

Review article

Exploring the Endeavors of CRISPR/Cas9 Gene-Editing Technology in the Therapy of Cancer and Other Human Diseases

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Abstract: Clustered regularly interspaced short palindromic repeats (CRISPR) and the associated Cas9 nuclease have become the dominant platform for genome editing, offering greater efficiency, simplicity and programmability than the zinc finger nucleases and transcription activator-like effector nucleases that preceded them. This review examines the application of CRISPR/Cas9 to cancer and to other human diseases, together with the obstacles that continue to limit its clinical translation. We outline the architecture and DNA-recognition mechanisms of the three programmable nuclease platforms, describe the classification of CRISPR/Cas systems into two classes and their constituent types, and summarise the pooled genome-wide screening formats knock-out, interference and activation libraries through which the technology has been used to identify essential genes, synthetic-lethal interactions and mechanisms of drug resistance. In cancer research, CRISPR/Cas9 has enabled the inactivation of tumour suppressor genes in animal models, the restoration of tumour suppressor function, and the dissection of resistance to targeted agents and cytotoxic chemotherapy. Its most advanced clinical application is in cell therapy, where multiplexed editing has been used to disrupt the endogenous T cell receptor, silence checkpoint genes such as PDCD1 and reduce the risk of graft-versus-host disease; early-phase trials in refractory malignancy have established feasibility and an acceptable safety profile, although objective responses have so far been limited. Beyond oncology, progress is reviewed in Duchenne muscular dystrophy, HIV-1 infection, sickle cell disease, haemophilia B and neurodevelopmental disorders. Off-target cleavage, delivery and vector immunogenicity remain the principal constraints on therapeutic use, and the strategies developed to address them are discussed.

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Introduction

Cancer is a genetic disease driven by an accumulation of diverse molecular alterations [1]. These mutations block programmed cell death, alter the expression of surface antigens so that transformed cells escape immune recognition, and promote proliferation through the activation or inactivation of multiple signalling pathways. Malignant cells also invade surrounding tissue and disseminate to distant sites through the vasculature and the lymphatic system. Although cancer therapy has advanced considerably

through surgery, chemotherapy, radiotherapy, molecularly targeted agents and combination regimens, rates of recurrence, treatment resistance and adverse effects remain high [2]. The identification of new cancer-specific vulnerabilities is therefore a priority. The number of validated cancer-driving mutations also remains limited, and new approaches are needed to identify functionally relevant driver genes.

Clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated protein 9

(Cas9) system have proved more efficient, simpler and more broadly applicable for genome editing than earlier gene-modification platforms [3, 4]. In cancer research, CRISPR/Cas9 is currently applied to the identification of oncogenic mutations and tumour suppressors, the construction of in vitro and in vivo cancer models, and the development of cancer gene therapy [5, 6].

Because it combines high editing efficiency with comparatively low off-target activity, CRISPR/Cas9 technology offers the prospect of modifying the biological behaviour of cancer cells at the genomic level while sparing normal tissue, and thereby of extending patient survival. This review introduces the CRISPR/Cas9 system as a flexible tool for genome engineering, summarises the in vitro and in vivo cancer

models that have been generated with it together with the available delivery strategies, outlines its current applications in cancer treatment and in other human diseases, and examines the limitations that constrain its clinical translation along with the strategies proposed to overcome them.

Overview of other genome editing tools

A number of techniques for knocking out and knocking in target genes have been developed to enable precise genome editing, and several have been applied therapeutically to specific disorders. The architectures and DNA-recognition mechanisms of the three principal programmable nuclease platforms are compared in Figure 1.

