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REVIEW

Recent Updates of the CRISPR/Cas9 Genome Editing System: Novel Approaches to Regulate Its Spatiotemporal Control by Genetic and Physicochemical Strategies

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Abstract: The genome editing approach by clustered regularly interspaced short palindromic repeats (CRISPR)/associated protein 9 (CRISPR/Cas9) is a revolutionary advancement in genetic engineering. Owing to its simple design and powerful genome-editing capability, it offers a promising strategy for the treatment of different infectious, metabolic, and genetic diseases. The crystal structure of *Streptococcus pyogenes* Cas9 (SpCas9) in complex with sgRNA and its target DNA at 2.5 Å resolution reveals a groove accommodating sgRNA:DNA heteroduplex within a bilobate architecture with target recognition (REC) and nuclease (NUC) domains. The presence of a PAM is significantly required for target recognition, R-loop formation, and strand scission. Recently, the spatiotemporal control of CRISPR/Cas9 genome editing has been considerably improved by genetic, chemical, and physical regulatory strategies. The use of genetic modifiers anti-CRISPR proteins, cell-specific promoters, and histone acetyl transferases has uplifted the application of CRISPR/Cas9 as a future-generation genome editing tool. In addition, interventions by chemical control, small-molecule activators, oligonucleotide conjugates and bioresponsive delivery carriers have improved its application in other areas of biological fields. Furthermore, the intermediation of physical control by using heat-, light-, magnetism-, and ultrasound-responsive elements attached to this molecular tool has revolutionized genome editing further. These strategies significantly reduce CRISPR/Cas9's undesirable off-target effects. However, other undesirable effects still offer some challenges for comprehensive clinical translation using this genome-editing approach. In this review, we summarize recent advances in CRISPR/Cas9 structure, mechanistic action, and the role of small-molecule activators, inhibitors, promoters, and physical approaches. Finally, off-target measurement approaches, challenges, future prospects, and clinical applications are discussed.

Keywords: CRISPR/Cas9, sgRNA, genome editing, off-target effects, spatiotemporal control, clinical translation

Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR)/associated protein 9 (CRISPR/Cas9) are a class of microbial defense mechanisms against mobile genetic elements, plasmids, and phage infections.[1,2] This endonuclease system has been proposed as an innovative tool for genome editing, genome imaging, epigenetic modulation, and transcriptional perturbation. The genome editing technology by using CRISPR/Cas9 is used to precisely manipulate

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functional genes involved in disease progression, correct disease-initiating mutations, activate tumor suppressor genes, and inactivate activated oncogenes.[3–5] CRISPR/Cas9 technology can be used to treat a variety of infectious and genetic diseases such as metabolic disorders, cardiovascular diseases, Alzheimer's disease, and human immunodeficiency virus (HIV) infections,[6,7] because of its simple design and ability to simultaneously edit multiple sites.[8]

Other DNA editing approaches such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are also used for DNA editing. The DNA editing approach of these techniques is based on tedious protein engineering, specific to each DNA-recognition domain to be targeted.[9] However, because these nucleases are specified by different proteins, these approaches are becoming less recognized.[10] However, the CRISPR/Cas9 genome editing system is a far simpler approach, as it is based on a 20-nt gRNA sequence.[4] The CRISPR/Cas9 system is swiftly implemented as a powerful tool in genetic engineering because of its simple structure and ability to edit any DNA sequence.[11]

Despite these promising developments, precise spatial dimensions and long-term control are still necessary for CRISPR/Cas9-mediated genome editing in complex biological systems.[12,13] Thus, CRISPR/Cas9 must be localized to certain cells at a specific time to prevent unnecessary gene disruption during certain phases of cell differentiation and tissue development.[14] Furthermore, the most significant concerns regarding CRISPR/Cas9 genome-editing therapy are off-target effects and a few other genotoxicity issues that worsen as Cas9 activity increases.[12,15] Thus, in complex biological systems, to maximize the therapeutic efficacy of CRISPR/Cas9 by minimizing its off-target activity and genotoxicity, spatiotemporal control of this genome-editing system must be precisely achieved.[16,17]

To date, spatiotemporal control of CRISPR/Cas9-mediated genome editing is still considered a formidable task in clinical applications.[16,18] To overcome this challenge, physical approaches and genetic and chemical regulations are constantly being studied to explore full control of the CRISPR/Cas9 system.[19] The roles of cell-specific promoters, small-molecule activators/inhibitors, bioresponsive delivery approaches, and control by thermal/optical/magnetic/ultrasonic activation of the CRISPR/Cas9 system have been studied.[20–22] Each approach has its limitations and most of the strategies are used by in vitro cell lines; however, their programmable applications under in vivo conditions continue to struggle. Thus, a thorough knowledge of precise spatiotemporal control over CRISPR/Cas9 by using different methods is needed to utilize this genome-editing tool for innovative applications.

This review discusses the current updates on CRISPR/Cas9 biology and its mechanisms of action. In addition, the innovative roles of genetic, physical, and chemical elements that can regulate spatiotemporal control of CRISPR/Cas9 activity are discussed. In addition, off-target measurement approaches, challenges, future prospects, and clinical translation are discussed.

Innovative Advances in CRISPR/Cas9 System

In 1987, Japanese researchers discovered numerous tandem repeats in the *E. coli* genome that had not been previously identified. They did not publicize these findings due to a lack of information about its biological significance.[23] However, these sequences were first named as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) in 2002, but their actual function was still unclear.[24] In 2005, three distinct research teams demonstrated that the CRISPR loci are important for bacterial adaptive immunity.[25] Some researchers reported that the resistance of bacteria to phages might be altered by the integration of viral gene sequences.[26] In 2008, it was found that small segments of CRISPR-incorporated non-coding RNA may lead the CRISPR-associated (Cas) proteins to the target-specific DNA region, enabling them to carry out a protective function.[27] Deltcheva et al unraveled some novel locations for crRNA development and showed a correlation between trans-coding crRNA (tracrRNA) and pre-crRNA maturation and processing.[28] Further studies conducted in vivo in 2012 revealed that when mature crRNA pairs base-wise with tracrRNA, it generated two distinct RNA structures that guided the CRISPR-associated protein Cas9 to cleave double-stranded (ds) DNA.[29] Later, the Cong and Mali research groups employed two distinct type II Cas systems to create DNA cuts in cell cultures and made genome editing with the CRISPR/Cas9 system feasible.[30] By 2020, a plethora of CRISPR/Cas9-based tools for DNA and RNA gene editing had been produced following the discovery of the technology, and the field has advanced quickly ever since[31] (Figure 1).

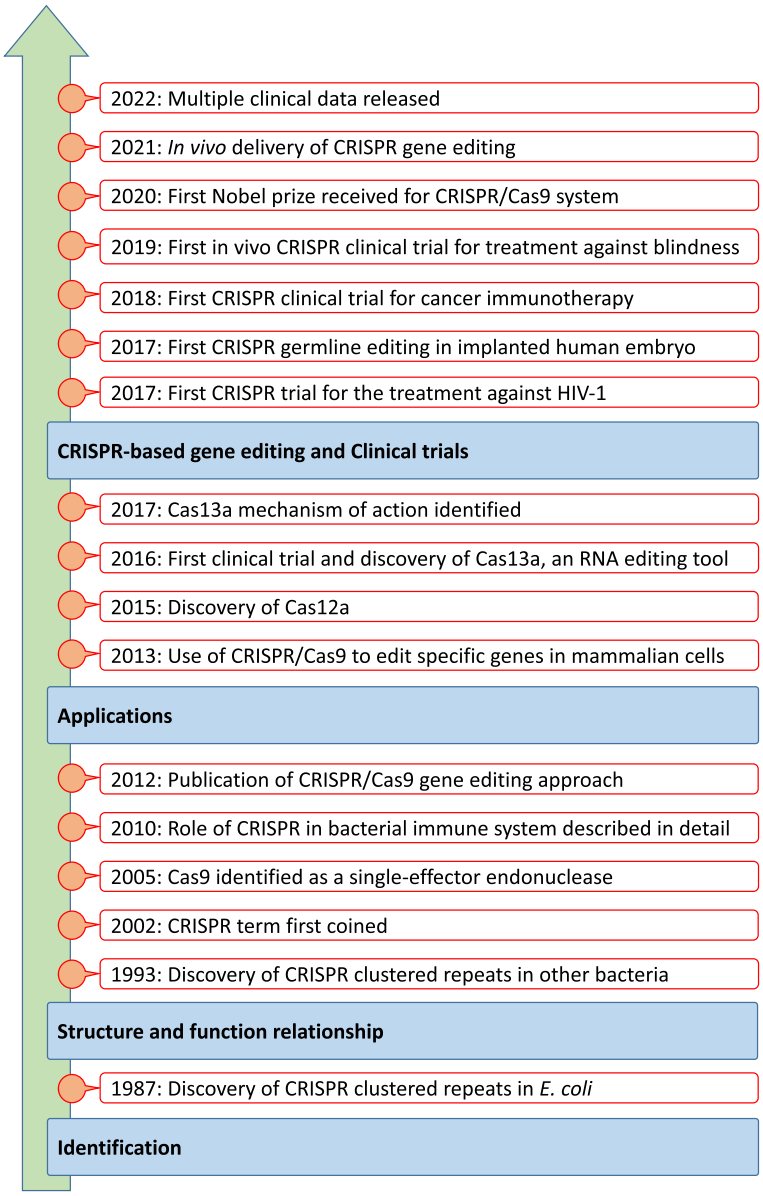

Figure 1 A timeline of the innovative discoveries in CRISPR/Cas9 system as identification, structure-functional relationship, and its role in different clinical trials.

CRISPR/Cas9 Biology and Its Mechanism of Action

The CRISPR/Cas system is a marvel of microbial defense approach. Different microbes (bacteria and most archaea) have evolved this smart RNA-guided adaptive immune system encoded by the CRISPR loci and CRISPR-associated (*cas*) genes. This system allows microbes to acquire immunity against infection using mobile genetic elements, plasmids, and bacteriophages[32,33] (Figure 2). The exposure of invading plasmids or phage genetic elements leads to the formation of new spacers through the integration of small fragments of their DNA into the CRISPR repeat-spacer array within the host chromosome.[34] This integration of new foreign genetic sequences provides the host access to the genetic history of all prior infections and helps it to defend similar invaders in the future.[35,36]

Short mature CRISPR RNAs (crRNAs) are key players in the CRISPR/Cas system, which are produced through endonucleolytic cleavage and enzymatic processing by subsequent transcription of the CRISPR array.[1] A short segment of RNA, known as a spacer, is followed by the crRNA at its 5′ end which complements the sequence from the foreign genetic element and the CRISPR repeat sequence is contained at the 3′ end. Hybridization between the protospacer

