vectors [152–154]. Two additional and significant safety concerns are directly linked to the induction of DSBs for genome editing by RGNs and could altogether be prevented by approaches that act independent of DSBs, such as base editing and transcriptional or posttranscriptional regulation. First, effective DSB induction may lead to P53-dependent apoptosis, and recent independent studies have pointed out that selecting for DSB-dependent editing events may thus select for P53-deficient cells and cell populations with elevated cancer risk [155–157]. Such enrichment may be prevented by suppressing P53 activity at the time of editing, and it remains to be shown whether the observations for both studies can be reproduced in clinically relevant cell types. As a second safety concern linked to DSB-dependent editing, even single DSB events have been demonstrated to induce unpredictable indel and interchromosomal recombination events [158]. While future research will show how far such events can be minimized by encouraging speedy and faithful DSB repair, they appear to be inherent to RGN activity and therefore represent a safety risk tied up with their application. Further safety concerns exist regarding the long-term safety of editing events. One such concern arises from the long persistence of AAV vectors in target tissue. A favorite vehicle for in vivo delivery of HDR donor templates, AAV vectors show long-term persistence of circular episomal concatemers and the occurrence of illegitimate recombination and rare random integration events [17, 159], which may represent a significant lifetime risk of insertional mutagenesis for treated patients. Another concern is common to all gene therapy approaches based on permanent modification of stem cells and relates to the natural turnover of stem cells actively contributing to tissue reconstitution. Exemplary work on HSCs and multipotent progenitor cells demonstrates that treated cells experience waves of quiescence and active contribution to repopulation [160, 161]. Disconcertingly, this implies that events of insertional mutagenesis or off-target-related genotoxicity in quiescent cells may remain hidden for an extended period of time until the affected cell may become active and experience pathological clonal expansion.

7.2 Efficiency

The frequency of HDR events is a major concern for many applications, particularly owing to bias of HDR events for less basic stem cells. This might partly be addressed by ex vivo selection of genome-edited cells [162], by refinement of additives for the maintenance of stemness in ex vivo procedures and culture [30, 163], by timing DSB induction
with suitable phases of the cell cycle, or by improving the selection procedure for enrichment of true stem cells [164], which would also help lower vector requirements and the cost of the procedure. Moreover, Cpf1 appears to have an advantage over Cas9 molecules in general for certain HDR-mediated knock-in or editing events, which might partly relate to the staggered DSB introduced by Cpf1, akin to that produced by TALENs [165, 166]. Additionally, the nature and symmetry of the HDR donor template affect HDR efficiency [166, 167]. Therefore, many factors allow optimization of HDR efficiency, from cell isolation, selection and culture to choice of RGN and donor sequence and type.

7.3 Utility

Several aspects of Cas9 are points of practical limitation for their employment. For one, Cas9 molecules are fairly large and difficult to deliver by AAV for systemic application, a problem that is aggravated for Cas9 fusion proteins, such as BEs, that include additional functional domains. Although this may be addressed by split delivery [168], the search continues for smaller Cas-type molecules that would more readily accommodate additional domains and efficient delivery. As a second point, PAM site requirement is an evolutionary necessity of the bacterial adaptive immune system and prevents self-cleavage of the host, so that the search for additional Cas variants with alternative PAM sites continues to extend the choice of target sites. However, true freedom to select the target site of DNA-targeting CRISPR/Cas approaches, would only be warranted by protein engineering and removal of PAM restriction altogether, as it already applies for RNA-targeting CRISPR/Cas molecules.

8 Of Disruptive Technologies

In any field of science, new discovery critically depends upon new technological advances and then, in turn, engenders new technological advances. Sometimes the resulting developments are so profound or their practical implications so wide-ranging that they cause a break with previous industry and scientific practice. A key example of such disruptive technologies in molecular biology is the polymerase chain reaction, with such versatility in its application that it had a rapid impact on virtually every aspect of molecular biology shortly after its publication in 1986 [169]. It then seemed that the discovery of RNAi as an antiviral defense and developmental regulatory system may also find wide-ranging biotechnological application [170]. However, more than 20 years after its first conceptualization [171], RNAi has simply turned into one of several arrows in our quiver for reverse genetics and functional analyses and only gradually is its potential for therapy coming to fruition [172, 173]. Given the RNAi experience, it was therefore hard to gauge early on whether the adoption of a prokaryotic antiviral defense system for genome engineering [25] truly heralded a new revolution in molecular biology or merely a further addition of tools for research and therapy development. However, and as we have summarized here, in the 6 years since its original application to mammalian cells, CRISPR/Cas technology has been adopted so widely and has been modified, functionally extended and adapted for so many purposes, that its utility to molecular biology and genetics has taken on a new quality altogether. In more senses than one, therefore, and going well beyond its application for design and delivery of effective therapeutic tools, CRISPR/Cas technology might prove a truly disruptive technology for many aspects of biomedicine and fundamental research.

Compliance with Ethical Standards

Conflict of interest CWL, PP and MK have no conflicts of interest that are directly relevant to the content of this article. No writing assistance was utilized in the production of this manuscript.

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