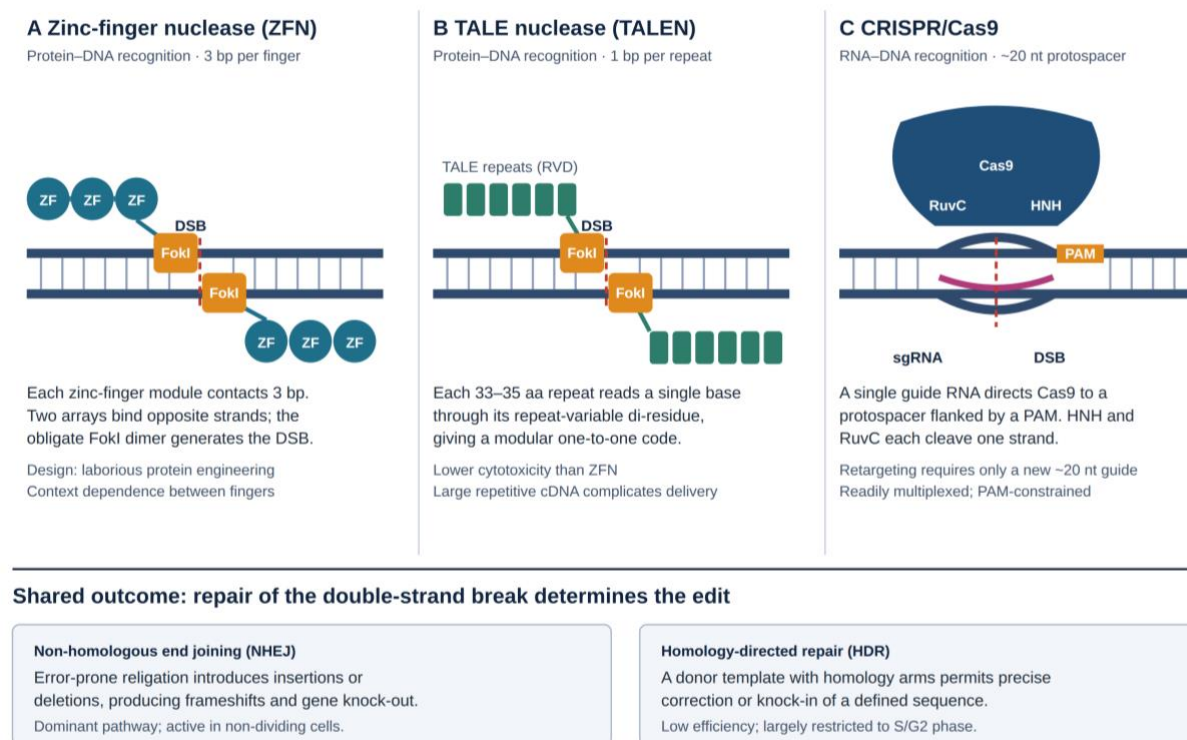


Figure 1. Architecture and DNA-recognition mechanisms of the three programmable nuclease platforms. (A) Zinc finger nucleases recognise DNA through protein–DNA contacts, each zinc finger module reading approximately three base pairs; two arrays bind opposite strands and the obligate FokI dimer generates the double-strand break. (B) TALE nucleases use tandem 33–35 amino acid repeats, each reading a single base through its repeat-variable di-residue, giving a modular one-to-one recognition code. (C) CRISPR/Cas9 is directed by a single guide RNA to a protospacer flanked by a protospacer adjacent motif (PAM); the HNH and RuvC domains each cleave one strand. In all three systems the biological outcome is determined by the repair pathway: non-homologous end joining introduces indels and produces gene knock-out, whereas homology-directed repair permits precise correction or knock-in when a donor template is supplied. DSB, double-strand break.

Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)

Zinc finger motifs were first identified in *Xenopus* oocytes as repeating zinc-binding domains [7], and comparable sequence-specific motifs are widely distributed among eukaryotic transcription factors. Crystallographic analysis showed that the $\beta\beta\alpha$ fold of each finger spans approximately thirty residues and is stabilised by a pair of cysteine and a pair of histidine residues (Cys2-His2) that together coordinate a zinc ion [8]. Zinc finger nucleases exploit these Cys2-His2 zinc finger proteins (ZFPs) as DNA-binding modules and fuse them to the non-specific restriction endonuclease FokI. Each finger recognises approximately three base pairs of the target DNA sequence [9-11].

Designing zinc finger arrays that bind long, specific DNA sequences has nonetheless proved difficult. Maeder et al. assembled engineered ZFPs capable of binding defined target sequences [12, 13], but gene repair remains complicated by binding at unintended genomic locations, and off-target cleavage across multiple loci is a further source of error [14, 15]. This limitation has been mitigated by the use of ZFN pairs, in which one nuclease binds the forward strand and the other the reverse strand. Each pair carries obligate heterodimeric FokI domains of opposing charge, so that a double-strand break (DSB) is produced only when both monomers are correctly positioned [16-18].

TALENs were subsequently developed and are generally regarded as more efficient and more readily programmed, with greater therapeutic potential [19, 20]. They are fusion proteins comprising a TALE DNA-binding domain and the FokI nuclease. The TALE domain consists of tandem repeats of 33 to 35 amino acids, each containing a repeat-variable di-residue that determines the single base recognised, giving a modular one-to-one recognition code [21, 22]. TALENs are considered more suitable than ZFNs for therapeutic use because of their lower cytotoxicity [23] and favourable DNA-binding affinity [24]. Their delivery, however, depends on viral and non-viral vectors, and vector design is complicated by the fact that the cDNA encoding a TALEN is considerably larger and more repetitive than that encoding a ZFN [25].

CRISPR

Constructing a tailored CRISPR system requires only the design of an appropriate single-guide RNA (sgRNA) [26]. The CRISPR/Cas system was first exploited as a genome editing tool in mammalian cells by Cong et al. [27] and has since been studied intensively in order to identify additional components capable of operating in

eukaryotic cells and, by extension, of treating human disease. Liang et al. applied this approach to correct a mutation in the β -globin (HBB) gene while working on the closely related δ -globin (HBD) gene [28].

Classification of CRISPR/Cas systems

CRISPR/Cas systems are divided into two classes, which are further subdivided into several types according to the composition of the surrounding cas genes and the nature of the nucleic acid targeted [29]. This classification has improved our understanding of bacterial phylogeny and of the emergence of phage-resistant strains. CRISPR/Cas immunity operates in three stages during plasmid or phage challenge: adaptation, expression and interference, each associated with distinct molecular features that together confer antiviral or antiplasmid immunity [30]. During adaptation, spacer sequences derived from the invading genome are incorporated into the CRISPR array. The array is then transcribed into CRISPR-associated RNA (crRNA) containing these spacers. Finally, an effector endonuclease uses the spacer as a guide to cleave the invading genome [31].

The functional properties of a CRISPR/Cas system are determined in large part by its cas1 and cas2 genes. The system was originally classified by taxonomic analysis, its two principal components being the CRISPR array, comprising spacers interspersed with identical repeat sequences, and the cas operon. On the basis of signature marker proteins, three types were initially recognised: type I (Cas3), type II (Cas9) and type III (Csm/Cmr) [32]. Further types were subsequently added [33]. The broader division into two classes rests on the architecture of the effector module. Class 1 systems, which include types I and III together with the putative type IV, employ multi-subunit crRNA-effector complexes such as Cascade and the Csm and Cmr complexes [34]. In class 2 systems, which include type II and the putative type V, a single large protein such as Cas9 performs all effector functions [35].

Type I systems are defined by the Cascade complex acting together with the Cas3 nuclease [36]. Cas6 cleaves the pre-crRNA transcribed from the CRISPR array to generate mature crRNA [37], which associates with Cascade and is responsible for recognising the protospacer in the target DNA.

