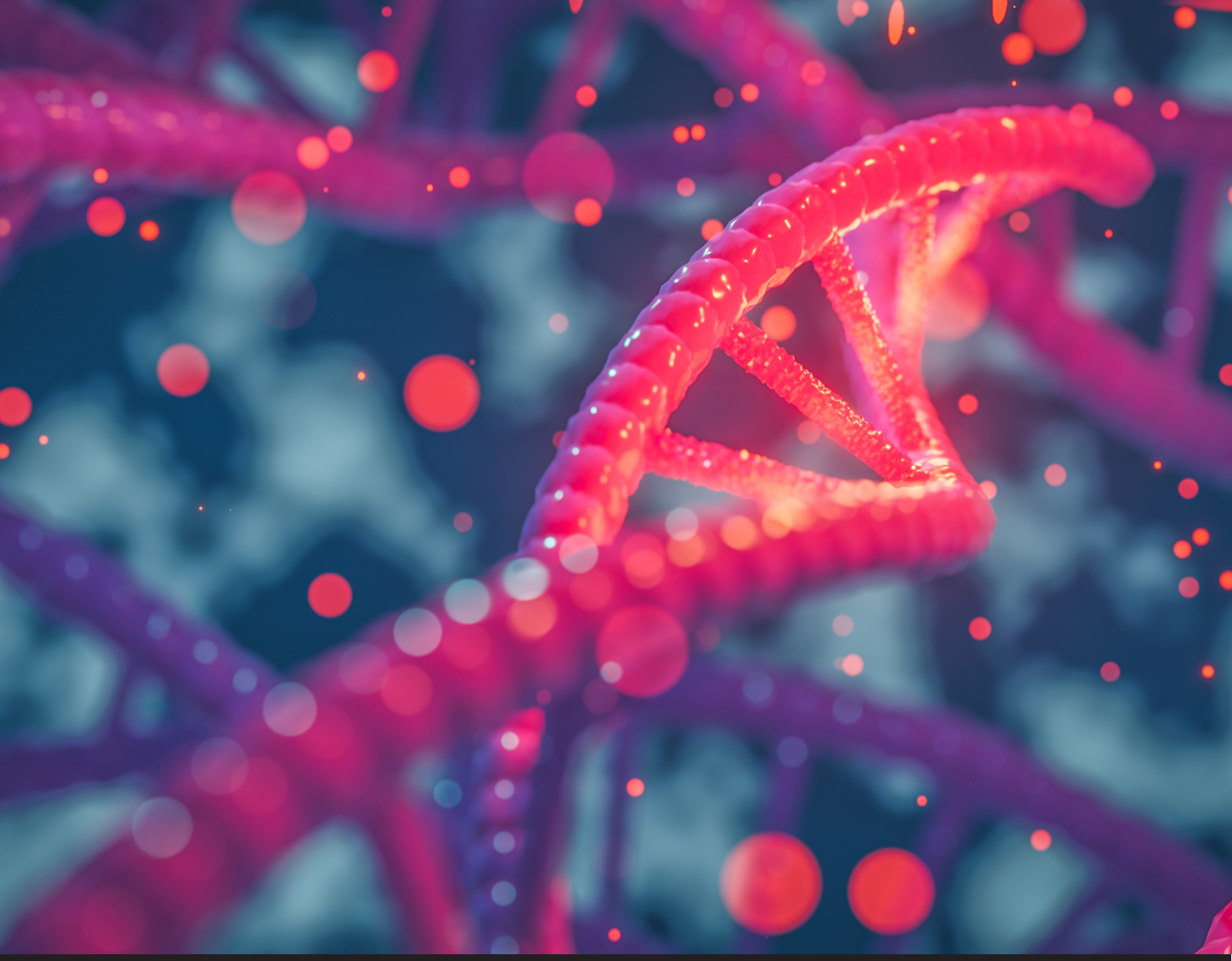

CRISPR and the Revolution of Cancer Research

ARTICLE COLLECTION

WILEY

CURRENT PROTOCOLS
A Wiley Brand

Sponsored by:
ThermoFisher SCIENTIFIC
The world leader in serving science

Biotech: Therapeutics | Immunotherapy

ThermoFisher SCIENTIFIC

Count on connections

A high-performing Cas9 protein for CAR T cell editing

Discover how our next-generation Cas9 proteins are designed to deliver maximum CRISPR editing efficiency. From basic research to preclinical process development, Invitrogen™ TrueCut™ Cas9 Protein v2, Invitrogen™ TrueCut™ HiFi Cas9 Protein, and Gibco™ CTS™ TrueCut™ Cas9 Protein offer exceptional performance and quality to support you at every step of CAR T cell development.

Learn more at **thermofisher.com/cas9proteins**

Intended use of the products mentioned on this page varies. For specific intended use statements, please refer to the product label.

Contents

Introduction

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a gene-editing technology that has revolutionized the field of cancer research. CRISPR enables researchers to make precise, targeted changes to the genome, allowing them to edit specific genes associated with diseases of interest. This technology has enabled scientists to study the role of individual genes in cancer development, as well as to develop new therapies and treatments for various types of cancer.

CRISPR has been used to study well-known cancer-causing genes, such as p53 and KRAS, and to identify new genes that are responsible for certain types of cancer. With this knowledge, researchers can develop targeted therapies to address the mutated genes and prevent cancer from progressing. For example, it has been used to create genetically modified T cells that can recognize and destroy tumor cells. This technology is being used to create personalized cancer therapy tailored to the specific needs of an individual patient. In addition, CRISPR is also being used to create animal models to study the disease in greater detail and to test potential therapies.

This article collection begins with a study by Afolabi et al. (2021) on the current use of CRISPR-Cas9 technology to revolutionize cancer research and immunotherapy. The technology is being used to modify autologous T and NK cells with antigen designs and chimeric antigen receptors, as well as to enhance their sensing circuits with sophisticated functionality. Despite the many potential applications, there are still challenges to overcome to make it suitable for clinical use, such as developing off-the-shelf, universal cellular products. The article also discusses the current advances and prospects for CRISPR technology in cancer research and immunotherapy, and its potential to expand cell-based therapies beyond immune-oncology.