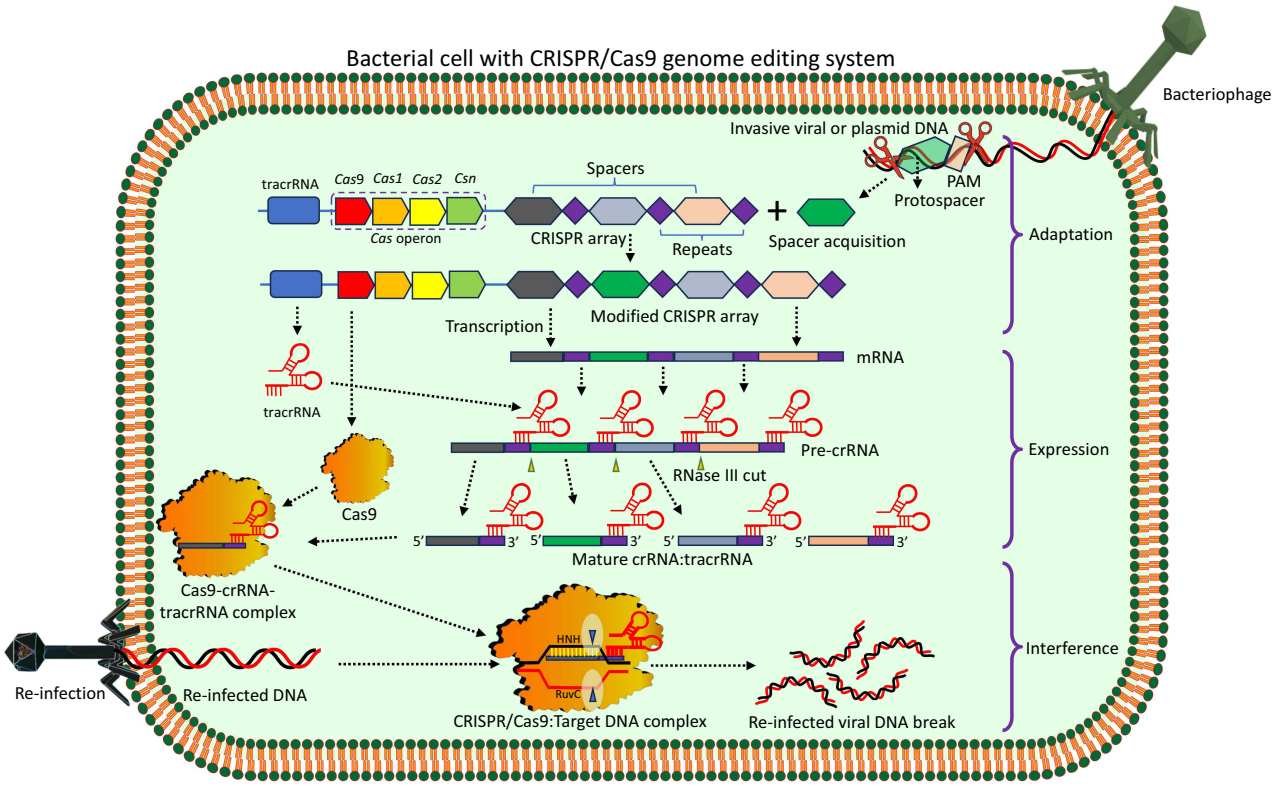

Figure 2 Diagrammatic representation of CRISPR/Cas9 action mechanism in a bacterial cell as adaptation, expression, and interference stages. Invasion of bacteriophage DNA or plasmid fragments are incorporated as a protospacer to bacterial CRISPR array as new spacers. It is followed by pre-CRISPR RNA transcription and mature crRNA: tracrRNA are formed subsequently. Activation of Cas9 protein occurs by binding with sgRNA for subsequent re-infection with bacteriophage or new plasmids.

(complementary foreign target sequence) and crRNA spacer initiates the destruction of sequence-specific invading RNA or DNA by Cas nucleases upon subsequent infection.[37,38] Thus, the CRISPR/Cas9 system is a smart assembly between the Cas9 protein and crRNA, forming crRNA-effector complexes that interact with foreign DNA targets and destroy matching sequences.[39,40] Furthermore, a protospacer adjacent motif (PAM), a short-conserved sequence (2–5 bp), is positioned adjacent to the crRNA-target sequence, that plays a critical role in DNA targeting.[41,42] In most CRISPR/Cas systems, it serves as a crucial double-check in the selection of the target DNA and its subsequent degradation.

According to the current classification of CRISPR/*cas* loci, CRISPR systems are divided into two classes (Class 1 and Class 2) divided into six types (I–VI) based on the effector Cas proteins.[43,44] Both crRNA and a distinct set of Cas proteins have been used for each type of CRISPR interference.[45] Unlike type I and type III CRISPR systems, which use a large multi-Cas protein complex for crRNA binding and target sequence destruction, type II CRISPR systems use a single DNA endonuclease as Cas9.[39]

During the expression process, an extra noncoding RNA called trans-activating crRNA (tracrRNA) is required (Figure 2). It is a small RNA molecule working together with crRNA to guide the Cas protein in targeting specific DNA sequence. In type II CRISPR systems, tracrRNAs are required for crRNA maturation.[46] A distinct dual RNA hybrid structure forms between tracrRNA and crRNA repeat sequences.[46] This dual RNA guide directs the Cas9 endonuclease protein to cleave any DNA sequence with an adjacent PAM and a complementary 20-nucleotide (nt) target sequence.[47,48] A novel innovation has been achieved in the history of the CRISPR/Cas9 endonuclease system by generating chimeric single-guide RNA (sgRNA), a combination of tracrRNA and crRNA, into a single RNA transcript. It maintains the functionality of Cas9-based sequence-specific DNA cleavage and streamlines the endonuclease system.[48] The guide RNA (spacer) sequence within the crRNA can be changed to target any desired DNA sequence by using this novel and straightforward two-component CRISPR/Cas9 system.[48]

CRISPR/Cas9 system indeed creates a blunt-ended double-stranded break (DSB) of invading double stranded DNA or any sequence of interest.[7,48] After a DSB is created in a DNA sequence, two repair pathways come into play including homology-directed repair (HDR) or by error-prone non-homologous end joining (NHEJ). These repair systems have varying levels of accuracy and efficiency.[49,50] HDR results in precise genome modification using a homologous repair template. Whereas, in contrast, NHEJ results in small random insertions and/or deletions (indels) at the cleavage site (Figure 3).

Cas9 Enzyme

SpCas9 consists of a single large multidomain multifunctional protein (160 kDa) with 1368 amino acid residues. This genome-editing tool requires Cas1, Cas2, and Csn2 during the foreign DNA acquisition step,[51] and RNase III is involved in the processing of pre-crRNA to mature sgRNA.[46,52] The three-dimensional computer modeling of CRISPR/Cas9 represented as mesh model, ribbon representation or space filling model has helped comprehensively to understand the structural and functional relationships between its different protein domains (Figure 4a–c). The SpCas9 protein in its apo form (pre-binding state) consists of two distinct lobes: a recognition (REC) lobe and a nuclease (NUC) lobe (Figures 4 and 5). The REC lobe is divided into three α-helix regions, a bridge helix spanning 60–93 residues, REC1 region (94–179, 308–713 residues), and REC2 domain spanning from 180 to 307 residues. The NUC lobe is composed of RuvC (residues 1–59, 718–769, 909–1098), HNH (residues 775–908), and a PAM-interacting (PI) domain spanning 1099–1368 residues[53] (Figure 4d). Additionally, a Cas9-specific fold with a PAM-interacting (PI) site is visible in the elongated C-terminal domain (CTD).

This DNA endonuclease system possesses two distinct domains that snip dsDNA 3bp upstream of the PAM. These two nuclease domains include the HNH-like nuclease domain and Ruv-C like nuclease domain. The HNH-like nuclease domain cleaves the target DNA strand complementary to the gRNA sequence, and the RuvC-like nuclease domain cleaves the nontarget DNA strand.[48,54] Additionally, Cas9 plays a role in the maturation of crRNA and acquisition of spacer sequences.[55]

In apo-Cas9 state, the PAM recognition region is largely disordered, indicating that apo-Cas9 is maintained in an inactive configuration and cannot recognize the target DNA before gRNA binding occurs[52] (Figure 5a). This finding indicates that in the presence of gRNA or hairpin, apo-Cas9 can also bind DNA nonspecifically and rapidly detach from nonspecific sites.[56] Thus, Cas9 endonuclease is inactive in the absence of bound gRNA,[48] thus supporting its function as an RNA-guided endonuclease system.[57]

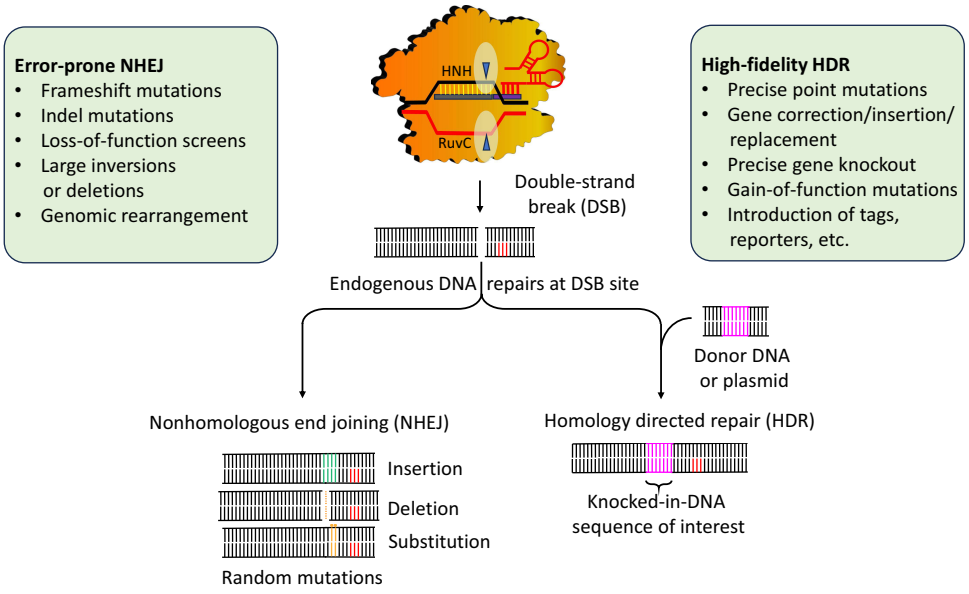

Figure 3 Diagrammatic representation of double-strand DNA break by CRISPR/Cas9 and the repair mechanism of DSB by NHEJ and HDR approaches. The NHEJ results in gene-knockout in the absence of any donor DNA and leads to the formation of random indels. The HDR approach works in the presence of donor DNA for precise nucleotide substitution.

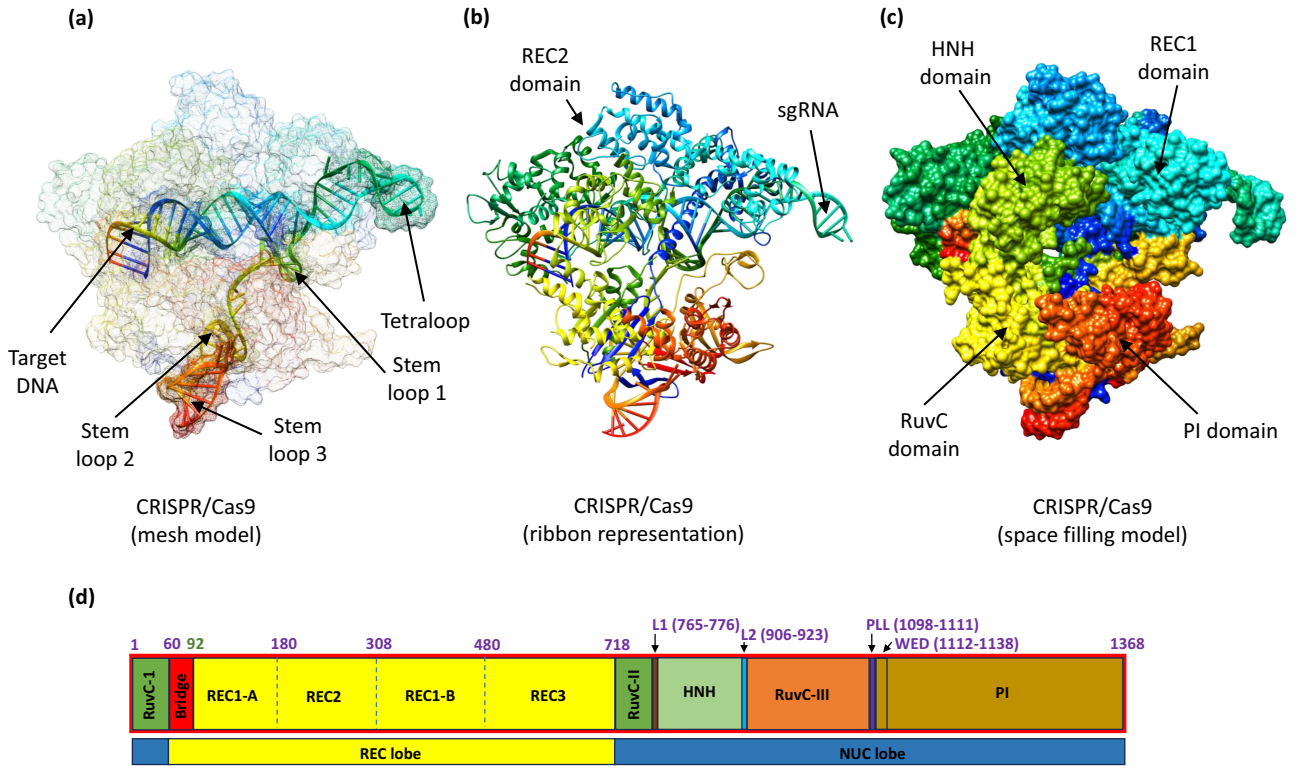

Figure 4 The Spy Cas9-sgRNA-DNA ternary complex three-dimensional structure, as found in the Protein Data Bank (PDB) (https://www.rcsb.org, PDB ID: 4OO8), edited by using software developed by UCSF Chimera. (**a**) Mesh model of CRISPR/Cas9 system to elucidate proper shape of sgRNA-DNA within protein interior. (**b**) Ribbon representation of CRISPR/Cas9. (**c**) Space filling model of CRISPR/Cas9 to show different domain organization. (**d**) Domain organization and their amino acid residue numbers.