Cas8, a further Cascade component, locates and identifies the protospacer adjacent motif (PAM), a short sequence positioned immediately next to the target site [38]. The PAM is essential to type I Cascade-Cas immunity: in its absence, Cascade proteins cannot

recognise the spacer within the target DNA [39, 40] and the R-loop between crRNA and target DNA does not form, allowing the virus to evade CRISPR surveillance [41, 42]. In a fully functional system, recognition activates the Cas3 nuclease, which nicks the single-stranded target DNA and brings about its degradation [43].

In contrast to type I, type II systems depend on a single Cas protein. Cas9 recognises the PAM and operates with two RNA species, the crRNA and the trans-activating crRNA (tracrRNA), which are processed into their mature forms with the assistance of host RNase III [44]. A double-strand break is produced when the HNH nuclease domain cleaves one strand and the RuvC domain cleaves the other [45]. Cleavage requires prior PAM recognition by Cas9, which drives local unwinding of the double-stranded DNA and the formation of an R-loop between the target strand and the crRNA; this conformational change allows the tracrRNA-Cas9 complex to engage the cleavage site [46, 47].

Type III systems are distinguished by the generation of crRNA through the endoribonuclease activity of Cas6 acting on pre-crRNA [48]. Unlike the preceding types, cleavage by Cas6 leaves an eight-nucleotide repeat-derived tag on the crRNA, which is trimmed to six nucleotides on maturation [49] and is located upstream of the spacer sequence [50, 51]. The crRNA is loaded into the Csm complex in type III-A systems and the Cmr complex in type III-B systems [52]. Type III systems can target both DNA and RNA, producing co-transcriptional crRNA-guided cleavage of the target [53, 54]: Csm3 in type III-A and Cmr4 in type III-B cleave the RNA transcript [55, 56], while the palm domain of Cas10 cleaves the DNA strand [49]. A further distinction of type III systems is that a PAM is not invariably required to initiate the immune response.

CRISPR/Cas9 library screens for drug target discovery

Genetic screening is routinely used to identify previously uncharacterised genes and to assign them a function. Mutagenesis screens have been applied successfully to define fundamental biological processes and signalling networks and can establish which genes are responsible for a given phenotype. Their principal limitation in the context of targeted drug development is that they generate heterozygous mutants carrying undefined random mutations. RNA interference (RNAi) was introduced as one means of overcoming this constraint. Although high-throughput RNAi library screens have yielded valuable information

on the causal relationships between loss of function and specific genes, they retain substantial drawbacks, in particular incomplete knockdown and appreciable off-target activity [57]. CRISPR/Cas9 offers several advantages over RNAi, including complete rather than partial loss of function, the ability to interrogate the entire genome, including introns, promoters, enhancers and intergenic regions, and greater reproducibility [58]. Experience gained in the construction of RNAi platforms has enabled the efficient synthesis of CRISPR/Cas9 libraries, which were first shown to outperform RNAi libraries in 2013 [59].

Three formats of genome-wide CRISPR library are currently in use: CRISPR interference (CRISPRi), used in loss-of-function screening [60]; CRISPR activation (CRISPRa), used in gain-of-function screening [61]; and CRISPR knock-out libraries, used to identify biological processes such as cell survival signalling and drug resistance [62]. Knock-out libraries employ catalytically active Cas9, whereas CRISPRi and CRISPRa libraries use catalytically inactive Cas9 (dCas9) fused to regulatory effectors, most commonly the Krüppel-associated box (KRAB) domain for repression [64] and VP64 [63] or the tripartite VP64-p65-Rta (VPR) activator for induction. These formats and the pooled screening workflow are summarised in Figure 2.

The adaptable architecture of CRISPR libraries supports both positive and negative selection screening. Positive selection identifies genes whose loss allows cells to survive a given pressure, such as cytotoxic drug exposure. In a typical experiment the library is introduced into the cell population, which is then treated with an anticancer agent; the sgRNAs recovered from the surviving cells are sequenced in order to identify candidate resistance genes [65]. Negative selection, by contrast, detects cells that die or grow slowly under defined conditions, and is therefore suited to identifying genes required for survival that may represent attractive targets for molecularly targeted therapy. When a pooled sgRNA library is used to generate a collection of random mutants, cells carrying guides directed against survival-essential genes are progressively lost, so that after several passages only cells carrying guides against non-essential genes remain. Sequencing the guide population at the start and at the end of the experiment by next-generation sequencing therefore identifies the genes essential for survival.

Applications of CRISPR technology in cancer research

CRISPR/Cas9 has assumed a substantial role in laboratory cancer research, where it is used to generate improved disease models and to identify therapeutic targets and mechanisms of treatment resistance. It is versatile, comparatively accessible, highly effective, inexpensive and straightforward to apply [60]. The technology has been used successfully to inactivate multiple tumour suppressor genes (TSGs) in the course of model development; CRISPR/Cas9-induced loss of Ptch1, Pten, Nf1 and Trp53 in the mouse brain, for example, generates glioblastoma and medulloblastoma [66].

Because cancer cells accumulate a succession of genetic aberrations over time, models carrying several defined mutations have been constructed with CRISPR/Cas9 in order to investigate the early events that give rise to genomic instability and mutagenesis. CRISPR-based approaches have been used to characterise the initiating aberration in the APC gene in colorectal carcinogenesis [67, 68]. In a further example, knockout of HER2 exon 12 produced a truncated HER2 protein and reduced full-length protein expression, indicating that partial knockout of an oncogene may be sufficient to achieve a therapeutic effect [69].