Next, Sadeqi Nezhad et al. (2021) discuss the potential of combining chimeric antigen receptor T (CAR-T) cell immunotherapy with CRISPR-Cas9 gene-editing technology to improve treatments for aggressive diseases like hematologic malignancies and solid tumors. Five types of CAR-T cell therapies have been approved by the FDA, and although they have produced promising results, they are not free from side effects and toxicities. CRISPR-Cas9 technology can be used to modify CAR expression and other cellular pathways to enhance CAR-T cells' antitumor function and persistence, as well as to reduce the toxicities and side effects of CAR-T cell therapy. The practical challenges and hurdles related to the accuracy, efficiency, efficacy, safety, and delivery of CRISPR-Cas9 technology to genetically engineered-T cells must be carefully investigated, but by combining these two technologies, this field could enter a new era of immunotherapy.

Finally, Karimian. et al. (2018) report why CRISPR-Cas9 is a powerful and cost-effective gene editing tool that offers advantages such as flexibility and ease of use compared to conventional methods. This review outlines the classifications and mechanism of action of CRISPR-Cas-based gene editing and discusses its potential therapeutic applications in mutational disorders, delivery systems, and cancer treatment. The advantages and limitations of CRISPR-Cas9 are also highlighted.

The article collection also features a white paper describing the importance of recognizing off-target effects and ways to help mitigate this risk. This is especially important to prevent undesired phenotypes or loss of functional gene activity, which can be detrimental for therapeutic applications. Additionally, the collection features an application note showcasing the importance of consistency in the Cas9 proteins to enable the advancement in CAR-T cell therapy. Overall, CRISPR is an incredibly powerful gene-editing technology that has revolutionized the field of cancer research. It has enabled researchers to study the roles of individual genes in cancer development and to develop new therapies and treatments.

Through the methods and applications presented in this article collection, we hope to educate researchers on new technologies and techniques in gene editing. For more information, we encourage you to visit Thermo Fisher Scientific to explore gene editing solutions to enhance your research.

Róisín Murtagh
Editor at Wiley Analytical Science

References

Afolabi, L.O., Afolabi, M.O., Sani, M.M., Okunowo, W.O., Yan, D., Chen, L., Zhang, Y. and Wan, X. (2021), Exploiting the CRISPR-Cas9 gene-editing system for human cancers and immunotherapy. *Clin Transl Immunol,* 10: e1286. https://doi.org/10.1002/cti2.1286

Sadeqi Nezhad, M., Yazdanifar, M., Abdollahpour-Alitappeh, M., Sattari, A., Seifalian, A., & Bagheri, N. (2021). Strengthening the CAR-T cell therapeutic application using CRISPR/Cas9 technology. *Biotechnology and Bioengineering,* 118, 3691– 3705. https://doi.org/10.1002/bit.27882

Karimian, A, Azizian, K, Parsian, H, et al. CRISPR/Cas9 technology as a potent molecular tool for gene therapy. *J Cell Physiol.* 2018; 234: 12267– 12277. https://doi.org/10.1002/jcp.27972

Clinical & Translational Immunology

Clinical & Translational Immunology 2021; e1286. doi: 10.1002/cti2.1286
www.wileyonlinelibrary.com/journal/cti

REVIEW

Exploiting the CRISPR-Cas9 gene-editing system for human cancers and immunotherapy

Lukman O Afolabi[1,2,3†], Mariam O Afolabi[4,5†], Musbahu M Sani[3†], Wahab O Okunowo[7], Dehong Yan[1,2], Liang Chen[1,2], Yaou Zhang[4,5,6] & Xiaochun Wan[1,2]

[1]Guangdong Immune Cell therapy Engineering and Technology research Center, Center for Protein and Cell-based Drugs, Institute of Biomedicine and Biotechnology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China
[2]University of Chinese Academy of Sciences, Beijing, China
[3]Department of Biochemistry, Faculty of Science, Federal University Dutse, Dutse, Nigeria
[4]Open FIESTA Center, Tsinghua University, Shenzhen, China
[5]State Key Laboratory of Chemical Oncogenomics, Graduate School at Shenzhen, Tsinghua University, Shenzhen, China
[6]School of Life Sciences, Tsinghua University, Beijing, China
[7]Department of Biochemistry, College of Medicine, University of Lagos, Lagos, Nigeria

Correspondence
Y Zhang, Open FIESTA Center, Tsinghua University, Shenzhen, 518055, China.
E-mail: zhangyo@sz.tsinghua.edu.cn

X Wan, Guangdong Immune Cell therapy Engineering and Technology research Center, Center for Protein and Cell-based Drugs, Institute of Biomedicine and Biotechnology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, 518055, China.
E-mail: xc.wan@siat.ac.cn

†These authors have contributed equally to this work.

Received 6 January 2021;
Revised 23 March and 19 April 2021;
Accepted 19 April 2021

doi: 10.1002/cti2.1286

Clinical & Translational Immunology
2021; **10**: e1286

Abstract

The discovery of clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9) technology has brought advances in the genetic manipulation of eukaryotic cells, which has revolutionised cancer research and treatment options. It is increasingly being used in cancer immunotherapy, including adoptive T and natural killer (NK) cell transfer, secretion of antibodies, cytokine stimulation and overcoming immune checkpoints. CRISPR-Cas9 technology is used in autologous T cells and NK cells to express various innovative antigen designs and combinations of chimeric antigen receptors (CARs) targeted at specific antigens for haematological and solid tumors. Additionally, advanced engineering in immune cells to enhance their sensing circuits with sophisticated functionality is now possible. Intensive research on the CRISPR-Cas9 system has provided scientists with the ability to overcome the hostile tumor microenvironment and generate more products for future clinical use, especially off-the-shelf, universal cellular products, bringing exciting milestones for immunotherapy. This review discussed the application and challenges of CRISPR technology in cancer research and immunotherapy, its advances and prospects for promoting new cell-based therapeutic beyond immune oncology.