Significant conformational changes in the three-dimensional structure of CRISPR/Cas9 occur for its mode of action, as shown by X-ray structures obtained at various active stages.[58,59] The different stages of the *S. pyogenes* CRISPR/Cas9 editing pathway have been resolved as free Cas9 with a protein data bank (www.rcsb.org) (PDB 4CMQ),[57] sgRNA-bound Cas9 (PDB 4ZT0),[59] Cas9 in association with target DNA and incomplete nontarget DNA with PAM sequence (PDB 4UN3),[58] and Cas9 in association with both target DNA and complete nontarget DNA (PDB 5F9R).[59] The sgRNA associated Cas9 from *S. pyogenes* (PDB 4CMQ and PDB 4ZT0) were determined at a resolution of 3.09 Å and 2.9 Å , respectively.[57,59] The helical REC domain undergoes a significant rearrangement upon sgRNA binding, resulting in a shift of the REC III domain of approximately 65 Å to accommodate the sgRNA[59] (Figure 5b). A further shift in the REC II domain occurs by binding of the target DNA and PAM containing an incomplete nontarget DNA strand to the Cas9:sgRNA complex (PDB 4UN3).[58] Melting of foreign DNA occurs via Cas9:sgRNA-based PAM recognition, resulting in a DNA:RNA hybrid formation (Figure 5c).[56,58]

For the cleavage of the nontarget DNA strand, RuvC most likely uses a two-metal-ion catalytic mechanism,[53,57] while the HNH nuclease domain adopts a characteristic ββα-metal fold and most likely uses a one-metal-ion mechanism for the cleavage of the target strand DNA. Nucleic acid cleavage by one-metal ion- and two-metal ion-dependent activities is conserved for aspartate and histidine residues.[60] This is consistent with mutating either the RuvC domain (D10A) or HNH (H840A), which converts Cas9 into nickase, whereas mutations in both these domains result in dead Cas9 (dCas9). This Cas9 variant exhibited intact RNA-guided DNA binding, whereas endonuclease activity was abolished.[60]

CRISPR/Cas9 Effector Complex Assembly

In order to accomplish site-specific identification and cleavage with full functionality, Cas9 must be assembled with sgRNA (crRNA-tracrRNA complex) (Figure 5b).[48,57] The positively charged groove at the interface between the NUC and REC lobes accommodates the negatively charged sgRNA target DNA heteroduplex[53] (Figure 4). Three motifs (RuvC

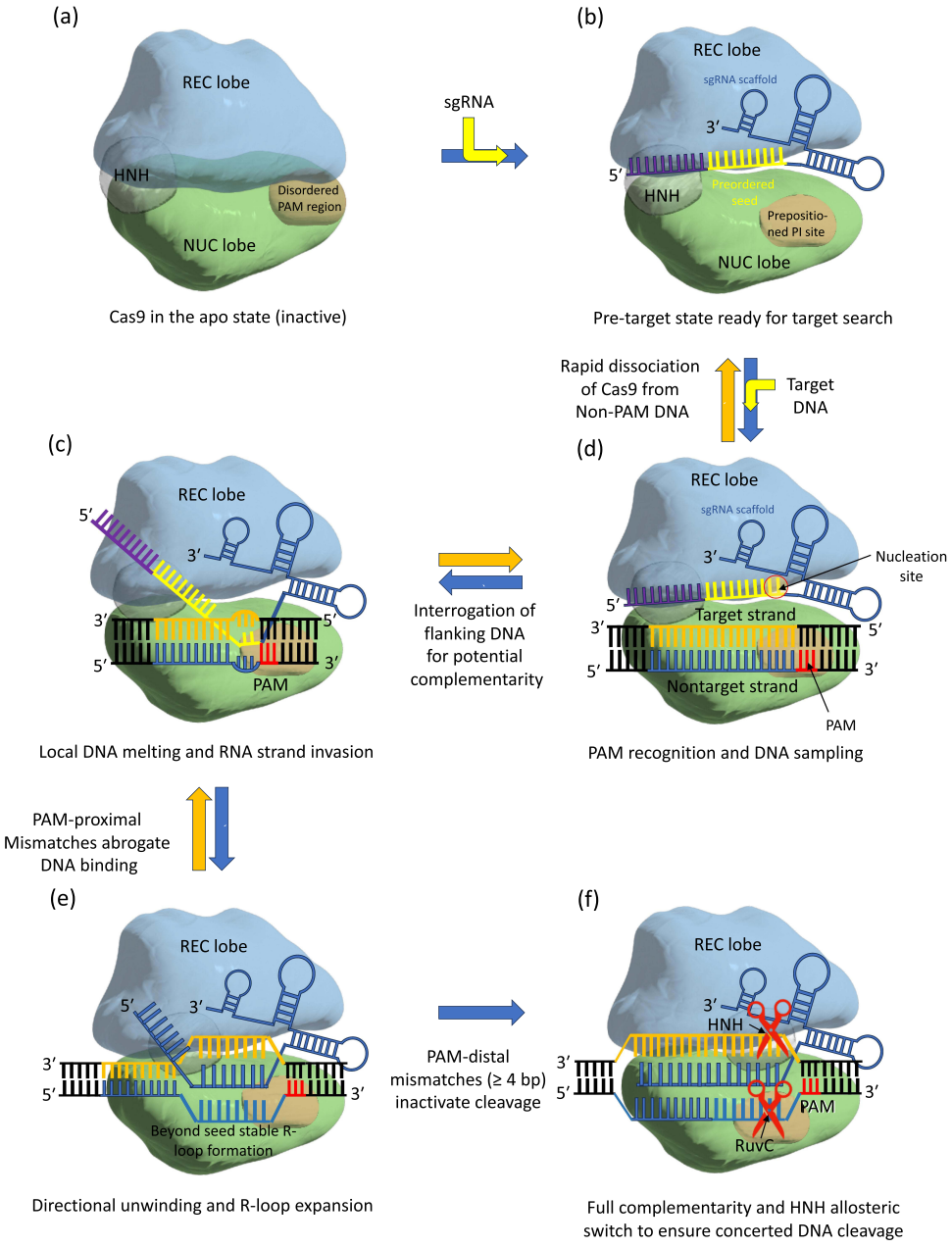

Figure 5 Proposed mechanisms of target DNA recognition and its cleavage by CRISPR/Cas9. (**a**) sgRNA, binding to apo-Cas9 state with largely disordered PAM-interacting cleft, undergoes large conformational changes to achieve the active target recognition state. (**b**) Functionally active Cas9. in pre-target state with REC and NUC lobes. becomes ready for target search. (**c**) Cas9 is further activated through PAM recognition. (**d**) Cas9 follows progressive R-loop formation, RNA strand invasion, and local DNA melting. (**e**) R-loop expansion is followed by directional unwinding of target DNA. (**f**) A complete process of concerted target DNA cleavage occurs by HNH allosteric switch.

I–III) make up the RuvC domain in the NUC lobe which interact with the PI domain to generate a positively charged surface that interacts with the sgRNA 3′ tail. The HNH domain lies between the RuvC II–III motifs and the rest of the protein forms only a few contacts.

DNA target specificity is conferred by the 20-nt crRNA spacer sequence, and tracrRNA plays a fundamental role in Cas9 recruitment.[4] Within the spacer region of crRNA lies a sequence, the so-called seed sequence of RNA nucleotides, which is crucial for target specificity.[61,62] While any close homology in the seed region with any DNA sequence frequently results in off-target binding events, any mismatch in the seed region either drastically reduces or completely eliminates the target DNA binding and cleavage.[63]

Cas9 undergoes substantial structural rearrangement upon guide RNA binding, from an inactive conformation to a DNA recognition conformation (Figure 5b). The most noticeable changes occur in the REC lobe, particularly in Hel-III, as it moves ~65 Å toward the HNH domain by sgRNA binding. However, fewer conformational changes occur when Cas9 binds to the target DNA and the PAM sequence. This suggests that Cas9 undergoes significant structural reorganization before binding to its target DNA, highlighting the idea that guide RNA loading largely controls Cas9 enzymatic activity.[57]

Because of its CTD and RuvC, Cas9 interacts with sgRNA stem loop 2 less frequently. However, several direct connections were seen between the repeat-antirepeat duplex and stem loop 1 as well as between the linker region and stem loops 1 and 2 via Hel-I (Figure 4). Furthermore, because sgRNA lacks a 3′ tracrRNA tail, no RNA–protein interaction was observed for stem loop 3 in the sgRNA-Cas structure.[53,64] In summary, stem loop 1 and the repeat-antirepeat duplex are necessary for the formation of the gRNA-Cas9 complex, but stem loop 2, stem loop 3, and the linker are not necessary for the function[65] but may play a role in the stabilization of gRNA binding to help active complex formation[66,67] (Figure 4).

Target Search and Recognition

The binding of gRNA to Cas9 complements the search for target DNA sites.[59] Target recognition depends on base pairing between the target DNA's protospacer and the 20-nt spacer region, as well as the conserved PAM next to the target site.[47,48] The PAM sequence[68] performs self- and non-self-sequence discrimination, and any mutation in PAM prevents Cas9 cleavage activity.[48] A host immune response can be evaded by bacteriophage by this mutation.[69,70] For commonly used SpCas9, the native PAM sequence is 5′-NGG-3′, where N represents any of the four DNA bases. After a three-dimensional collision, Cas9 dissociates swiftly from the DNA with an inappropriate PAM sequence, and vice versa.[71,72] Local DNA melting is triggered adjacent to the nucleation site at an appropriate target site with a proper PAM match (Figure 5c). RNA strand invasion creates an RNA-DNA hybrid and a displaced DNA strand (R-loop) from the PAM proximal to the distal end (Figure 5d).[73,74]

The middle channel that divides the REC and NUC lobes encases the RNA-DNA hybrid, which Cas9 detects regardless of its sequence. This implies that instead of recognizing the nucleobases of the guide target heteroduplex, Cas9 recognizes its shape. Different SpCas9 variants have been shown of the induced fit mechanism, Cas9 recognizes the noncanonical PAM sequence via an induced-fit mechanism.[75,76] Additionally, some modified Cas9 variants showed structural plasticity for PAM recognition, highlighting the critical function of PAM recognition in initiating target DNA unwinding.[73] Cas9-PAM interactions promote Watson–Crick base pairing between the target DNA strand and guide RNA and enable local structural modifications to stabilize the nearby DNA duplex.[58,64]

Straight upstream of PAM, a sharp kink turn in the target strand is observed; this kink-turn configuration is important for driving the transition of the target DNA strand from guiding RNA pairing to pairing with a nontarget strand (Figure 5d). Additional biochemical and single-molecule investigations have demonstrated a connection between PAM acknowledgment and the local destabilization of nearby sequences.[48,56] This suggests that the interaction between the phosphate lock loop and +1 phosphate upon PAM recognition contributes to the stabilization of RNA-DNA hybridization and local DNA duplex melting.[58] Previous studies have shown that a mismatch of two base pairs adjacent to PAM fully inhibits binding and the introduction of DNA bubbles into the target DNA, hence promoting strong cleavage and removing the requirement for the formation of DNA-RNA heteroduplexes.[56] Cas9 significantly bends the DNA helix and change the duplex trajectory. Thus, a bend from 180° to almost 150° in bound DNA is observed (Figure 5d). Cas9 mediated DNA bending facilitates strand separation and prevents rehybridization (R-loop collapse) (Figure 5e).