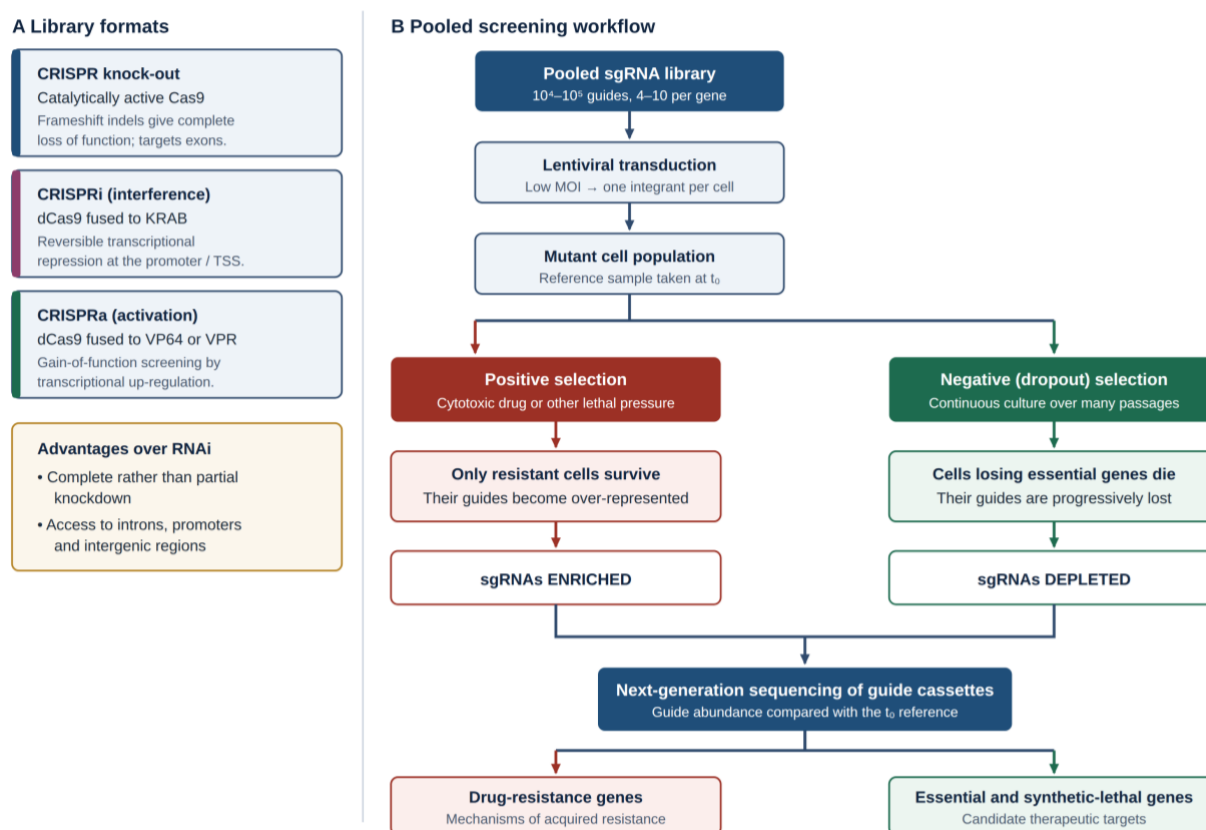


Figure 2. Pooled genome-wide CRISPR screening for target and resistance-gene discovery. (A) The three library formats. CRISPR knock-out libraries use catalytically active Cas9 to generate frameshift indels; CRISPR interference (CRISPRi) uses dCas9 fused to the KRAB domain to repress transcription reversibly; and CRISPR activation (CRISPRa) uses dCas9 fused to VP64 or VPR to achieve gain of function. (B) In the pooled workflow, a sgRNA library is delivered by lentiviral transduction at low multiplicity of infection so that most cells acquire a single integrant, and a reference sample is taken at t_0 . Under positive selection, only resistant cells survive and their guides become enriched, identifying drug-resistance genes. Under negative (dropout) selection, cells losing essential genes are progressively lost and their guides become depleted, identifying essential and synthetic-lethal genes. Guide abundance in both arms is quantified by next-generation sequencing and compared with the t_0 reference. MOI, multiplicity of infection.

CRISPR/Cas9 has also been used to restore tumour suppressor activity, for instance by demethylating the BRCA1 and PTEN promoters, which is associated with improved responses to chemotherapy [70, 71], and by correcting mutations responsible for TSG inactivation [72]. PTEN expression is lost in many malignancies; in triple-negative breast carcinoma and melanoma cell lines, CRISPR-mediated restoration of PTEN expression was associated with reduced downstream oncogenic MAP kinase signalling [73].

Drug resistance remains a major problem in oncology, and CRISPR/Cas9 has enabled investigators to study its mechanisms by introducing or correcting the alterations that confer resistance to specific agents. CRISPR/Cas9 models harbouring EGFR exon 20 variants have been used to examine the activity of the EGFR tyrosine kinase inhibitor osimertinib [74], and deletion of Aurora kinase B has been shown to restore cisplatin sensitivity in resistant lung cancer cell lines [75].

In breast carcinoma, CRISPR-based approaches have identified a range of alterations associated with treatment resistance, including ESR1 mutations conferring resistance to fulvestrant and novel mechanisms of resistance to PARP inhibitors [76, 77]. Toxicity arising from combinations of anticancer agents is an important constraint on drug development. Despite the mechanistic rationale for combining PARP inhibition with platinum chemotherapy, concurrent administration has generally failed in practice, chiefly because of high-grade myelosuppression. In ovarian carcinoma, CRISPR-mediated PARP1 inhibition acted synergistically with cisplatin cytotoxicity [78], suggesting that genetic rather than pharmacological inhibition may provide a route to concurrent PARP and platinum targeting. CRISPR/Cas9 can also be used to knock out kinases such as KRAS, BRAF and PIK3CA, even where pharmacological inhibition of the activating kinase domain is not feasible or would be prohibitively toxic.