Keywords: CRISPR-Cas9, genetic manipulation, cancer, immunotherapy, T cells, natural killer cells

INTRODUCTION

Despite concerted global efforts to control this disease, cancer continues to be a significant health burden, in spite of the advancements in treatment options such as radiotherapy, surgery, chemotherapy and, more recently, immunotherapy. Cancer is the world's second leading cause of death

due to constant metastasis and relapse.[1] Therefore, the fight against cancer is a global concern, which calls for new treatment strategies.

In the past, attempts to edit eukaryotic cells, particularly immune cells using the available genetic tools, have yielded little success. The ability of deoxyribonucleic acid (DNA) to repair itself after a double-stranded break provides an avenue for genetic manipulation. The clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9) technology represents one of the high-throughput gene-editing technologies that have revolutionised available treatment options for many human diseases, including cancer.[2,3] CRISPR-Cas9 offers a flexible and advanced gene-editing capability compared with other gene-editing technologies such as ribonucleic acid interference (RNAi), transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs).[4] Besides, CRISPR offers the potential to multiplex multiple gene targets, program its guide RNA (gRNA) and ease of *in vivo* delivery with low cytotoxicity.[5] The CRISPR toolkit has been applied to multiplex genetic research with great success.[6] Other research areas that have benefited from the CRISPR-Cas9 system include neurological, skin and genetic disease therapies.[7]

Here, we describe the CRISPR-Cas9 gene-editing system and discuss how it has been exploited for cancer research and immunotherapy. We also highlight its challenges and prospects for the creation of new cell-based non-immuno-oncology therapy in the future.

The CRISPR–Cas9 biology and mechanism

The CRISPR-Cas9 concept originated from the adaptive immune system of prokaryotes against foreign or invading DNA from bacteriophages.[8–10] Prokaryotes (bacteria and archaea) acquire short genome segments (spacers) from the invading phage, which they integrate into their genetic code to serve as molecular memory during any subsequent infection by the same invading organism.[10,11] The acquired short sequence is then transcribed after maturation as part of the CRISPR array to form the CRISPR RNA (crRNA), which serves as a guide to the Cas9 endonuclease to scan and cleave any invading genetic material that matches the genetic target.[7,12] Cleavage of the genetic target is usually at the site that predates the protospacer adjacent motif (PAM). This biological defence system has been widely adapted for genomic engineering across various species from microbes, plants and animals.[7,13]

CRISPR-Cas9 mechanism of action

The CRISPR-Cas9 system can be regarded as an RNA-guided endonuclease (RGEN), which involves recognising specific short target sequences (~20-bp). The system employs a guide RNA to recognise its target nucleotide, followed by Cas9 nuclease activity.

In principle, the CRISPR-CAS system works following two crucial steps:

- Sequence recognition (foreign nucleotide sequence)
- Nuclease cleavage (on identified target sequence), assisted by gRNA and Cas9 effector proteins.

The PAM, a 2–6-base pair nucleotide sequence, is highly essential for the gRNA to recognise its target nucleotides (~20-bp), followed by the recruitment of the Cas9 protein.[11] The gRNA then guides the recruited Cas9 through its specific sequences related to a transactivating crRNA (tracrRNA) to form the complementary DNA target sequence for the site-specific double-strand break. Interestingly, CRISPR-Cas9 can simultaneously cleave multiple genes,[14] thus serving as an ideal tool for cancer research and the advancement of various therapeutic options, such as immunotherapy.

In endogenous systems, nuclear cleavage begins when mature CRISPR RNA (crRNA) fuses with transactivating crRNA (tracrRNA), which gives rise to a Cas9-guided complex that leads to the target site of the invading DNA (protospacer).[9] However, researchers have developed a gRNA as an artificial replacement for the endogenous crRNA complex.[15]

Ideally, DNA repair in the cell can occur via the non-homologous end-joining (NHEJ)-mediated DNA pathway or by homology-directed DNA repair (HDR).[16] The former (NHEJ) involves direct ligation of the two single-stranded ends, with resultant small random insertion or deletion mutations (indels)[17] while the latter (HDR) requires a template donor DNA sequence with homologous arms to generate DNA repair,[18] where programmed single-strand DNA fragments are introduced to achieve insertion of a specific gene, also known as gene knock-in. Lately, another repair mechanism known as

microhomology-mediated end joining (MMEJ) has been Identified.[19,20] It involves repairing DNA breaks through elongation from substantial microhomology arms (5- to 25-bp sequences), usually generating indels.[21] One unique advantage of the Cas proteins is that single- or dual-guide RNAs can be designed and generated easily.

Advantages of CRISPR over ZFNs and TALENs

The CRISPR system, when compared to other genetic tools such as ZFNs and TALENs, offers many advantages which include the following.

First is the design simplicity. Since the CRISPR system target recognition relies on forming a ribonucleotide complex rather than protein/DNA recognition, gRNA design is easier for any genomic targets.[2] The second is its efficiency. The CRISPR system is highly efficient in terms of its actual genetic editing workflow; for example, mouse embryos can easily be modified by the direct delivery of RNAs encoding the Cas protein and its gRNA into them, thus eliminating the hurdles and difficulty associated with the classical homologous recombination techniques.[5] The third is its multiplex potential. The CRISPR system offers the ability to modify several genomic sites simultaneously by injecting with multiple gRNAs, and this has been used to simultaneously introduce five different gene mutations in mouse ES cells.[22] Recently, it is now easy to also predict its off-target sites, thereby maximising its efficiency.[4]

CRISPR-Cas9 for cancer research and drug targets

Cancer remains a global burden, with an unprecedented annual death. Cancer is characterised by several point mutations leading to an altered genome, and DNA damage, resulting in abnormality in cell division. However, the CRISPR-Cas9 system has shown immeasurable success for studying normal and aberrant genes in cancer cells in various mouse models. For example, by combining the Cre–LoxP technology with the CRISPR–Cas9 system, a phenotypic deletion of tumor suppressor genes such as p53 and PTen was induced. This deletion could be accomplished either individually or in combination using CRISPR's hydrodynamic injection of a designed DNA plasmid expressing Cas9 and targeting these genes in the liver.[23] Another study involving adeno-associated virus (AAV) delivery of CRISPR plasmid to model p53, KRAS and LKB1 genes in lung adenocarcinoma caused mutation in p53 and LKB1, resulting in loss of function in these genes, followed by the formation of adenocarcinoma pathology mediated by homology-driven repair of KRAS G12D mutations.[24]