Target Cleavage

The Cas9 enzyme is activated to cleave DNA upon PAM recognition, followed by DNA-RNA duplex formation.[56] The RuvC domain of Cas9 consisted of three split RuvC motifs and the HNH domain is located in the middle portion of the protein. Specific nuclease domains cut each strand of target DNA at a specific position; three base pairs distant from the NGG PAM sequence (Figure 3). Target cleavage of DNA causes most blunt-ended double-strand breaks (DSBs).[47,48] However, Cas9 nickase (D10A or H840A), a variant of wild-type (WT) Cas9, can cut only one strand of a DNA duplex,

resulting in a single-stranded break.[47] A double nick-induced DSB can be performed on target DNA to enhance genome-editing specificity by pairing sense and antisense sgRNAs targeting Cas9 nickases on opposite strands.[77]

Proposed Model for CRISPR/Cas9-Based DNA Targeting and Cleavage

Based on the current structural and systematic research, a detailed Cas9 mechanistic model was generated based on the activation of gRNA binding and recognition of target DNA (Figure 5). In this model, a major conformational rearrangement of Cas9 occurs via guide RNA binding.[57,59] This conformational change shifts the inactive Cas9 state to the DNA recognition state[6,59] (Figure 5b). For target binding and strand invasion, the RNA seed sequence A-form conformation is preordered and prepositioned for PAM interrogation and recognition.[59] The Cas9 binding to PAM allows the enzyme to quickly search nearby DNA for the target sequence.[73,78] Once Cas9 discovers a specific target with a suitable PAM, duplex unwinding is initiated[73,79] (Figure 5d). The phosphate lock loop stabilizes unwound target DNA. To pair the bases, it positions the first base of the target DNA to flip and rotate upward toward the gRNA.[58,64] The flipped base interacts with Cas9 on the nontarget strand to enable duplex unwinding.[64,80]

The Cas9 conformational changes that result from base pairing between the guide RNA and target facilitate the invasion of the gRNA strand beyond the seed region.[53] The base pairing is propagated to the 5′end of the guide sequence once the non-seed regions are sequentially released from the constraint.[56,73] This advanced base pairing further promotes strenuous conformational changes in Cas9 until an active state is achieved.[81,82] After extensive annealing of the target DNA and gRNA, HNH eventually achieves a stable active conformation for target strand cutting.[64,80] These HNH conformational changes instantaneously lead to conformational changes in loop linkers, which direct the RuvC catalytic center to interact with the nontarget strand for concerted cleavage[64,83] (Figure 5f). After the target DNA is cleaved, Cas9 remains closely linked until other biological components are removed from the enzyme, allowing for additional activity.[56]

Targeting Approaches of CRISPR/Cas9

The Delivering CRISPR/Cas9 components into desired cells is a crucial step in the gene editing process. It allows the Cas9 nuclease and sgRNA to reach their target location in the genome and make the desired edits. It is highly significant to consider the appropriate, safe, and accurate delivery method to transport the CRISPR/Cas9 system to specific cellular location, particularly in vivo, and aiming for the correct sequence inside the nucleus. Different approaches including viral, nanomaterial-based, and physical methods are used to target CRISPR/Cas9 system within target cells. The viral vectors include lentivirus (LV), adenovirus (AV) and adeno-associated virus (AAVs), nanomaterial-based nanoparticles (NPs) and physical methods by microinjection and electroporation are commonly used to target this genome editing tool within specific cells.[84,85]

Delivery by Viral Vectors

Some viral vectors are commonly used to deliver CRISPR/Cas9 genome editing components to desired cells. Adeno-associated viruses (AAV), including lentivirus and adenovirus, have been effectively employed in in vivo studies[86] and only very rarely induce immunological responses in humans.[87] The genomic size of SpCas9 alone is around 4.3 kbp, whereas the maximal size that a single AAV vector can transport is approximately 4.7 kbp.[88] AAVs do not cause any other disorders; however, the primary drawback of AAV is its small package size, which necessitates the use of many AAVs in order to contain all CRISPR components, including gRNA and Cas protein.[87]

A viable solution to the packaging problem is to divide the Cas9 protein into two AAV (AAV-split-Cas9) vectors rather than one.[89] Large size vectors raise the possibility of off-targeting and mutation, as previously mentioned.[90] To decrease off-targeting and boost delivery effectiveness, a smaller Cas9 protein must be used and spliced into two AAV vectors.[91] The use of ribonucleoprotein (RNP) complexes, such as recombinant CRISPR-Cpf1 Ribonucleoprotein (CRISPR-Cpf1-RNP) inhibited off-target activity in mouse cells,[92] is another method to lessen the risk of off-targeting associated with delivery modalities. Additionally, based on these findings, Mout and associates used Cas9-RNP techniques, which have about 95% success rate in cultivated cells.[93] Consequently, there is a much lower chance of subsequent mutations and off-targeting that arise from the ongoing expression of viral vectors.[94]

Delivery by Non-Viral Vectors

Non-viral delivery vectors including lipid and inorganic nanoparticles (NPs) offer a more strategy of CRISPR/Cas9 transport.[95] The non-viral delivery strategy such as nanoparticle-based delivery permits more frequent gene therapy injection with less immunogenicity risk, reduced exposure to nucleases, and improved targeting precision.[95] Moreover, non-viral vectors are more capable of integrating into the conveyed genome than viral vectors without compromising their capacity.[96]

Cas9 mRNA/sgRNA delivery has also been investigated by using alternative methods, including extracellular vehicles (EVs),[97] nanogels,[98] and polyplex micelles.[99] Membrane-related vehicles (EVs) consisting of proteins, lipids, and nucleic acids have gained popularity as RNA carriers because of their unique signal transmission capacity and innate biocompatibility. Conversely, extracellular vesicle-based systems have been effectively applied in both in vitro and in vivo settings and are less expensive and safer than the other method.[100] The transport and efficiency percentage of the CRISPR system into the targeted area is another difficulty, particularly in cancer therapy where entire editing efficiency is required.[88]

Regulation of CRISPR/Cas9 Activity

Recent comprehensive knowledge about the role of PAM and the role of crRNA and tracrRNA, which are now often engineered as single guide RNA (gRNA), has uplifted the applications of CRISPR/Cas9 to new heights. The CRISPR/Cas9 system currently has enormous potential for treating HIV, cancer, genetic, metabolic, Alzheimer's, and cardiovascular diseases.[101] Despite these inspiring possibilities, more precise control over spatial dimensions and time is a basic requirement for handling CRISPR/Cas9 activity in complex biological systems.[12,13] Additionally, CRISPR/Cas9-based genome-editing perturbations at undesirable genetic loci must be avoided during specific phases of cellular differentiation and tissue development. Thus, strict localization of this endonuclease-based genome editing tool at certain time in specific cells or tissues is of utmost importance.[14] Furthermore, genotoxicity and off-target effects of CRISPR/Cas9 are the most important issues that can be enhanced by increasing the activity of Cas9.[12,15] Therefore, to minimize the off-target activity and genotoxicity of CRISPR/Cas9 and expand its therapeutic efficacy, proper spatiotemporal control of this endonuclease system must be achieved.[16,102]

To date, precise spatiotemporal control over CRISPR/Cas9 remains a formidable challenge for robust implementation in clinical applications.[18] For this purpose, thorough knowledge of the different factors responsible for the genetic and physicochemical regulation of CRISPR/Cas9 is of principal importance in strengthening its conditional control. Figure 6 provides a brief diagrammatic presentation of various methods, including chemical, physical, and genetic strategies, for the spatiotemporal control of CRISPR/Cas9 in biological systems.[103]

It is crucial to have complete knowledge of the functions of these small-molecule promoters that are specific to cells, inhibitors, and bioresponsive delivery vehicles. In addition, spatiotemporal control by thermal/optical/magnetic/ultrasonic activation of the CRISPR/Cas9 system maximizes its application in different clinical applications.[20–22] However, all of these strategies are suboptimal and have some shortcomings (Table 1). Therefore, the majority of CRISPR/Cas9 regulation strategies have only been tested in cell lines in vitro. It is unclear if this genome editing system is suitable for in vivo applications given the abundance of genetic and spatiotemporal regulators. Therefore, systematic comparisons of various approaches to precisely regulate the spatiotemporal activity of CRISPR/Cas9 gene editing are essential.

Genetic Regulation

Various techniques have been used to engineer new Cas9 variants with a high fidelity. These approaches include the rational, nonrational, and combined methods. A rational method involves structural and/or functional expertise, particularly point mutations or computational modelling, to engineer new Cas9 variant designs.[108] Nonrational methods are evolution-based approaches that involve high-throughput screening through random mutagenesis.[109] Using both structure-guided and direct evolution, new Cas9 variants are being engineered.

Different high-fidelity Cas9 variants have been generated through simple amino acid substitutions in the wild-type (WT) Cas9 protein. These variants included eSpCas9, evoCas9, HypaCas9, Sniper-Cas9, SpCas9-HF1, SpCas92Pro and xCas9 3.7.[20,110,111] These engineered Cas9 variants exhibited reduced off-target effects and/or potential cleavage activities to different degrees. Consequently, choosing the correct Cas9 variant is crucial, but difficult. WT Cas9 has

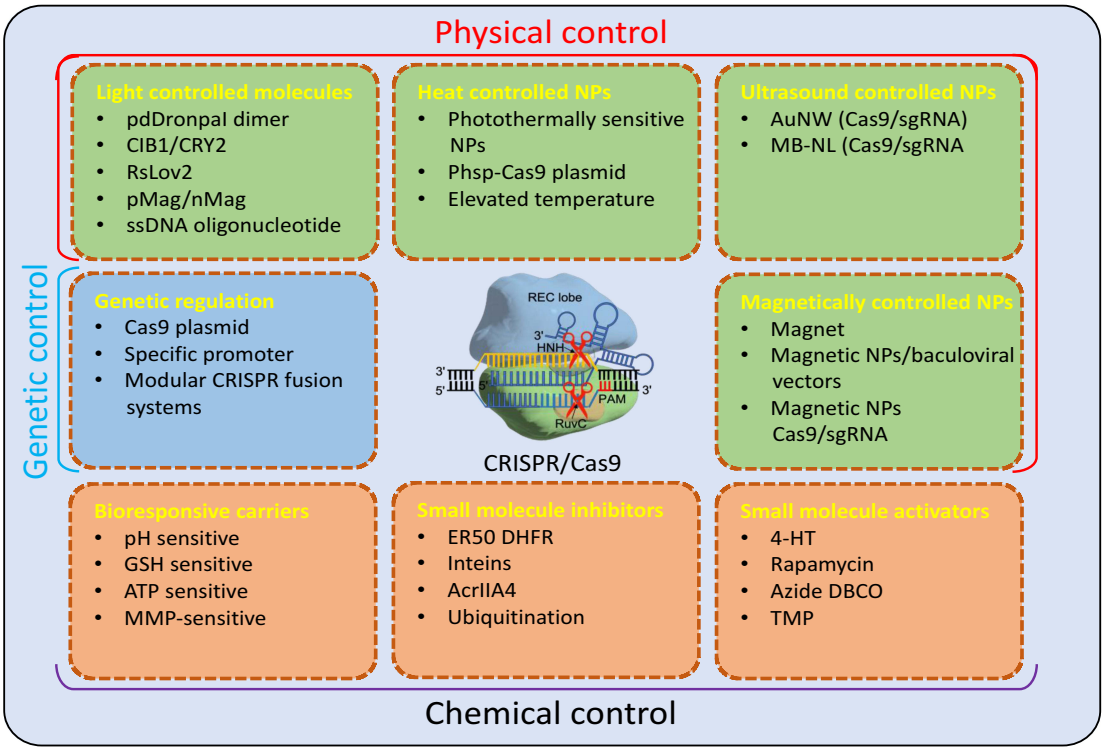

Figure 6 Different strategies of spatiotemporal (physical, chemical, and genetic) control used in CRISPR/Cas9 genome-editing approach.

been designed to accommodate nuclease-dead (dCas9), catalytically inactive, or Cas9 nickase form.[112] This Cas9 form binds the DNA precisely and specifically under the control of sgRNA, but does not cleave it.[113] For the purpose of genetic regulation, different transcriptional activators or repressors are recruited by dCas9 for targeted gene activation or repression (Figure 7).