Current status of CRISPR/Cas9 in cancer treatment

Over the past decade the treatment of patients with cancer has changed substantially, with immunotherapy at the forefront of this shift. Immune checkpoint blockade, chimeric antigen receptor T cell therapy and adoptive cell therapy have all entered clinical practice or late-stage clinical evaluation.

CRISPR/Cas9 and CAR-T cell therapies

Chimeric antigen receptor T cells (CAR-T cells) are genetically modified T cells engineered to express receptors that recognise neoantigens displayed on the surface of malignant cells [79]. A CAR comprises an extracellular domain that binds the target surface antigen and an intracellular signalling domain that initiates T cell activation [80]. In later-generation constructs, additional co-stimulatory intracellular domains support T cell survival, proliferation and expansion [81, 82]. Most CARs recognise surface antigens independently of the major histocompatibility complex (MHC). Their performance depends on several parameters, including the position of the epitope on the target cell surface antigen [83], the composition of the hinge and transmembrane regions, and the fine design of the extracellular and intracellular domains [84].

CAR-T cell therapy has produced marked responses in haematological malignancies, particularly B cell neoplasms [85, 86], but has been largely ineffective in solid tumours, for several reasons. The most important is the relative scarcity of suitable surface antigens: in solid tumours the majority of neoantigens are intracellular and therefore inaccessible to conventional CAR-T cells [87]. Considerable effort has been directed at improving the affinity and specificity of target engagement [88, 89]. Tumour heterogeneity gives rise to heterogeneous antigen expression and immune escape [90], and in some malignancies conventional CARs are further limited by low MHC expression and low tumour mutational burden. Peptide-centric CARs (PC-CARs) have been developed to target tumour-specific oncoproteins presented in this setting [91].

A further obstacle is T cell exhaustion arising from continuous antigen exposure [92]. Several strategies have been used to improve T cell fitness, including inactivation of inhibitory molecules, transgenic cytokine production and the introduction of additional co-stimulatory signals [93]. Exclusion of T cells from the tumour is another significant barrier in solid malignancies, and CAR-T cell trafficking into solid tissue is influenced by the tumour vasculature, the density of the tumour stroma, chemokine gradients and the composition of tumour-infiltrating immune cells [94, 95]. Suppressive immunomodulatory factors within the tumour microenvironment, including inhibitory cytokines and tumour-associated fibroblasts, further impair T cell fitness and promote exhaustion [96, 97]. These constraints, together with a high incidence of toxicity such as cytokine release syndrome (CRS) and

the risk of graft-versus-host disease (GVHD), have impeded the application of CAR-T cell therapy to solid tumours [98].

CRISPR/Cas9, and in particular its capacity for multiplexed editing, offers a means of addressing several of these difficulties. Disruption of the endogenous T cell receptor (TCR) and silencing of HLA class I in allogeneic T cells reduce the risk of graft rejection and of GVHD, and thereby support the development of off-the-shelf allogeneic CAR-T products [99]. Specific cytokine genes can also be edited directly in order to enhance cytokine production and immune cell responsiveness while avoiding the T cell exhaustion and autoimmune reactions associated with other methods of cytokine augmentation, such as viral transduction [100, 102]. In addition, the CRISPR/Cas9 platform can be used to knock out checkpoint genes such as PDCD1 and CTLA4 in order to achieve more durable T cell engagement [101, 103].

CRISPR/Cas9 and adoptive T cell therapies

Adoptive cell therapy (ACT) offers a means of circumventing the requirement for tumour-infiltrating lymphocytes (TILs), which are not present in all tumours. The TCR complex, which is responsible for recognising foreign antigen, lies at the heart of these approaches. In ACT, patient-derived T cells are engineered to express defined TCRs that recognise antigens presented by tumour cells and direct their destruction. Transgenic TCRs designed in this way can recognise NY-ESO-1 in certain cancers and permit targeted killing of cells presenting this immunogenic antigen.

Two limitations have constrained the approach. Mispairing between the α and β chains of the endogenous and therapeutic TCR complexes reduces effective engagement of the target antigen, and PD-1 expression exerts a negative regulatory effect on T cells, diminishing antigen responsiveness and therefore the efficiency of T cell-directed tumour killing. Improved efficacy has been demonstrated in preclinical murine studies combining transgenic TCR T cells with PD-1 blockade against NY-ESO-1-expressing tumour cells [104].

These findings led to the first phase I clinical study to evaluate the feasibility and safety of multiplexed CRISPR/Cas9 editing in this setting, in which the PDCD1 locus encoding PD-1 was deleted and mispairing was reduced by deleting the loci encoding the endogenous TCR α and β chains in cells carrying a recombinant transgenic TCR. This triple-edited product was

administered to three patients with refractory malignancy, one with advanced metastatic liposarcoma and two with treatment-refractory multiple myeloma [105]. All three received lymphodepleting chemotherapy with fludarabine and cyclophosphamide three to five days before infusion of the edited T cells.

The investigators reported that treatment-related adverse events, in particular grade 3 and 4 haematological toxicity, were attributable principally to the myelosuppressive conditioning regimen. The most frequent non-haematological events were gastrointestinal disturbance, cough, headache, paraesthesia and pain. Cytokine release syndrome was not associated with administration of the modified transgenic TCR. Edited T cells persisted in the blood of all three patients for up to nine months after infusion, with a decay half-life of up to 293 days, and trafficked effectively to tumour tissue and bone marrow at levels comparable with those in the blood. Off-target nuclease activity gave rise to translocations and mutations, although these were not associated with any detectable effect on cell growth over 300 days of *in vivo* expansion [105].