CRISPR-Cas9 was used to assess putative and novel targets, including the functional roles of cancer-associated mutations in the spliceosome genes. The Degron-KI system consisting of CRISPR-Cas9-mediated knock-in of inducible degron tags was used to determine the causal link between the splicing changes of the SF3B1 hotspot mutations.[25]

The CRISPR-Cas9 has also provided unparalleled usefulness in mimicking structurally aberrant chromosomes, which were previously tricky to model. This approach is relatively easy for insertion or deletion of DNA fragments of varying sizes in the human and mouse genome by the NHEJ/HDR pathways of CRISPR. Likewise, CRISPR technology has made it possible to generate duplication and deletion of DNA fragments by trans-allelic recombination, creating double-strand breaks (DSB) induced by Cas9 on homologous chromosomes, providing a model for the study of millions of gene clusters as well as many regulatory DNA clusters.[26]

It is now possible through a virally assisted CRISPR-Cas9 delivery system to specifically induce *in vivo* chromosomal rearrangement in somatic cells in animals. The generation of an echinoderm microtubule-associated protein-like 4 gene fused to the anaplastic lymphoma kinase gene (Eml4-Alk), which drives lung cancer mouse model, expressing the Eml4-Alk fusion gene, shows the typical molecular and histopathological features of the human ALK^{+} non-small-cell lung cancer (NSCLC)[27]; such an approach can be modelled to investigate other genes implicated in the aetiology of other cancer types.

Furthermore, the use of CRISPR-Cas9 for investigating multiple gene targets has led to the synthesis and creation of a genetic circuit that can aid cancer cell identification with strict specificity and efficacy of cancer gene therapies.[3] This circuit approach involves integrating two promoters as input in a cell, and the output gene is activated only upon the dual activation of the input genes; this has been established for genes such as p21, E-cadherin

and hBAX, which inhibited cell growth, cell motility and induced apoptosis as a result of its corresponding genes.[28]

Chemotherapy represents one of the most common cancer treatment options, and drug resistance is a stumbling block to the success of many therapies; therefore, the search for novel antineoplastic drugs has become imperative. The CRISPR system has been employed as part of the approach to predict and validate novel drug targets. One of such approach is the Drug Target SeqR, designed to find physiological drug targets, which involves the combination of computational mutation discovery, high-throughput sequencing and genome editing mediated by the CRISPR-Cas9. The process consists of inducing protein mutation, which confers drug resistance and reduces cell activity when tested in biochemical assays. An example of such a drug target discovered by this approach is ispinesib (kinesin-5 inhibitor) – an anticancer inhibitor that causes cell death in actively dividing tumor cells.[29]

Another potential cancer drug (selinexor) target was identified and validated by the CRISPR-Cas9 system. Selinexor is an exportin-1 (XPO1) inhibitor, and the CRISPR-Cas9 system was used to show that resistance of cancer cells to this drug was because of mutations at the cysteine-528 in the XPO1 gene.[30]

Besides drug target discoveries, other chemotherapy problems such as multidrug resistance against anticancer drugs are also challenges. The CRISPR-Cas9 system can help identify the gene(s) responsible for drug resistance and test whether any single mutation in such gene(s) or knock-in/out of target genes can confer drug resistance in different tumors. Such an approach will be convenient to reliably generate *in vitro* and *in vivo* models for thorough and high-throughput basic research and preclinical investigation on candidate genes and elucidate the responses of cells in the presence or absence of such target gene(s) (Figure 1).

Another significant benefit of the CRISPR technique is identifying which proteins cancer cells depend on for survival, thus identifying other potential drug targets. This process involved the identification of functional protein-coding exon, which could serve as new targets. For example, Shi *et al*. screened 192 regulatory chromatin domains in mouse acute myeloid leukaemia (AML) cells, 19 new drug targets and six known drug targets were identified.[31] Similarly, the CRISPR-Cas9 system targeted at the promoter of the human papillomavirus (HPV) (E6 and E7 transcript region) resulted in the accumulation of p21 and p53 proteins, leading to a reduction in the proliferation of cancerous cells (both *in vivo* and *in vitro*), thus demonstrating the usefulness of CRISPR for high risk-HPV oncogenes and HPV-related cancer treatment.[32]

CRISPR-Cas9 studies targeting multiple genes may hold the key to treating multiple mutations involved in heterogeneous tumor mass in NSCLC. This system is a better alternative to lung cancer therapy involving histone deacetylase or DNA methyltransferase (DNMT) as it does not have many of the after-effects of DNMT inhibitors.[25] It also enables the target of epi-enzymes to study the epigenetic modulation, control and expression status of cells by recruiting effector domains, including any major chromatin remodelling complexes.

It is also possible to construct a CRISPR-Cas9-based sequence to probe and identify novel regulatory gene clusters unique to specific cancer features. Based on this approach, a novel mutation that elicits resistance against the PLX-4720 (a potent and selective inhibitor of $BRAF^{V600E}$) in melanoma cells was identified.[33] These genome screenings have a lot of potential because they allow for the identification of epigenetic marks within the cancer genome when combined with bioinformatics approaches. Other cancer therapeutic areas that CRISPR-Cas9 can exploit for genetic transcripts include RNAs, antisense transcripts, polymerase III transcripts, non-coding RNAs, nuclear-localised RNAs, microRNAs, polymerase III transcripts with such large variety sequences that can be targeted, including promoters and introns.[34] Employing this technology in genome and epigenome editing is expected to lead to numerous new treatment options in one of the deadliest human diseases.