In addition, genetic modifiers have been employed to achieve specific epigenetic modifications at some genomic locations using dCas9. General examples of some key molecules or structures that are used as modular CRISPR fusion systems include AcrIIA 1–4, VP16 multiple copies, VP64, numerous sgRNAs, KRAB, p65, SID4X, etc.[114,115] Some cell-specific promoters

Table 1 Advantages and Disadvantages of Different Regulators of CRISPR/Cas9 Activity

Control Type	Advantage	Disadvantage	Reference
Cell-specific promoters	Efficient specificity of gene editing in the spatial dimension and low level off-target potential	Cumbersome in assessment of more promoter with high cell specificity and activity	[104]
Small molecule activators	Easy synthesis with higher cell permeable and nonimmunogenic and good gene editing efficiency	Higher cytotoxicity with substantial background activity and lack of broad dynamic range	[8]
Small molecule inhibitors	Easy to synthesize with good cell permeability capacity and nonimmunogenic	The identification and screening is difficult with high background activity and lacks easy tunability in clinical application.	[8]
Light control	Easy tunability, low toxicity and noninvasive with reversible properties	Tissues demonstrate strong absorption of light, limited to a narrow spectral range and require long exposure time	[12]

(*Continued*)

Table 1 (Continued).

Control Type	Advantage	Disadvantage	Reference
Ultrasound control	Potential gene editing specificity, noninvasive and has good transfer efficiency	Lacks easy tunability in clinical applications, is less reversible and is not fully explored	[105]
Heat control	Potential gene editing specificity, easy tunability and is noninvasive	It has low photothermal conversion potential with long exposure time and face difficulty in screening of more heat shock promoters	[106]
Magnetic control	It possesses potential gene editing specificity, has low cytotoxicity and is not disturbed by biological tissues	The screening of magnetic sensing material is difficult and have not been fully explored	[107]

used to achieve genetic regulation of CRIPR/Cas9 include the CD4 promoter, egg cells, liver cells, macrophage-specific promoters, and erythrocyte-specific gata1 promoters[104,116] (Figure 7).

Transcription Activation of CRISPR/Cas9 Through Modular Fusion Systems

The regulatory activity of the CRISPR/Cas9 system can be demonstrated through the attachment of protein-interacting RNA aptamers. This allows the system to recruit a variety of transcription activation domains, such as p65 and VP64, to improve gene expression (Figure 7a). It has been noted that sgRNA constructs with various RNA hairpin structures, such as Com, MS2, or PP7 RNA hairpins, significantly drive reporter gene expression. These RNA hairpins can recruit protein-interacting RNA aptamers such as Com, MCP, or PCP attached to the VP64 activation domain, which causes target gene expression[117] (Figure 7d).

Owing to mutations in the RuvC and HNH nuclease domains, dCas9 is unable to cleave DNA strands, but can still target loci with the assistance of sgRNA.[118] A target gene can be basically transcriptionally activated by binding dCas9-sgRNA to certain small-molecule activation domains like p65AD, Rta, and VP64[119,120] (Figure 7c). For instance, dCas9 was fused with VP64 or p65 activation domains to create dCas9-p65 and dCas9-VP64, respectively.[119,121] Targeting the Gal4 upstream activation sequence (UAS), these sgRNA-bound fusion proteins were concurrently transfected into HEK-293 T cells, which expressed a green fluorescent protein (GFP). However, HEK-293T cells transfected with dCas9-p65 or dCas9-VP64 fusion proteins showed a 12- or 25-fold increase in GFP fluorescence intensity, respectively, when expressing dCas9 alone.[119]

Different optimization approaches can also be used to further improve the transcription activation efficiency. Activation domain VP16 can be added to multiple copies (dCas9-VP48, VP96, or VP192) (Figure 7b). It has been documented that the insertion of multiple sgRNAs or the tripartite activator domain VPR occurred when 12 VP16 repeats were fused with dCas9 (dCas9-VP192). This system efficiently activated and enhanced the expression levels of target genes 70-fold.[122] dCas9-VP64 was used as a scaffold connected to Rta and p65 to create a dCas9-VP64-p65-Rta tripartite activator, also known as dCas9-VPR (Figure 7c).[123,124] The transcriptional activity was enhanced by this three-part fusion system.

Transcription Repression

The dCas9/sgRNA complex inhibits the RNA polymerase to prevent transcriptional elongation, resulting in sequence-specific gene suppression.[113,125] Enhanced transcriptional repression efficiency can be achieved by fusing dCas9/sgRNA with small-molecule transcription repression domains such as KRAB, MXII, and SID4X (Figure 7f).[117,119] The CS domain of HP1, KRAB domain of Kox1, and WRPW domain of Hes1 are examples of inhibitory chromatin modifier domains that, when coupled with dCas9/sgRNA, effectively suppress expression of the GFP reporter gene in HEK-293-GFP reporter cells.[119] Nonetheless, in contrast to the dCas9-expressing cells, the dCas9-KRAB fusion protein-expressing cells displayed a five-fold decrease in fluorescence signal, whereas the dCas9-WRPW or dCas9-CS fusion protein-expressing cells showed a two-fold reduction in the GFP signal.[119] Combining sgRNA with small-molecule transcriptional repression domains is another method of transcriptional repression to suppress the expression of a particular gene.

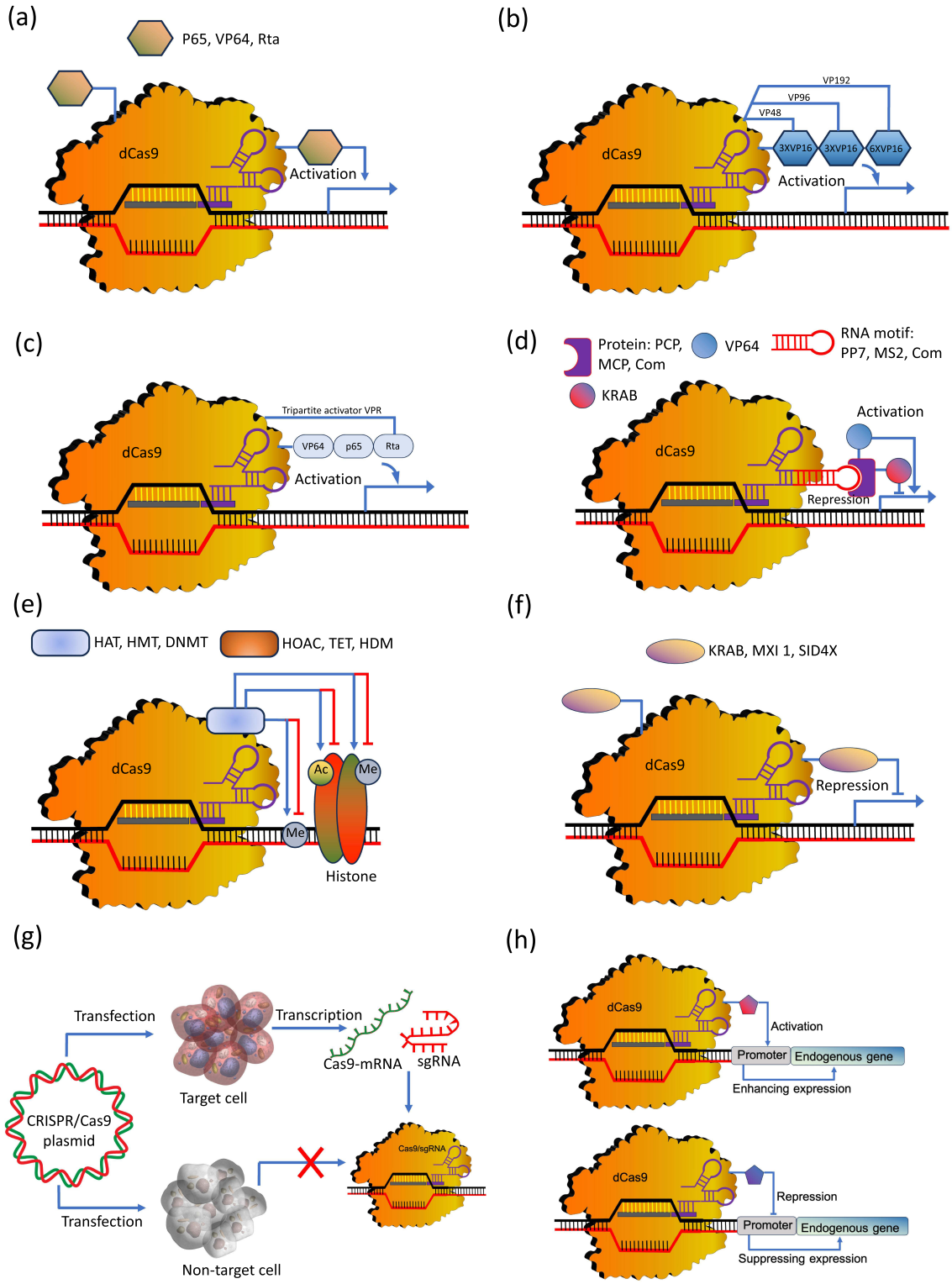

Figure 7 Different strategies used to genetically regulate the CRISPR/Cas9 genome-editing tool. (**a**) Small molecule activation domains for example p65, Rta, and VP64 can be used as target gene transcriptional activators when fused with dCas9. (**b** and **c**) For improved transcription activation, VP16 multiple copies or a tripartite activator domain fusion as VPR (a fusion of Rta, p65AD, and VP64) can be added to dCas9. (**d**) RNA binding polypeptides (Com, PCP, or MCP) are recruited via RNA aptamer (PP7, MS2, or Com) in order to facilitate sgRNA engineering. (**e**) Interaction of dCas9 with target site epigenetic regulatory enzyme domains (HTD/HMT, TET/DNMT, or HDAC/HAT). (**f**) Fusion of various repressor domains (KRAB, MXI1, and SID4X) for transcriptional repression of dCas9. (**g**) Cas9-mRNA and sgRNA transcription using a CRISPR/Cas9 plasmid driven by a specific promoter occurs in target cells but not in nontarget cells. (**h**) Induction of up-regulation or some endogenous loci by targeting the enhancer or promoter region of endogenous genes by dCas9 activator/inhibitor fusion.

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An sgRNA construct was obtained by fusing the RNA-binding domain, Com, with the repression domain, Com-KRAB, to target the transcription start site. This approach led to significant GFP repression compared to dCas9 alone.[117]

Epigenetic Modification

dCas9 participates in epigenetic modification by fusing epigenetic modifying enzymes, such as histone acetyltransferase (HAT)/deacetylase (HDAC) and histone demethylase (HDM)/methyltransferase (HMT). These Cas9-epigenetic modifying enzyme constructs have been used to expand the action on the target genome to enhance functional tasks and control different epigenetic states[126] (Figure 7e).

Cell-Specific Promoter

The construction of cell-specific promoters is a novel strategy to counteract undesired side effects of CRISPR/Cas9 in nontarget cells. gene editing and reduce the undesirable side effects of this genome editing tool in nontarget cells, is to directly construct cell-specific Cas9-nuclease promoters in target cells[116] (Figure 7g). Using organ-specific promoters, the CRISPR/Cas9 system has been developed to induce gene editing in zebrafish, *C. elegans*, macrophages, hepatocytes,[127,128] and monocytes.[129,130] To create the Cas9 macrophage-specific expression plasmid pM330 (CD68-Cas9 plasmid), the original chicken β-actin promoter of the Cas9 expression plasmid, pX330, was substituted with a macrophage-specific promoter (CD68).[104] To create a CLANpM330/sgNtn1 platform, sgRNA targeting Ntn1 (sgNtn1) was encoded in plasmid pM330 and encapsulated using cationic lipid-assisted PEG-b-PLGA nanoparticles (CLAN).[104] Without interfering with other cell types, these CLAN nanoparticles (NPs) effectively led to Ntn1 knockout in monocytes and Cas9 protein expression.[104]

Additionally, the promoter or enhancer region can be specifically activated via dCas9-activator fusion, which can also cause the target gene to be upregulated. This represents another method for conducting spatiotemporal control of CRISPR/Cas9 gene editing (Figure 7h).[131] By fusing the dCas9 protein to the catalytic core of the human acetyltransferase p300, a programmable CRISPR/Cas9-based acetyltransferase component was created. This component could catalyze the acetylation of histone H3 lysine 27 at its target site, resulting in robust expression of the target gene by activating enhancers and promoters.[132]