The patient with advanced treatment-resistant metastatic liposarcoma showed a mixed response, with a 50 per cent reduction in the size of a large abdominal lesion sustained for four months, while other metastatic deposits persisted. The best overall response across the cohort was stable disease, and all three patients subsequently progressed. The study was nonetheless the first to demonstrate the feasibility and safety of CRISPR/Cas9 gene editing in patients with cancer, including one with a solid malignancy.

A second clinical study evaluated CRISPR/Cas9-edited PD-1-disrupted T cells in advanced, treatment-resistant non-small cell lung carcinoma. Twelve of the twenty-two enrolled patients received the edited T cell infusion. The investigators reported that treatment was safe and that all treatment-related adverse events were of low grade. Editing efficiency in the infused product was low, which may reflect the delivery method rather than a limitation of the editing chemistry itself, and the edited T cells consequently persisted for only a short period. The frequency of off-target mutations, assessed by next-generation sequencing at eighteen candidate off-target loci, was low at 0.05 per cent (range 0 to 0.25 per cent). No objective responses were observed, although one patient with high PD-1 editing efficiency maintained stable disease for 76 weeks, suggesting that greater editing efficiency may be associated with greater antitumour activity [106].

Taken together, these two trials provide early evidence on the safety of CRISPR/Cas9 in patients and establish proof of concept for its feasibility as a therapeutic strategy in both haematological and solid malignancies. A number of clinical studies are now under way to evaluate the safety and anticancer activity of CRISPR/Cas9-edited T cells across a range of tumour types.

Applications of CRISPR/Cas9 in other human diseases

Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscle disorder caused by mutations in the DMD gene at Xp21. These mutations reduce the quantity of dystrophin, the protein required for normal muscle membrane integrity [107]. Males are affected because they carry a single X chromosome [108]. Most of the mutations identified in the DMD gene are exonic deletions [109]. Haematopoietic approaches using stem cells derived from the ex vivo expansion of myoblasts from satellite cells were developed in an attempt to regenerate damaged muscle, but proved of no benefit to patients with DMD [110], since myoblasts largely lose their capacity to engraft into muscle tissue [111]. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can generate large numbers of skeletal myogenic progenitors with in vivo regenerative capacity that are able to produce dystrophin [112, 113], and genome editing in ESCs has been shown to reduce the features of DMD [114]. Alongside cell-based strategies intended to restore DMD gene expression, anti-inflammatory treatments have been developed to mitigate the consequences of dystrophin deficiency [8]. The most effective of these approaches are based on genome editing tools, principally the CRISPR, TALEN and ZFN systems.

CRISPR and TALENs have both been used to restore dystrophin production in vitro by knocking in exon 44 of the DMD gene, with limited off-target activity. iPSCs derived from the fibroblasts of a patient with DMD lacking exon 44 were transfected independently with CRISPR and with TALEN constructs in order to insert the missing exon by generating indels in the flanking exons [115]. Rather than restoring a single exon, exons 45 to 55 of the DMD gene were subsequently excised in patient-derived iPSC-derived skeletal myotubes and cardiomyocytes using CRISPR/Cas9 with appropriate sgRNAs, restoring dystrophin production and normalising the elevated creatine kinase levels

associated with muscle instability and degeneration [116].

A comparable deletion has been shown to modulate miR-32, which reduces dystrophin levels in muscular dystrophies such as Becker muscular dystrophy [117]. These observations point to the potential value of large multi-exon deletions in correcting defects in other factors that influence dystrophin function. CRISPR/Cas9 has also been used in vivo to correct DMD mutations in mdx mice, which carry a point mutation in the *Dmd* gene and lack dystrophin expression. A single-stranded oligodeoxynucleotide (ssODN) donor was used to repair the lesion by activating homology-directed repair (HDR), with sgRNA-expressing plasmid vectors targeting exon 23. The findings nonetheless revealed a higher frequency of repair by non-homologous end joining (NHEJ), resulting in the generation of indels [118].

Human immunodeficiency virus infection

HIV-1 enters host immune cells through the CD4 receptor in conjunction with the chemokine co-receptors CCR5 and CXCR4. Combination antiretroviral therapy has substantially improved quality of life, but it does not eliminate the virus from the body, and morbidity and mortality remain appreciable [119]. A 32-base-pair deletion in the CCR5 allele inactivates the gene and confers resistance to HIV-1 infection [120, 121]. A patient with HIV-1 infection and acute myeloid leukaemia who underwent allogeneic stem cell transplantation from a donor homozygous for CCR5 Δ 32 showed no detectable HIV-1 and cessation of viral replication [122].

The generation of homozygous CCR5-null cells by genome editing has therefore been investigated extensively [123, 124]. Transfection of human CD34+ haematopoietic stem and progenitor cells (HSPCs) from umbilical cord blood with ZFNs produced CCR5 $-/-$ clones by knocking out the CCR5 locus on chromosome 3 [125, 126]. These cells were engrafted into non-obese diabetic severe combined immunodeficient interleukin-2 receptor γ -null (NOD/SCID/IL2R γ null, NSG) mice, an established model for the study of haematopoiesis and HIV-1 infection [127], and control of HIV-1 replication was demonstrated [128]. Delivery of CCR5-targeting ZFNs by adenoviral vector produced more efficient CCR5 knockout and fewer off-target events in CD34+ HSPCs than delivery of plasmid DNA [129].