Exploiting genome-wide CRISPR-Cas9 screening for cancer therapeutic

The genome-wide CRISPR-Cas9 screen entails disrupting gene functionality with sgRNA to uncover novel yet unidentified targets and pathways that influence many biological processes.[35,36] Since its emergence, many studies have developed genome-wide CRISPR knock-out (GeCKO) libraries harbouring arrays of sgRNAs targeted towards a set of genes implicated in cancer aetiology. Since the designed sgRNAs alter and modulate the targeted gene's role in cell

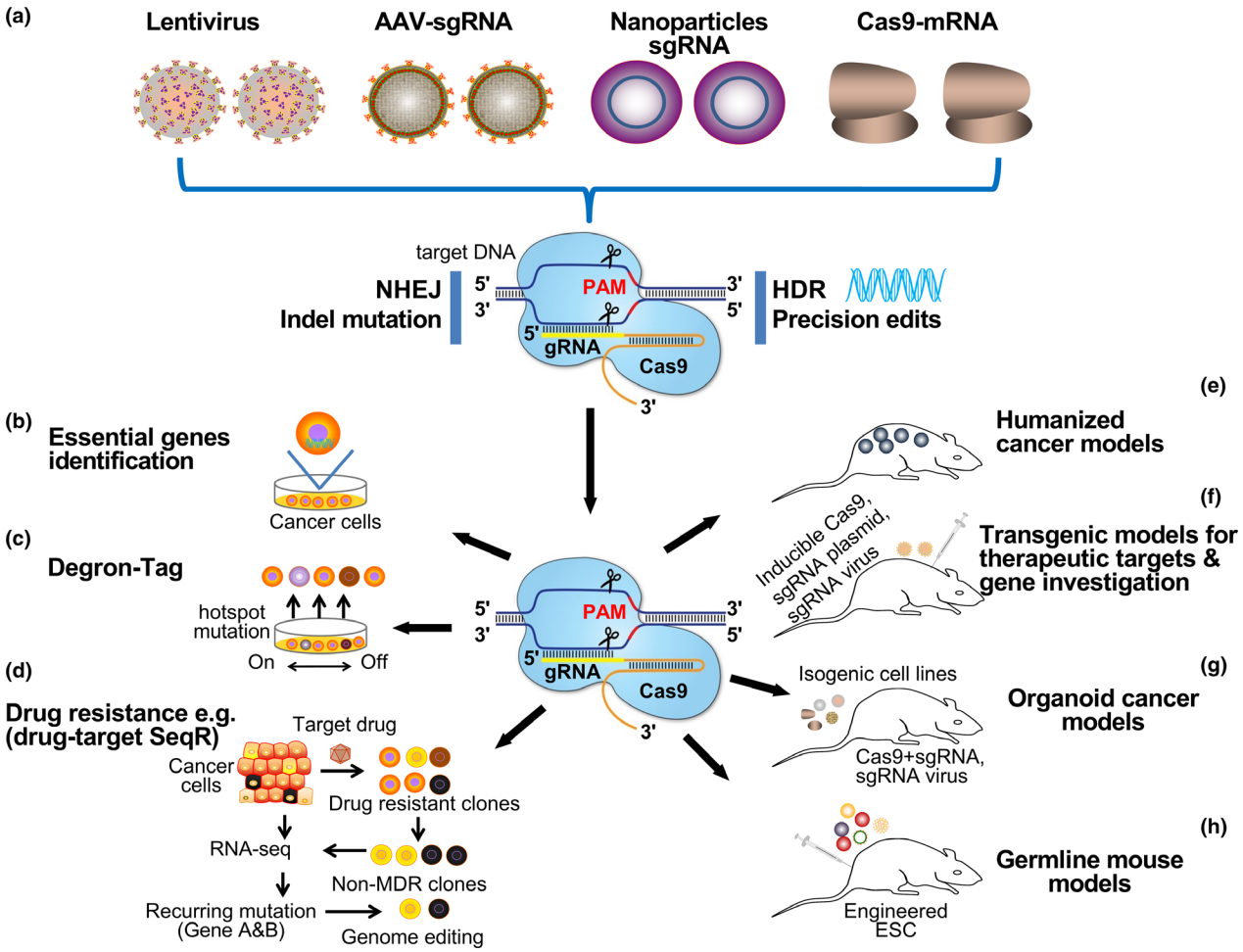

Figure 1. Application of the CRISPR-Cas9 system in cancer research and therapeutics. **(a)** Various delivery methods of the CRISPR-Cas9 material. They range from lentivirus, adeno-associated virus (AAV), nanoparticles and Cas9-mRNA. The CRISPR system can employ either the non-homologous end joining (NHEJ) or the homology-directed repair (HDR) for gene knockout and knock-in, respectively. **(b)** The identification of essential genes or gene clusters peculiar to individual cancer cells. **(c)** Target validation mediated by degron tag knock-in in a gene subjecting its expression to the presence of a small molecule. **(d)** Schematic workflow of DrugTargetSeqR application in identifying a drug's direct target gene curated from recurring gene mutation between parental cancer cells and non-MDR clones, which can be validated by biochemical assays to ascertain whether mutations are sufficient to confer resistance. **(e)** CRISPR-Cas9 mediated generation of humanised mouse strains carrying physiological levels of gene expression. Their endogenic gene expression levels make them essential components for human biology and pathology modelling, including the study of dosage-sensitive genes such as aggregate sensitive proteins and RNA-binding proteins **(f)** Cas9 mediated transgenic mouse models mediated by the delivery of viral sgRNA. Co-expressing and/or inducible Cas9 enzymes can cause tissue-specific gene knockout in different organs. **(g)** CRISPR-Cas9 generation of mutation (point or compound) by chromosome translocation or deletion in different mouse tissues, generating a panel of isogenic cell lines with a variety of oncogenic lesions. **(h)** Generation of germline mouse models harbouring several genetic mutations mediated by CRISPR-Cas9 engineered embryonic stem (EM) cells.