Chemical Control

Chemical methods primarily consist of three approaches to enhance the spatiotemporal control of CRISPR/Cas9-based gene editing: (a) Cas9 activity regulation through the fusion of WT Cas9 or dCas9 with self-splicing inteins, (b) Cas9 inhibition using anti-CRISPR proteins or degrons, and (c) designing bioresponsive delivery carriers to regulate CRISPR/Cas9 release in particular cells or tissues.[133]

Small-Molecule Activators

Conformational changes in some proteins are controlled by molecules such as rapamycin and 4-hydroxytamoxifen (4-HT). Hence, Cas9 spatiotemporal control is also attained by conformational changes through the addition of small molecules (Figure 6). In this vista, an intein (412 amino acid residues) was inserted at two different sites (Ser219 and Cys574) of Cas9. The addition of 4-HT resulted in the removal of intein through a conformational and self-cleavage reaction, which eventually caused reactivation of the Cas9 protein.[134] Furthermore, compared with WT Cas9, the efficiency of protein activation is determined by the protein insertion site and can range from three- to ten-fold. Additionally, the ratio of on-target to off-target effects produced by this strategy can increase up to 25 times.[134] In another study, 4-HT was used to turn Cas9 variant activity on or off in human cells.[135] Cas9 is sequestered in the cytoplasm by the hormone-binding domain of the estrogen receptor (ERT2) in conjunction with a Cas9 variant. However, the presence of 4-HT causes this fusion product to move within the nucleus, and the Cas9/sgRNA complex shows evidence of gene editing.[135]

In one study, split fragments of Cas9 protein were produced, and chemically induced dimerization of these fragments by small molecules was a good choice to control the conformational changes of this endonuclease system.[136,137] Two distinct sites (Arg535 and Glu573) produce split Cas9 fragments. As a result, Cas9 C- and N-terminal fragments were formed, which subsequently bound to the FKBP rapamycin binding domain (FRB) and FK506 binding protein 12

(FKBP).[138] Rapamycin-induced heterodimerization leads to the activation and conditional reconstitution of split-Cas9.[138] Furthermore, to prevent spontaneous reconstitution, the Cas9 fragments were spatially separated into different cellular compartments. The N- and C-terminal Cas9 fragments were linked to a nuclear export signal (NES) and a nuclear localization signal (NLS), respectively, resulting in reduced basal activity of Cas9 in the absence of rapamycin.

Oligonucleotide Conjugates

Recently, research has been conducted on site-specific oligonucleotide-Cas9 conjugates to increase the precision and effectiveness of gene editing.[139] For HDR-dependent gene editing by Cas9, sufficient donor DNA templates (single-stranded oligodeoxynucleotides or ssODNs) are typically needed and insufficient donor DNA templates reduce the efficiency of HDR. To create a new Cas9 mutant, azide-containing noncanonical amino acids have been used to alter Cas9.[139] These variations can bind to DNA adapters modified by dibenzyl cyclooctyne (DBCO) or ssODNs modified by DBCO. HDR-based gene editing efficiency can be increased by recruiting ssODNs to the cleavage complex in both the forms.[139]

Suffice it to say, small-molecule activators are important for the spatiotemporal regulation of CRISPR/Cas9 activity. However, some challenges still exist that need to be overcome to further use this genome editing strategy. These challenges include: (a) some small molecules, such as doxycycline and rapamycin, induce strong gene toxicity in both nontargeted and targeted cells;[120] (b) small-molecule activators have a restricted dynamic range because dCas9 exhibits some background activity when certain small-molecule activators are absent,[67,140] thereby interfering with precise spatiotemporal control of CRISPR/Cas9-based gene editing.

Small-Molecule Inhibitors

Inhibition of Cas9 Activity by Anti-CRISPR Protein

Elevated and persistent Cas9 activity often leads to off-target effects, genotoxicity, and chromosomal translocations. Thus, Cas9 nuclease activity must be curbed precisely within a narrow time frame after proper target editing.[8] Recent studies have demonstrated that a class of small molecules, such as anti-CRISPR (Acr) proteins, obtained from a furious co-evolutionary arms race between phages and bacteria, can mediate deactivation of the CRISPR/Cas9 genome editing system.[141,142] Small molecules called anti-CRISPR proteins are delivered into cells via plasmids or proteins that act as on/off switches. This is an important way to control the spatiotemporal editing of the CRISPR/Cas9 gene (Figure 6).

To date, various Acr proteins have been identified, including AcrIIAs, AcrIIIB, AcrIICs, AcrIFs, AcrVAs and AcrVIA.[143,144] Among these, AcrIIAs and AcrIICs have the greatest potential to control gene editing by inhibiting Cas9 activity.[145] Furthermore, AcrIIA4 cannot bind to Cas9 alone; it can only bind to the assembled Cas9/sgRNA complex.[146] The weaker binding of Cas9/sgRNA to target DNA caused by the fusion of Cas9/sgRNA and AcrIIA4 inhibits Cas9-mediated gene editing in human cells.[147]

Degradation of Cas9 by Small Molecules

In some circumstances, inhibition of Cas9 activity is overruled by its timely degradation by small molecules.[148,149] There are two strategies for degrading Cas9: (1) colocalization of the target protein and specific ubiquitination, which results in the proteasomal degradation pathway, and (2) direct fusion of degrons with the target protein, leading to its degradation.[150,151] In one study, Cas9 was bound to the FKBP12F36V variant, which can bind to the E2/E3 ubiquitin ligase through dTAG addition. This causes the fusion protein to be ubiquitinated and degraded.[152] However, it is more practical to fuse Cas9 directly with degrons (ER50 and DHFR) (Figure 6). When the VEGF gene was edited using the Cas9-ER50 or Cas9-DHFR systems and treated with different concentrations of 4OHT or TMP, an increased on-target to off-target ratio was observed.[153] These small-molecule inhibitors limit the CRISPR/Cas9 gene editing activity in particular cells or within a short-time window.[140,153]

Physical Control

The efficient spatiotemporal precision and non-invasiveness of CRISPR/Cas9 by physical control have increased in popularity in recent years.[114,120] This approach uses the CRISPR/Cas9 gene-editing platform in addition to some physically responsive components, such as heat-responsive, optical-responsive, magnetic-responsive, and ultrasound-responsive

components (Figure 6). Proper stimulation by different physical factors leads to a wide range of controls over CRISPR/Cas9, such as structure, function, activity, expression, transport, and release, to precisely control the time and space dimensions. Some physical strategies, such as light, heat, ultrasound, and magnets, are discussed below which significantly affect the CRISPR/Cas9 spatiotemporal activity.

Light

Over the past decade, a wide variety of photoresponsive molecules have been screened to optically control the CRISPR gene editing technique.[153,154] Some photoresponsive molecules, such as spiropyrans, azobenzene derivatives, and molecules with *o*-nitrobenzyl moieties, readily undergo ester bond cleavage or photoisomerization in the presence of light.[155,156] These characteristics of photoresponsive molecules have been used to control the CRISPR/Cas9 gene-editing system. To date, three main approaches have been devised for photocontrol of the CRISPR/Cas9 system.

The first method modifies Cas9 nuclease activity using light. In this case, photoinducible dimerization domains (pMag and nMag) were fused to a photoactivatable split Cas9 (paCas9) containing N- and C-terminal fragments (Figure 6).[157,158] In the absence of light, the split Cas9 fragments were inactive. In contrast, blue light stimulation promotes and reinstates heterodimerization of Cas9 fragments via pMag-nMag interactions.[157–159] The specificity of paCas9 nuclease targeting was comparable to that of WT Cas9. After exposure to blue light, paCas9 can be effectively used to control spatiotemporal editing of CRISPR genes. In contrast, psCas9 is an engineered photoswitchable Cas9 with a single-protein architecture.[160] A light-dissociable dimeric fluorescent protein (pdDronpa1) was inserted into the PI and REC2 domains of this Cas9 type (Figure 6).[161,162] The inserted pdDRonpa1 domains homodimerized and sterically inhibited psCas9 activity in the absence of light (500 nm). However, 500 nm wavelength light illuminated the complex and dissociated pdDronpa1, resulting in transcriptional regulation by the restoration of Cas9 activity and its gene editing function.[160,162]

Similarly, the Cas9-RsLOV2 monomer was created by combining the *R. sphaeroides* LOV domain (RsLOV2) with Cas9 (Figure 6).[163] Two Cas9-RsLOV2 monomers homodimerize in the absence of light, thereby strongly inhibiting Cas9 activity. In contrast, Cas9-RsLOV2 dimer dissociation and reversion to monomer forms were facilitated by blue light. This form exhibits a high nuclease activity and strong target specificity.[161,163]

Another method for controlling the effect of light illumination on Cas9 activity involves inserting a photocaged lysine into a particular endonuclease domain, which is important for RNA binding, making Cas9 inactive.[164] After two minutes of UV light exposure, the photocaged group was eliminated, and Cas9 activity was restored, leading to subsequent gene editing and transcriptional upregulation.

An optogenetic two-hybrid system comprising two independent components is the second method used to expand the functionality of the Cas9 protein by photoinduction. It consists of a cryptochrome circadian clock 2 (CRY2) linked to a different effector domain (activating effectors) to form a CRY2-activator complex, and the dCas9 system as a genomic anchor fused to the photosensitive cryptochrome-interacting CIBI protein (Figure 6).[158,165] Blue light (~450 nm) irradiation attracts the CIBI-effector complex, which then assembles to form the dCas9-CIBI-CYR2-effector complex.[158,166] Nevertheless, the activation of treated cells is reversed by dark incubation.[158,167]

The third approach used light to control sgRNA activity. In this strategy, a photocleavable ssDNA oligonucleotide, termed the protector, is coupled to sgRNA at a specific site (Figure 6).[168] This photocleavable protector can hybridize with sgRNA in the presence of UV light, which prevents sgRNA:DNA base pairing until the ssDNA oligonucleotide is photolyzed. Consequently, sgRNA is released to bind the target DNA once more and carry out subsequent gene editing; this tactic is irreversible.

Ultrasound

The application of ultrasound (US) to regulate payload release from various carriers has garnered increasing interest, along with light and heat applications.[105,127,169] Different nanomotors convert external energy or chemical fuels into mechanical motion that can be used for propelled motion or cargo transportation. This property has led to attractive characteristics that enable their implementation in drug delivery and biosensing.[170,171] US-driven nanomotors can swiftly penetrate plasma membranes and intracellular spaces to sustain acoustic activity. This property makes them suitable vehicles for intracellular drug delivery.[172] In this scenario, US-propelled nanomotors were employed as carriers to

introduce the Cas9/sgRNA system, within cells, under US activation.[105] Cas9/sgRNA-AuNW complexes were created by attaching a Cas9/sgRNA expression plasmid via disulfide bonds to the surface of gold nanowires (AuNWs). This complex produced an active movement to facilitate internalization into the cytoplasm under US activation (Figure 6).

Microbubble conjugate nanoliposomes (MB-NLs) were effectively employed as Cas9/sgRNA complex carriers, greatly enhancing local delivery to target sites upon US activation.[169] Similarly, androgenic alopecia therapy was performed using MB-NLs to deliver Cas9/sgRNA.[169] The delivery of Cas9/sgRNA complexes in dermal papilla cells is enhanced by the sonoporation of carrier particles by MB cavitation at high acoustic wave US frequencies (1–5 MHz).[169] The safe transportation of gene editing systems at specific sites has been demonstrated by the local delivery of MB-NL and Cas9/sgRNA-AuNW complexes through US stimulation.

Heat

A recent review examined the use of heat shock and photothermal effects to remotely control gene expression.[173] This method is separated into two groups based on heat shock. In one category, the spatiotemporal release of CRISPR/Cas9 from carriers is regulated by heat shock. Lipid-encapsulated gold nanoparticles (AuNPs) were used to create an engineered thermosensitive CRISPR/Cas9 release system[173] (Figure 6). In contrast to cationic AuNPs, nucleus-targeting TAT peptides and cations were added to AuNPs via sulfhydryl linkage. Electrostatic interactions were then used to abridge the negatively charged Cas9-sgPlk-1 plasmid (CP) on the cationic AuNPs, resulting in the formation of the AuNP/CP (ACP) complex. Localized surface plasmon resonance (LSPR) of AuNPs has the potential to produce heat through photothermal effects. Under 514 nm laser irradiation, the AuNPs could localize the heat to cause TAT/CP release from the AuNPs. To potentially knock down the target gene (Plk-1) and prevent cancer in vivo, the TAT moiety guides the TAT/CP complex into the nucleus.[173]

Using a photothermal trigger system, a semiconductor polymer brush (SPPFs) was created using the CRISPR/Cas9 release mechanism. This system has a photothermal transducer and NIR-II imaging capabilities.[174] Using its NIR-II imaging capabilities, this system could monitor in real time the distribution of gene-editing instruments under the control of a remote photothermal trigger. The system consists of three components: (a) SPPFs, which act as photothermal transducers, payload carriers, and NIR-II imaging agents; (b) dexamethasone, which binds to nuclear glucocorticoid receptors and wraps the core of SPPF NPs; and (c) CRISPR/Cas9 cassettes, which attach to PF to edit target genes.[174] LACP or SPPFs-CRISPR/Cas9 systems enable a novel method for spatiotemporal control of CRISPR gene editing through spatiotemporal control of the photothermal-trigger system.