These studies were extended to patients with HIV, who received autologous CD4+ T cells in which CCR5

had been disrupted by ZFN [130]. Large numbers of modified CD4⁺ T cells were recovered, but viral replication was greater in cells retaining a wild-type CCR5 allele than in cells in which both alleles were disrupted, indicating that biallelic deletion is required for therapeutic benefit [130]. Long-term engraftment of ZFN-modified cells was not observed in vivo, and the persistence of unmodified cells limited suppression of HIV-1 replication [131]. The potential adverse consequences of adenoviral vectors must also be taken into account.

Wild-type iPSCs have been used to generate homozygous CCR5 Δ 32 cells [132] by transfection with a CRISPR/Cas9 plasmid encoding a CCR5-specific sgRNA [133]. The edited iPSCs differentiated into monocytes and mature macrophages that were resistant to HIV-1 infection [133-135]. A further study reported that CD4⁺ T cells from patients with HIV-1 edited with Cas9 and a specific gRNA acquired resistance to infection [136]. The same work used Cas9 and gRNA directed at the RSBN1 locus to excise proviral HIV-1 DNA from T cells without disrupting the normal function of the gene, which encodes a histone demethylase required for chromatin organisation [136]. In a related approach, astrocyte cell lines were transfected with Cas9 protein and a gRNA, and a dual-fluorescent HIV-1 reporter was used to map proviral excision sites; loss of fluorescence in the edited cells indicated successful excision with restoration of normal astrocyte morphology and function [137].

Sickle cell disease

Sickle cell disease (SCD) is an autosomal recessive disorder of haemoglobin and is among the most common monogenic conditions worldwide [138]. Modified CRISPR/Cas9 systems have been applied to SCD using human kidney cells and guide strands directed at the CCR5 and HBB loci delivered on the pX330 plasmid [139]. A direct relationship has been described between the presence of adjacent acquisition motifs in the PAM sequence and off-target activity, which reduces or abolishes cleavage at the intended target [140]. The frequency of Cas9-mediated correction was also found to depend on the distance between the PAM and the region of the protospacer recognised by the sgRNA [141].

In order to correct the causative mutation in cells from patients with SCD, a truncated sgRNA targeting the first exon of HBB (trG10) was delivered as a ribonucleoprotein complex with Cas9 protein, together with an ssODN donor, into HSPCs isolated from patient blood samples [141]. Compared with earlier work using ssODNs with ZFN mRNA

electroporation, this approach produced more efficient gene correction with fewer off-target events and more favourable activation of HDR [142].

Haemophilia

CRISPR/Cas9 delivered by adeno-associated virus (AAV) vectors has been evaluated as a therapeutic strategy for X-linked genetic disorders [143]. Haemophilia B is an X-linked bleeding disorder caused by variants in coagulation factor IX (FIX) and is associated with considerable morbidity. An AAV8 vector carrying a codon-optimised sgRNA and *Staphylococcus aureus* Cas9 cDNA was delivered to the hepatocytes of a murine model of haemophilia B in order to generate double-strand breaks in an intron adjacent to the mutated region of the F9 gene and to insert a corrective cDNA, thereby restoring F9 function [144]. The findings demonstrated genotypic and phenotypic correction in treated mice without morphological or functional injury to the liver [145].

Subsequent work extended the therapeutic design to vectors capable of carrying larger constructs. A low dose of adenovirus (Adv) expressing Cas9 and a specific sgRNA, together with a double-stranded DNA donor template, was delivered to the hepatocytes of haemophilia B mice and achieved more efficient repair of the F9 gene than earlier vectors [146]. Restoration of circulating coagulation factor was nonetheless incomplete, since the adenoviral vector provoked an immune response attributable to vector genome immunogenicity and the rate of HDR was low; recombinant adenovirus was therefore considered more suitable [147]. Integration of donor cDNA into the host genome may in addition cause genotoxicity by disrupting functional genes or activating proto-oncogenes [148], and the use of patient-derived iPSCs rather than direct vector delivery may consequently be preferable.

In vitro and in vivo CRISPR studies using iPSCs derived from patients with haemophilia B have produced encouraging results. iPSC lines derived from patient peripheral blood mononuclear cells were corrected by CRISPR/Cas9-mediated insertion of the full human F9 cDNA; these cells were differentiated into hepatocytes and transplanted into NOD/SCID mice, which subsequently secreted human FIX [149]. TALENs have also shown promise in this setting. An in vitro comparison of TALENs and CRISPR targeting canine FIX (cFIX) found that TALENs produced fewer off-target events, generating fewer double-strand breaks than CRISPR [150]. Codon-modified TALEN and Cas9 binding sites were cloned into a pscAAV-cFIX construct together with HDR cassettes containing 471

base pairs of wild-type coding sequence spanning the cFIX mutation; the modified cassettes increased HDR efficiency and reduced the off-target impact of both editing approaches [151].

Autism spectrum disorder and related neurodevelopmental conditions

Autism spectrum disorder (ASD) is a largely heritable neurodevelopmental condition characterised by impairments in social interaction, communication and language, usually identified in early childhood. Several genetic lesions have been associated with the phenotype, including 2q37 and 22q13.3 deletions and maternal 15q11-13 duplication [152], as well as fragile X syndrome [153]. AAV vectors, and AAV9 in particular, are of interest in this context because of their capacity to cross the blood-brain barrier and transduce central nervous system cells efficiently [154].

To improve the gene-carrying capacity of the vector, a self-complementary AAV9 construct was combined with a codon-optimised methyl-CpG binding protein 2 (MECP2) transgene; mutations in MECP2 cause Rett syndrome, which accounts for a small proportion of patients with an ASD phenotype [155]. MeCP2 regulates transcription by both activating and repressing neuronal gene expression, and intravenous delivery of its brain-predominant isoform to Mecp2-mutant mice improved behavioural outcomes [156]. At high doses, however, an off-target effect produced elevated MeCP2 levels in hepatocytes, disturbing liver metabolism and function [157]. Other AAV serotypes have also been evaluated: AAV8 was used to deliver enhanced green fluorescent protein to astrocytes in the mouse striatum and achieved a high rate of transduction owing to its penetration of brain tissue [158], a property that might be exploited to deliver target genes to astrocytes in patients with ASD.