viability during proliferation, the depletion or enrichment of these sgRNAs identifies the genes implicated in the observed cell phenotype[37–41]. The CRISPR genome-wide screening was shown to identify a novel target in AML tumor cell lines. The knockout of the transcriptional activator KAT2A alters their growth. Although KAT2A is not an essential gene for hematopoietic progenitor cells, targeting this gene represents a novel strategy for AML treatment, including the use of MB-3 – a potent inhibitor of KAT2A for AML treatment.[42]

Moving forward, the genes responsible for bortezomib (BTZ) resistance in multiple myeloma (MM) were uncovered via a genome-scale positive selection assay involving culturing MM cells

harbouring various sgRNA targets in the presence of a lethal BTZ dosage. PSMC6 was identified as conferring resistance to BTZ in this cell after surviving-conferring genes were enriched in sgRNAs sequencing.[43]

Another exciting study employing sgRNAs targeted at 2368 murine genes unravelled the protein tyrosine phosphatase non-receptor type 2 (Ptpn2) as a resistance-conferring gene to programmed death ligand 1 (PD-L1) blocking and its loss improves PD-L1 immunotherapy.[44] Another study showed that the loss of GRB2, IRF4, SOS1 and STAT3 in ALK^+ anaplastic large-cell lymphoma cells dampened PD-L1 expression and restored T-cell and NK-cell antitumor functions.[45]

The identification of novel immunomodulatory compounds can be used to augment conventional chemotherapy care. For example, the mechanism of MM cell lines susceptibility to immunomodulatory imide drugs (IMiDs) was explored by loss-of-function genome-wide screening and found that COP9 signalosome complex subunit 9 mediates the regulation of cereblon, which serves as the main factor responsible for sensitivity of MM cells to IMiDs.[46]

CRISPR-Cas9 genome screening for TCR and CAR-T cells

The roles of cytotoxic T cells in the control of tumors have been well established. However, despite the advances in adoptive T-cell immunotherapies and other novel T-cell-based therapeutics, malignant refractory and immune escape by some tumor cells remains a significant burden. In the past, gene knockdown attempts have been made using RNA interference libraries to identify targets that enhance T-cell functions and understand how T cells respond when they encounter their target antigens.

CRISPR-Cas9 ushered in a new gene perturbation approach known as CRISPR-Cas9 genome-scale screening. This functional genetic perturbation approach has been applied in many genetic studies, including primary T cells, to identify intrinsic T-cell factors vital for an enhanced T-cell cytotoxicity by employing an unbiased genetic screening approach.[47]

The CRISPR-Cas9 genome screening involves generating a large pool of T cells (mediated by lentiviruses or other retroviruses encoding large libraries of perturbed genes) harbouring diverse edited genes traceable by their sgRNA sequences in the integrated CRISPR cassette. The CRISPR genome screen can then be coupled with single-cell RNA sequencing (scRNA-seq) to provide a powerful approach to evaluating each gene perturbation effect on the cell state and key signalling signature for its effector functions.

In principle, the CRISPR-Cas9 genome screen is based on three components – (1) gene perturbation, (2) an applicable model and (3) an appropriate assay – to investigate the curated top hits genes.[47]

CRISPR genome screens in human T-cell-based therapies have been used to unravel target genes, including key signalling pathways that modulate the effector function of T cells. For instance, one way the Genome-wide CRISPR screens have been used to enhance the effector function of CAR-T cell is through a comprehensive study that identifies targets that can be translated to novel immunotherapies or an enhancement of existing therapy with gene-engineering, biologics and small molecules.

Based on large-scale CRISPR screens, a new method termed 'SLICE' was developed by Shifrut *et al*. to discover new regulators in primary human T cells that impacted its stimulation responses. This genome-wide loss-of-function screen identified certain critical T-cell-positive genes – LCP2 and negative genes – CBLB, CD5– signatures as important for TCR signalling. Additionally, the authors identified genes resistance to adenosine-mediated immunosuppression, which enhanced T-cell proliferation in the presence of adenosine agonist (CGS-21680) when the identified genes are knocked out.[48] Evidently, the described approach will significantly improve TCR-based T-cell therapies.

About 10–20% of patients with acute lymphoblastic leukaemia (ALL) show resistance after CD19-directed CART19 treatment without a clear understanding of the development of such resistance. Using the CRISPR screen approach, an inherent impaired death receptor signalling in ALL patients was identified to directly correlate to failed CAR-T therapy through impairment of T-cell cytotoxicity, ultimately resulting in CAR-T cell dysfunction. This study demonstrates a novel antigen-independent mechanism of resistance to CART19 therapy.[49]

In another closely related report, the use of CRISPR-Cas9 loss-of-function screens with a systematic investigation of druggable mechanisms

of CAR-T cell cytotoxicity of over 500 small molecules revealed some tyrosine kinase inhibitors that transcriptionally impede T-cell signalling, thereby impairing CAR-T cell cytotoxicity. Interestingly, the identification of death receptor signalling mediated via the FADD and TNFRSF10B (TRAIL-R2) signatures was also implicated as a key mediator of CAR-T cell cytotoxicity, which further elucidate the RIPK1-dependent mechanism of SMAC mimetic sensitisation of diffuse large B-cell lymphoma cells and B-cell acute lymphoblastic leukaemia to anti-CD19 CAR T cells.[50] Since death receptors have varied expression profiles across genetic subtypes of B-cell malignancies, this highlights a direct link between the mechanistic cytotoxicity of CAR-T cells and cancer genetics.

In another interesting study, using a reciprocal CRISPR screening approach, Wang *et al*. revealed genes in both CAR-T and tumor cells regulating cytotoxicity of CAR-T cells while identifying the target genes critical for patient-derived cancer stem cells susceptibility to such CAR-T-mediated killing. In their study, they discovered a novel CAR-T cell- and tumor-intrinsic target that improved *in vitro* and *in vivo* cytotoxicity against Glioblastoma stem cells (GSCs). Genetic ablation of identified hits in CAR-T cells enhanced the cytolytic activity, long-term activation and improved *in vivo* antitumor cytotoxicity against GSCs. Similarly, the knockout of identified targets hits in GSCs sensitised them to *in vitro* and *in vivo* CAR-mediated cytolysis.[51] This reciprocal CRISPR screening can be used to design and find a potential combinatorial inhibitory treatment strategy that would augment CAR-T cell tumor clearance efficacy and promote advanced immuno-oncotherapy.