Heat shock can spatiotemporally activate the heat shock promoter (Phsp) in CRISPR/Cas9 cassettes. This results in conditional gene editing at different developmental stages in different cell types.[120,175] In this regard, heat shock exposure led to the successfully controlled expression of the CRISPR/Cas9 plasmid and conditional knockout of genes in *C. elegans*. The CRISPR/Cas9 system showed powerful and time-bound target gene editing under the control of Phsp, led by a heat-shock system.[106]

Recent innovations have led to the generation of more convenient switches to control the on/off activity of Phsp through photothermal effects by combining Phsp with photothermal carriers.[120] In this case, a Cas9 plasmid driven by HSP, heat shock factor (HSF), and cationic polymer-coated Au nanorods (APCs) were combined to create a nano-CRISPR system.[120] By inducing a conformational change in the HSF monomer to form an HSF trimer, APC serves as both a local heat source and a carrier of the plasmid delivery system. It activates HSP to promote Cas9 endonuclease gene expression.[120] The off-switching of the NIR-II irradiation system led to the disappearance of the photothermal effect. A drop in temperature induces HSF decomposition and inactivation of the CRISPR/Cas9 plasmid transcription process. This method fine-tunes gene editing by NIR-II irradiation at several in vivo and in vitro time points, with ease and precision.[120]

Magnetic Field

Certain molecular behaviors can be altered by magnetic nanomaterials, both in vivo and in vitro, when exposed to an external magnetic field.[176,177] A CRISPR/Cas9 carrier system for non-invasive delivery and on-demand release in target cells or tissues was built using these magnetic nanomaterials (Figure 6).[178] To deliver a Cas9/gRNA gene-editing

nanoformulation system affixed to magneto-electric nanoparticles (MENPs), a magnetically guided system was developed.[178] This nanomaterial (MENPCas9/gRNA) can pass through the barrier (BBB) when driven by a magnetic field. This system has the potential to modify HIV-1 and decrease latent HIV-1 infection in HIV (HC69)/microglial cells.[178] These 20–30 nm-sized ferromagnetic MENPs are safe (up to 50 μg). An AC magnetic field can induce polarization changes in these molecules. This breaks down the linkages between MENPs and Cas9/gRNA, allowing Cas9/gRNA to be released in target tissues when needed. This is followed by mutations or gene knockouts.[178]

A recombinant MNP vaculoviral vector (MNP-BV-CRISPR) was designed to mediate CRISPR/Cas9 system-based gene editing using a magnetic field at a specific site.[107] Once exposed to a magnetic field, these nanoparticles disperse and migrate as nanomagnets in the aqueous buffers.[107] Furthermore, these NPs can be rendered inactive by the serum complement system, which functions as an off switch for gene editing. An external magnetic field provides an on-switch by locally regulating the margination and cell entry of NPs, thereby enabling targeted gene editing.[107] Although not thoroughly investigated, MNP-BV-CRISPR and MENPs-Cas9/gRNA are magnetically stimulated to carry out the spatiotemporal regulation of CRISPR in in vivo gene editing.

The spatiotemporal control over CRISPR/Cas9 activity by genetic regulation, chemical control and physical control have tremendously advanced the applications of this genome editing tool but all these approaches still have some drawbacks which are listed in Table 1.

CRISPR/Cas9 Facilitated DNA Base-Editing and Prime-Editing

A Cas9-induced DSB is created during CRISPR-mediated genome editing, and it can be repaired using HDR or NHEJ methods.[179] HDR is a mechanism that can be used to precisely restore the DNA sequence by inserting a specific DNA template, however it is not very efficient approach and has a high rate of unwanted indel mutations that outweigh any possible benefits from fixing the mutation.[7] Moreover, because HDR-mediated editing depends on homologous recombination, it can only target dividing cell types, which narrows the spectrum of disorders that can be treated.[180] CRISPR/Cas-mediated single-base-pair editing techniques have been developed recently to get circumvent these restrictions.[181]

Base-pair changes in the genomic DNA are the primary cause of many hereditary illnesses and undesired features. By using recent innovative approach of CRISPR/Cas technologies for base-editing, point mutations can be introduced into cellular DNA directly without causing a DSB. So far, adenine base-editors (ABEs) and cytosine base-editors (CBEs) have been identified as two groups of DNA base-editors. All the four transition mutations (A→G, G→A, C→T, and T→C) are installable by these base editors (Bes). In addition to base-editing, CRISPR/Cas system is used to induce prime-editing (PE) as well and now all twelve transition and transversion changes, as well as minor insertion or deletion alterations are performed by this genome editing tool.[182] In short, DNA base-editing and prime-editing technologies allow for exact, programmable substitutions of nucleotides without the need for donor templates.

As a therapeutic tool, DNA base-editing and prime-editing hold great promise for correcting disease-causing mutations in the human genome. By focusing on the four transition mutations, over 25% of human pathogenic single nucleotide polymorphisms (SNPs) can be fixed, and up to 89% of known genetic variations linked to human disease could potentially be fixed using prime editing.[183] Since viral vectors have a limited capacity for packaging, DNA base-editing and primer-editing may be especially well suited for the repair of large genes in cases when vector-mediated delivery of the target gene is not practical.[184] Additionally, base-editing can be used to treat autosomal dominant diseases, for which gene augmentation is not a good strategy because the affected gene must be silenced or eliminated.

Some of the potential applications of DNA base-editors and prime-editors include editing of large genes, targeting autosomal dominant diseases, editing of premature stop codons, and editing of splice-site variants.[182] However, before this platform can reach its full potential, there are still a lot of obstacles to be solved. The field of base-editing technologies is very young, and in order to facilitate therapeutic applications, more in vivo characterization of BEs and PEs is necessary. To further understand and refine base-editing and prime-editing over a wide spectrum of cell types and organisms, a great deal more study is required.

Methods to Identify Genome-Wide CRISPR/Cas9 off-Target Sites

It is now well known that gRNA determines the target DNA specificity of the CRISPR/Cas9 system and several noncanonical base-pairing interactions are observed between Cas9 and guide off-target heteroduplexes. This results in binding and cleavage of partially complementary off-target sequences.[185,186] This off-target cleavage issue raises safety concerns for comprehensive clinical application. Thus, there is a critical need to identify off-target events properly. There are three general categories of off-target CRISPR/Cas9 nuclease sites. These categories include, (a) in vitro recognition of cleavage sites using genomic template DNA, (b) capture of off-target editing events by in cellulo capture method, and (c) sequence homology and in silico bioinformatics tool prediction[187] (Figure 8). The accurate prediction of off-target editing events is not possible using a single tool, especially those at low frequencies. Furthermore, targeted deep sequencing is necessary to validate the presumed off-target sites. It is the gold standard for confirming the existence of mutations and indels caused by nucleases.[188] However, other validation approaches, such as TIDE/TIDER and CUT-PCR,[189] have notable limitations owing to their detection sensitivity and limitation scales regarding Sanger sequencing.[190]

Cas-OFFinder is a widely used in silico software used to predict Cas9-induced off-target effects.[191] Several other web-based algorithms have been established to predict the potential off-target sites for specific gRNA, these include E-CRISP, CasOT, CROPIT, COSMID, Bowtie2, CCTOP, Elevation CNN std, CRISPR, FlashFry, Synergizing CRISPR, Crisflash, MOFF, and CRISPRitz (Figure 8). Although useful and powerful, computational algorithms may require a richer biophysical framework and excellent training data for complete prediction of off-target potentials. In addition, for effective identification of true off-target locations, currently available in silico predictions require further supplementation with experimental methods.

Challenges, Ethical Considerations and Future Prospects

Because of its simplicity and versatility, the CRISPR/Cas9 system presents incredible opportunities for the management of various genetic and infectious diseases. However, the precision of the genome editing process is severely affected by the lack of spatiotemporal control over this tool in complex biological tissues. Researchers working on this system are constantly looking for better spatiotemporal controls using genetic regulatory elements, in addition to physical and chemical strategies to minimize undesired genome targeting and improve its precision at target sites. Spatiotemporal control over CRISPR/Cas9 in dimensions of space and time has been verified by its conditional expression by chemical induction, heat, light, magnetism, ultrasound, and bioresponsive delivery strategies. Although these methods have yielded

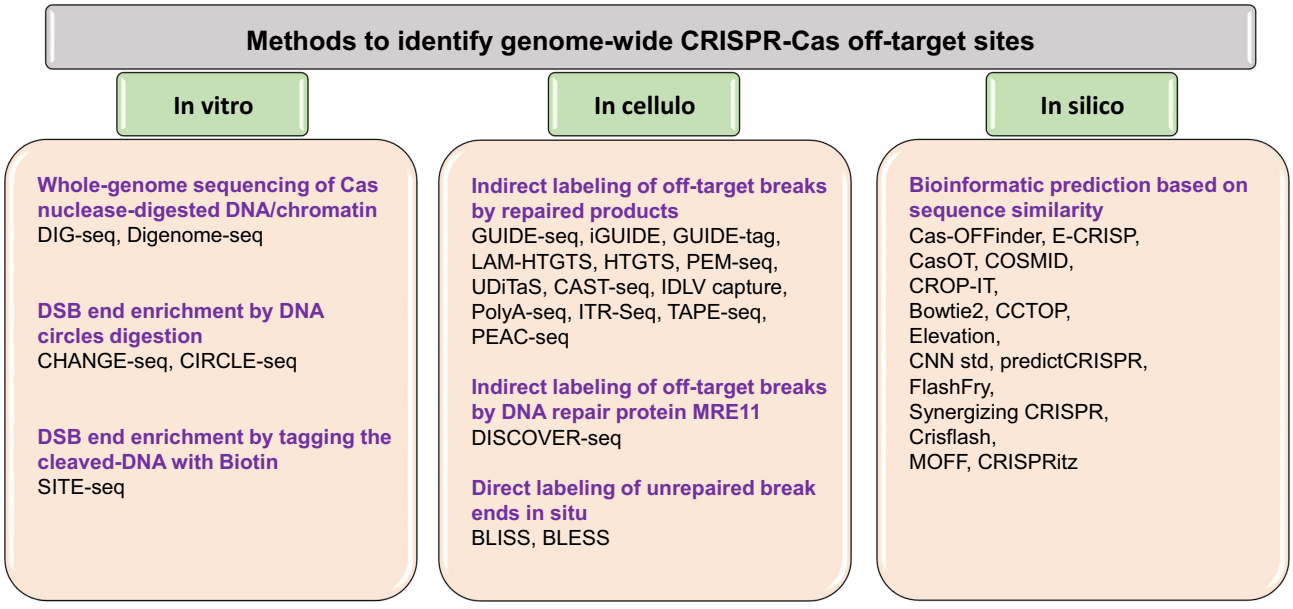

Figure 8 Examples of some currently used methods to identify genome-wide CRISPR/Cas off-target sites.

encouraging preclinical results, more obstacles must be overcome before this system can be fully controlled for clinical applications.

Major challenges regarding this genome-editing tool include minimizing off-target effects and maximizing targeting approaches to specific cells. To attain the maximum delivery of this system to a specific location, some challenges are related to the construction of novel nanoformulations with suitable surface properties, size, shape, design, and stability during circulation. Additional challenges include in vivo toxicity of CRISPR/Cas9-loaded NPs, immunogenicity, and proper delivery and clearance at specific sites after job completion.