CRISPR/Cas9 has been used to deliver specific edits to neural and brain cells. Cas9 ribonucleoprotein carrying the SV40 nuclear localisation signal was delivered to neural progenitor cells in the hippocampus, striatum and cortex of Ai9 tdTomato reporter mice and demonstrated substantial editing activity both in vitro and in vivo, indicating that neuron-specific targeting is achievable and may be applicable to future ASD therapies [159].

Work in a related neurological disorder illustrates the therapeutic potential of the approach. In Huntington's disease, CRISPR/Cas9 has been used to suppress the mutant HTT (mHTT) gene on chromosome 4, which encodes the mutant huntingtin protein. In vivo targeting of HTT in HD140Q knock-in

mice using an AAV vector carrying CRISPR/Cas9 with four gRNAs produced a substantial reduction in mHTT expression and a corresponding reduction in mutant huntingtin protein [160, 161]. It may therefore be feasible to develop effective genome editing strategies that treat ASD or reduce the severity of its symptoms.

Among the most successful experiments in this field, studies in Thy1-YFP and Ai9 mice modelling fragile X syndrome found that CRISPR-Gold, in which the editing machinery is complexed with gold nanoparticles, delivered Cas9 or Cpf1 to the striatum by local intracranial injection and produced a marked reduction in repetitive behaviour. In contrast to AAV-based delivery, the CRISPR-Gold system produced limited off-target activity and no detectable effect on the immune system. The gene encoding metabotropic glutamate receptor subtype 5 was selected as the editing target [162, 163] because its signalling is overactive in fragile X syndrome and in other ASD-related disorders [164, 165]. Adverse effects were minimal, indicating that delivery and editing were both effective. The same work exploited CRISPR-Gold to edit additional cell types, including glial cells, whose dysfunction is implicated in a range of neurological disorders [166, 167]. Such results offer novel insights into CRISPR-based genome editing and suggest that the approach may be extended to further rare diseases.

Off-target effects of CRISPR/Cas9

Although additional classes of CRISPR/Cas system have been developed, their broad application may be limited by a number of difficulties [168]. The principal disadvantage of CRISPR/Cas9-mediated editing is the difficulty of predicting off-target activity accurately [169]. Off-target effects are defined as unintended cleavage and mutation at genomic sites that are similar, but not identical, to the intended target. The most serious concern is the high reported frequency of off-target cleavage by RNA-guided endonucleases at sites other than the intended one [170]. Editing efficiency is a further consideration, since effective therapy requires both an efficient endonuclease and a stable delivery system [170].

Several strategies have been developed to improve on-target activity while reducing off-target effects, and considerable effort has been directed at engineering CRISPR/Cas variants with higher fidelity and precision [171]. The genomic context of the target DNA, the secondary structure of the sgRNA and its GC content, ideally between 40 and 60 per cent, all influence cleavage efficiency, and tools for designing sgRNAs

with high on-target activity are therefore required [172]. Analysis of the cleavage potential of 218 sgRNAs by in vitro mismatch cleavage assay showed that the nucleotides at both PAM-proximal and PAM-distal positions of the guide are strongly associated with on-target efficiency, with particular base preferences at defined positions relative to the PAM [173, 174]. The distance between the PAM site and the start codon also influences cleavage efficiency and target specificity.

A further means of reducing off-target activity is to titrate the delivered amounts of Cas9 and sgRNA and thereby modulate the concentration of the ribonucleoprotein complex [175]. Increasing specificity by reducing the quantity of transfected nucleic acid may, however, reduce on-target cleavage, so that the balance between on-target efficacy and off-target activity must be evaluated in each application [176]. Fusion of catalytically inactive Cas9 to the FokI nuclease domain (fCas9) has been reported to increase specificity by approximately 140-fold relative to wild-type Cas9 [177]. In a complementary approach, wild-type Cas9 was replaced with the D10A nickase, which cleaves a single strand, and paired with two sgRNAs; this paired-nicking strategy reduced off-target activity in vitro by 50- to 1500-fold [178]. Over the past decade, work on identifying off-target mutations and limiting off-target activity has focused on the rational engineering of designer nucleases [179], the development of computational prediction algorithms and databases [180], and high-throughput sequencing-based detection [178]. Collectively, these measures strengthen the genotype-phenotype relationships derived from CRISPR/Cas9 experiments and support the clinical application of the technology [181].

Conclusion

CRISPR/Cas9 permits precise editing of target sequences in model organisms and in human cells, and holds therapeutic promise across hereditary, viral and malignant disease. Its adaptability and comparative simplicity have made genome-wide library screening practicable in cancer research, and the first clinical studies have established that edited cell products can be manufactured and administered safely. Continuing efforts are directed at improving specificity and thereby limiting off-target cleavage, and recent refinements of the CRISPR/Cas9 platform have reduced the frequency of unintended mutations. Irrespective of the extent to which off-target activity can be minimised, efficient delivery systems that improve efficacy while restricting immune activation remain to be established, and investigators are actively

working to direct CRISPR reagents to defined cell populations in vivo. Cas9 ribonucleoprotein complexes are now widely used in place of plasmid vectors, an approach that increases efficiency, confers more transient Cas9 activity and avoids the integration of vector sequences, although it does not eliminate the risk of chromosomal rearrangement. Optimizing efficacy, safety and specificity therefore remains a prerequisite for the wider clinical application of CRISPR/Cas9.

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