Besides the CRISPR-Cas9 genome-scale knockout approach, Roth *et al*. demonstrated a widely adaptable non-viral DNA CRISPR-Cas9 genome-scale knock-in screens in primary human T cells. In their approach, dozens of uniquely barcoded large non-viral DNA templates construct were knocked-in into the TCR locus to unravel the candidate constructs that enhanced the fitness and functionality of the engineered T cells both *in vitro* and *in vivo*. Their pooled knock-in sequencing (PoKI-seq) combined with single-cell transcriptome analysis was used to identify a novel transforming growth factor b (TGF-b) R2-41BB chimeric receptor constructs that significantly improved solid tumor clearance.[52] Such laudable pooled knock-in screen approach will allow the gene knock-in of a large multiplexed library of DNA constructs to endogenously modify genetic sequences to generate and accelerate the discovery of more effective T-cell therapies.

The CRISPR-Cas9 genome-scale knockout offers the platform to knock out canonical checkpoint genes such as PD-1 or other immune-suppressive genes, followed by an extensive assay to identify critical elements/pathways responsible for such negative immune signals which could be targeted via gene ablation or pharmacologically. Additionally, CRISPR genome-scale pooled knock-in (such as PoKI-seq) offers the ability to rewrite the endogenous genetic signatures of immune cells, particularly T cells, to improve tumor specificity and resistance to exhaustion, homing to the tumor site with augmented tumor cytotoxicity. Employing such an approach for adoptive TCR and CAR-T-cell therapies holds much promise in developing functional and clinically relevant T-cell-based therapies.

CRISPR-Cas9 in immunotherapy

The hallmark of failed cancer therapies is immune escape by tumor cells that circumvents the numerous antitumor immune responses. Hence, cancer immunotherapy seeks to understand the immune system's complexities in relation to cancer cells in order to harness and augment natural immune mechanisms to combat the disease. Simply put, cancer immunotherapy entails innovative treatment options, unlike traditional cancer treatments such as radiotherapy and chemotherapy. It offers an incomparable advantage with extended progression-free survival and overall survival in patients. Its dynamic and innovative therapies entail reinvigorating the endogenous antitumor immunity against cancers via several directions.[53] Therefore, immunotherapy seeks to fortify components of the immune systems and modulate the complexity of the hostile tumor microenvironment (TME) such that immune cells can target tumor cells with high specificity and penetrate tumor sites to exert their antitumoral functions.[54] Immunotherapy has shown to be highly efficacious with tumor-targeting specificity when combined with conventional treatment options or designed with multiple immune checkpoint blockades (ICB). To achieve this, it is imperative to modify cytotoxic lymphocytes such as T and NK cells that are not easy to manipulate, considering the available

genetic editing methods. The CRISPR-Cas9 gene-editing system provides a viable and safe alternative to generate clinically safe engineered T and NK cells for cancer immunotherapy.

CRISPR-Cas9 in chimeric antigen receptor (CAR) immunotherapy

The emergence of chimeric antigen receptor T-cell (CAR-T) therapy as a promising treatment option for cancer, particularly for haematological malignancies, is laudable.[55] Engineered CAR-T cells can be activated, infiltrate tumor sites, secrete cytokine and licensed to kill tumors in a manner that ensures complete tumor regression. Since CARs are usually designed for a specific tumor-associated antigen, they consist of one or all of the following: an extracellular antigen binding domain, a hinge domain, a transmembrane region and an intracellular signalling domain.

Interestingly, most current CAR-T cell clinical trials utilise autologous T cells from the patient's own peripheral blood mononuclear cells. Although this is ineffective, attempts have been made to create a universal CAR-T cell.[56,57] The CRISPR-Cas9 system offers many alternatives to enhance the current CAR-T and facilitates efficient and straightforward multiplex genomic modification of T cells to enhance its activation, tumor specificity and infiltration to improve the overall efficacy and safety of CAR-T cells (Figure 2).

Engineering CAR-T cells with CRISPR-Cas9

The therapeutic efficacy of CAR-T cell has been shown especially for B-cell lymphoma and other malignancies.[58,59] Currently, the standard CAR-T treatment procedure required the autologous transfer of cells, which are often detailed, expensive and sometimes challenging to obtain