Off-target effects (a form of genotoxicity) frequently impede the application of this genome-editing tool for better clinical translation.[101] Off-target alterations can lead to unusually large deletions and unexpected rearrangements.[15] Complex genomic rearrangements and deletions, including insertions and inversions, have been reported in mouse and human cell lines in close proximity to target cut sites.[192]

The ethical considerations for human genetic modifications have been always in consideration, but CRISPR/Cas9-mediated genome editing has given it a fresh viewpoint. A careful analysis of the ethical and societal consequences of this technology is necessary, given its unpredictable nature and wide-ranging consequences in appealing applications. The fundamental principles about genetic modifications are held by the general population and scholars who practice religion. Another controversial idea is to permanently alter the human genome to remove mutations that cause disease or, in some cases, to introduce or enhance desirable traits in progeny by introducing beneficial genes.[193] Genome alterations in non-reproductive cells are not heritable, while those in germ cells can be inherited by the following generation. Because of this, there are moral, ethical, and safety issues with the appealing applications of this strategy.[194] Human safety and dignity are at risk, and the possibility of genocide has been brought up by human germline modification via CRISPR/Cas9-based gene editing. A move was made to put a stop to research on the human genome until a decision was made at the national or international level regarding how society would embrace this new technology.[195] The range of applications of CRISPR/Cas9 is growing at an astonishing rate to almost all biological fields such as production of resistant crops, enhancement in antibiotic resistance and desired genetic changes in some tamed animal. Thus, more and more reforms of ethical considerations need to be implemented in near future.[196]

Some other future prospects are related to new variants of Cas9 with a better gRNA design recognized by broad-range PAM sequences and improved targeting efficiency within specific cells.[197] In addition, new inhibitors have been identified for better genome editing regulation and some new related compounds are expected to be discovered in the near future.[141] Furthermore, more advanced tools, in addition to GUIDE-seq, BLESS, and HTGTS, for the identification of off-target sites and gene-editing outcomes, are expected to be discovered soon.[198] The field of base-editing technologies by CRISPR/Cas9 system is very young, and in order to facilitate therapeutic applications, more in vivo characterization of BEs and PEs is necessary. To further understand and refine base-editing and prime-editing over a wide spectrum of cell types and organisms, a great deal more study is required. In addition, future perspectives on the CRISPR/Cas9 system are related to better cancer management through a comprehensive understanding of oncogenes and approaches targeting this molecular tool. Furthermore, some noncoding region manipulations using this system may increase our knowledge of the relationship between these genes and different cancers.

Clinical Trials

The innovative applications of CRISPR/Cas9 technology in human clinical trials for the management of different diseases include diverse cancers such as hematological malignancies (β-thalassemia, multiple myeloma, $CD19^+$ leukemia, non-Hodgkin lymphoma, T-cell lymphoma etc.) and other solid tumors. In a clinical trial, autologous CD19-directing CAR-T treatment demonstrated a significant persistent tumor remission (86–89%) over a median follow-up of 28.6 months in patients who had an initial response in a clinical study for $CD19^+$ relapsed or refractory lymphoma.[199] While CAR-T cell therapy has shown impressive outcomes in treating B-cell lymphoblastic leukemia, its efficacy in treating other solid and hematological cancers has been lower.[200] The CAR-T product was used to treat patients with relapsed or refractory B-cell non-Hodgkin lymphoma in a Phase 1 clinical trial (NCT04213469). It was very effective even at low infusion doses with a low percentage of CAR+ cells.

Eight patients (87.5%) experienced complete remission (87.5%) and partial remission (12.5%) during the 12-month follow-up trial without experiencing any major side effects.[201]

Another clinical trial (NCT03398967) was based on chimeric antigen receptor (CAR) T-cell therapy for refractory hematological malignancies caused by CD19 cancer cells. The interrogation of two CARs (CD19 and CD20/CD22) was performed at the TRAC locus of T cells to recognize CD19 cells.[202] Furthermore, a clinical trial (NCT03166878) was performed using gene-disrupted CD19-specific CAR T cells by lentiviral delivery of CARs in patients with refractory $CD19^{+}$ lymphoma or leukemia. CRISPR RNA electroporation was performed to disrupt the B2M and TCR genes to reduce host-versus-graft disease (GVHD).

Clinical trials have been conducted to treat solid tumors using CRISPR-engineered CAR-T, TCR-T, and TIL therapies in an effort to evaluate the safety and effectiveness of these treatments. The first ex vivo clinical trial performed by using CRISPR/Cas9 genome editing (NCT02793856) was on a patient with non-small cell lung cancer.[203] This trial was performed by electroporation of the Cas9 plasmid and sgRNA targeting PD-1 on T cells present in the peripheral blood and infusing it back into patients. Although feasible and safe, this method has made a significant contribution to advanced gene editing clinical trials. In another clinical trial, CAR-T cell therapy was performed in conjunction with disruption of CD70, TRAC, and B2M produced stable disease in nine patients (69.2%) and a lasting full remission in one patient (7.7%) in one clinical trial (NCT04438083) treating renal cell carcinoma.[204]

Recently, another clinical trial (NCT03399448) was performed on three subjects suffering from advanced-stage refractory cancer.[205] In this trial, the CRISPR/Cas9-based approach was used to remove the TRAC and TRBC genes that encode TCR and PDCD1, respectively, from T lymphocytes to enhance anticancer immunity. In addition, a transgene (NYESO-1) has been introduced into T cells to recognize cancers in a superior manner.

Besides, this genome editing technology is applied to manage the sickle cell anemia. Beam Therapeutics Inc. led the first clinical trial using a base editor in the United States, NCT05456880, a phase 1/2 clinical research that sought to replicate HPFH in patients with severe sickle cell disease by generating an A to G base swap in the HBG1/2 promoters through base editing.[206]

Additionally, some other diseases such as respiratory diseases, endocrine, metabolic system disorders such as diabetes mellitus, heterozygous familial hypercholesterolemia, hereditary transthyretin amyloidosis have been checked by CRISPR/Cas9 clinical trials. Within the respiratory system, the coronavirus disease-2019 (COVID-19) is the subject of five out of eight CRISPR-based clinical studies. This disease is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has resulted in a global pandemic during the last three years.[207] The exact gene editing targets are unknown, and no results have been reported from the two clinical trials (NCT05210530 and NCT05565248) currently being conducted in type I diabetic subjects to assess the safety and tolerance of gene-edited cell replacement therapy. Through in vivo TTR gene editing, CRISPR/Cas9 gene editing technology offers an alternate method for treating hATTR. A clinical evaluation of the in vivo gene editing therapeutic drug NTLA-2001, which used LNPs to encapsulate Cas9 mRNA and a sgRNA targeting the TTR gene, was one of the clinical trials that we reviewed (NCT04601051).[208]

Furthermore, the elimination of some immune system diseases such as acquired immunodeficiency syndrome (AIDS) is seriously in consideration by different clinical trials involving CRISPR/Cas9 genome editing technology. A model of HIV infection in non-human primates produced similar outcomes.[209] The success of these studies provided the impetus for human clinical trials aimed at assessing the long-term safety of EBT-101 for 15 years (NCT051443307) and evaluating the safety and efficacy of a similar approach, EBT-101, in which the CRISPR/Cas9 system was delivered by AAV9 for intravenous administration to target HIV-1 pro-viral DNA in HIV-1-infected adults on stable antiretroviral therapy (NCT05144386). The first patient to receive a single-dose intravenous infusion of EBT-101 is being monitored, and in due course, their eligibility to cease ART and experience a viral rebound will be assessed. A brief description of some more registered clinical trials based on the CRISPR/Cas9-mediated gene editing approach for the management of different diseases are mentioned in Table 2.

Table 2 Registered CRISPR/Cas Based in vivo and ex vivo Genome Editing Clinical Trials (ClinicalTrials.gov)

Disease	Target Gene and Effect	Intervention	Phase	NCT Number
Gastrointestinal cancer	CISH protein inhibition	TILS inhibited immune checkpoint CISH	I/II	NCT04426669
Multiple myeloma	CTX120 (BCMA)-directed T cell immunotherapy	Biological safety and efficacy of CTX120 in multiple myeloma	I	NCT04244656
Renal cell carcinoma	CTX130 CD70-based T cell immunotherapy comprised of allogeneic T cells	Safety and efficacy of CTX130 in relapsed or refractory renal cell carcinoma	I	NCT04438083
Refractory B cell malignancies	Creation of a CD19-directed T cell	CD19-directed T cell immunotherapy	I/II	NCT04035434
Severe sepsis	Target adjustment of antibiotics	Detection of alveolar lavage fluid changes the choice of early antibiotics in patients with pneumonia	–	NCT04178382
Refractory B cell malignancies	Disruption of HPK1	CD19-CAR-modified T cells with CAR delivered by lentivirus and Cas9 knockout of HPK1	I	NCT04037566
Hepatocellular carcinoma	PD-1 knockout engineered T cells	TACE combined treatment to block the blood supply of the tumor	I	NCT04417764
Leber congenital amaurosis 10	Removal of alternative splice site in CEP290	ZFN-mediated removal of intronic alternative splice site in retinal cells	I	NCT03872479
B-thalassemia	Correction of the hemoglobin subunit b-globulin gene	Ex vivo-modified hematopoietic stem cells	I	NCT03728322
Mesothelin-positive solid tumors	PD-1 and TCR knockout	CAR T cells to mesothelin with added PD-1 and TCR knockout	I	NCT03545815
HPV-related malignancy	E6 and E7 oncogenes of HPV16 and HPV18 deletion	Plasmid in a gel containing a polymer to facilitate delivery	I	NCT03057912
Gastrointestinal infection	Stem cell-derived human intestinal enteroids	Duodenal biopsies, followed by differentiation into mini-guts	–	NCT03342547
B-thalassemia	Disruption of the erythroid enhancer to the BCL11A gene	Ex vivo-modified hematopoietic stem cells	I/II	NCT03655678
Mesothelin-positive solid tumors	PD-1 knockout	CAR T cells to mesothelin with PD-1 knockout	I	NCT03747965
B cell leukemia	Cas9-mediated creation of CD19 and CD20 or CD19 and CD22 CAR T cells	CAR T cells to CD19 and CD20 or CD19 and CD22	I/II	NCT03398967
Sickle cell anemia	Disruption of the erythroid enhancer to the BCL11A gene	Ex vivo-modified hematopoietic stem cells	I/II	NCT03745287
HIV	CCR5 knockout	Modified $CD34^+$ hematopoietic stem cells	–	NCT03164135
B cell leukemia	Btcra, tcrb, B2M knockout	CD19-CAR-modified T cells with CAR delivered by lentivirus and Cas9 knockout B2M and TCR to create universal T cells	I/II	NCT03166878
EBV-positive, advanced stage malignancies	PD-1 knockout	Modified T cells selected for those targeting EBV-positive cells	I/II	NCT03044743

Abbreviations: CISH, Cytokine-induced SH2; TILS, Tumor-infiltrating lymphocytes; BCMA, B cell maturation antigen; PD-1, Programmed cell death protein 1; B2M, b_2-microglobulin; TACE, Trans-arterial chemoembolization.

Conclusion

CRISPR/Cas9's remarkable versatility and ease of use have revolutionized the genome-editing technology. This molecular toolbox offers unprecedented applications in almost every branch of biological science for the induction of genetic modifications, including the treatment of various genetic and infectious diseases. However, novel applications of CRISPR/Cas9 are severely affected by the lack of spatial and temporal precision when using this genome-editing tool in complex biological systems. Researchers working on this molecular toolbox are constantly making efforts to understand the different parameters that regulate spatiotemporal control. Different parameters such as genetic, physical and chemical strategies significantly affect this molecular toolbox in terms of time and space. The conditional expression of CRISPR/Cas9 is significantly affected by heat, light, magnetism, ultrasound, bioresponsive delivery, and new challenges that need to be addressed in the near future for comprehensive clinical applications. Furthermore, some concerns need to be addressed in the near future about nanoparticle composition, loading capacity and cost-effective targeting approaches in specific cells only, dodging endocytosis by some immune system cells, prevention of immune responses, and minimization of off-target effects. Furthermore, CRISPR/Cas9-mediated DNA base-editing and prime-editing hold great promise in near future for the management of different human diseases. In addition, a potential breakthrough is looked forward by engineering some more CRISPR/Cas9 variants, which show high fidelity in their applications. However, systematic research on these parameters has provided many novel opportunities for advanced research on application transformations using this toolbox.

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Disclosure

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