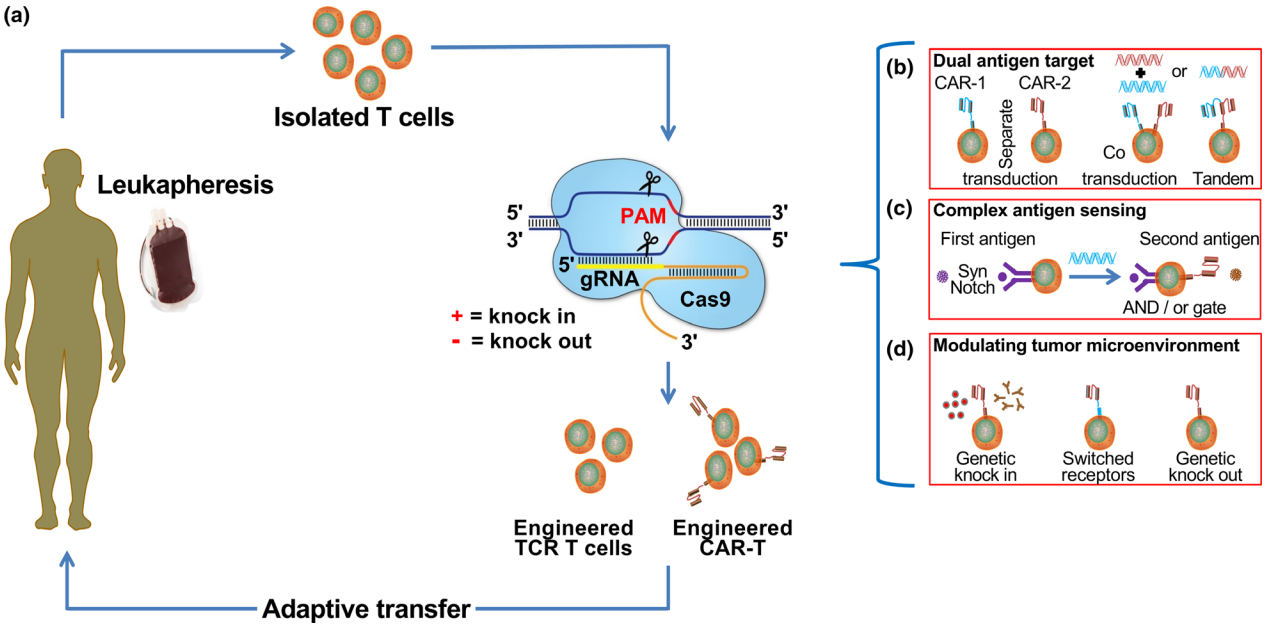

Figure 2. CRISPR-Cas9 genome-editing strategies in adoptive T-cell immunotherapy for cancer. Applications of the CRISPR-Cas9 in T-cell cancer immunotherapy. **(a)** Isolated patient-derived T cells are genetically engineered with CRISPR-Cas9 to knockout endogenous genes, for example PD-1, and knock-in therapeutic TCR, and CARs, followed by *ex vivo* expansion and adoptive transfer. **(b)** CRISPR-Cas9 inspired dual-specific tumor recognition to overcome tumor heterogeneity or antigen loss. This can be achieved by transducing a single CAR molecule into two T-cell populations (separate transduction), incorporating two CAR molecules into a single-cell population either individually or by bicistronic (co-transduction) and linking two separate CAR molecules to produce a single signalling chain (tandem transduction). **(c)** To surmount the off-target effect and fine-tune antigen sensing of tumor-specific T cells, incorporating a synNotch receptor specific for a first antigen that can trigger the production of CAR upon interaction with a second antigen – this triggers its activation with a licence to kill the tumor. **(d)** Genetically reprogrammed T cells to overcome the hostile tumor microenvironment. The incorporation of genes capable of local cytokines or antibody release. Similarly, switched receptor strategies enhance sustained antitumor response and the deletion of inhibitory molecules or immune checkpoints to generate off-the-shelf T-cell therapies.

sufficient qualitative T cells, especially in neonates and elderly, to generate patient-specific CAR-T cells.[60]

CRISPR-Cas9 offers the potential to develop a universal CAR-T (obtained from healthy donors) for allogeneic transfer, which has many advantages over autologous CAR-T. The success of such an approach will be to delete the human leukocyte antigens class I (HLA-Is) and subunits of the T-cell receptor (αβ) – (*TRA* and *TRB*) on the allogenic CAR-T cells.[60] Mutation in the T-cell receptor (TCRα) subunit constant (TRAC gene) of the T cell can lead to loss of its surface αβ TCR[61]; similarly, a mutation in the beta-2 microglobulin (B2M) gene led to the loss of expression of HLA-I heterodimers on the T-cell surface [62]. The generated $B2M^{-/-}$embryonic stem cells (ESCs) could serve as universal donor cells where the transplanted cells do not express HLA class II genes.[62]

In another modified approach, Liu *et al*. showed that two (B2M and TRAC) and three (PD-1, B2M and TRAC) genes could be effectively disrupted by the CRISPR technique to generate universal CAR-T cells. By designing two sgRNAs each specific for the first exon of B2M and PD-1, and another for the TRAC gene. The in vitro antitumor function of these multiplex double-knockout (DKO) (TRAC and B2M) and triple-knockout (TKO) (TRAC, B2M, and PD-1) CAR-T cells revealed higher cytokine production and potent cytotoxic activity against tumor cells compared to standard CAR-T cells.[60]

Using a xenograft lymphoma mouse model, similar results were obtained for the *in vivo* effector function of these CAR-T cells where a DKO and TKO was induced, leading to a significant reduction in tumor size, indicating that the CRISPR-mediated multiplex gene deletion of HLA-1 and TCR from CAR-T cells retained their CD19-specific antitumor function.[60]

In a closely related report, CRISPR-Cas9-mediated allogeneic CAR-T cells show multiplex gene editing, the authors combined CAR lentivirus delivery with CRISPR RNA electroporation for co-introduction of gRNA (specific for B2M, TCR and PD1 deletion). This approach describes the concept of engineering CAR-T cells devoid of the TCR, programmed death protein (PD1 – immune checkpoint) and the HLA class 1 molecule, with potent *in vitro* and *in vivo* antitumor activity, compared to the unmodified CAR-T cells. The DKO CAR-T cell showed significantly reduced alloreactivity and did not elicit graft versus host diseases.[63]

Other promising studies include a CRISPR-Cas9-mediated CD19-specific T-cell targeting the α-TCR subunit constant (TRAC); the method employed in this study resulted in the uniform expression of the CD19-specific CAR on human peripheral blood-derived T cells.[64] By targeting the first TRAC exon, the gRNA and a repair matrix of AAV harbouring a self-cleaving P2A peptide followed by cDNA of CAR were electroporated together with the Cas9-mRNA to generate the engineered TRAC-CAR-T cell. The efficiency of these engineered CAR-T cells (with TCR knockout) could be compared to other sequence-specific strategies often employed to target different loci (CCR5, AAVS1, CD40L).[65–67] Finally, the engineering of CAR-T cells should use endogenous regulatory elements such as TRAC to avoid tonic signals, T-cell exhaustion and delayed T-cell differentiation while the CAR molecule can be re-expressed after repeated antigen exposure.

Based on the above reports, it is evident that the generation of CAR-T cells on a custom-made patient basis is not sustainable. Such autologous T-cell production remains the bottleneck for the large-scale clinical application of CAR-T therapies, considering the invested resources, cost and time. However, the inherent production failure associated with autologous T-cell production, together with its restricted application on different cancer types, is enough to push for the development of universal 'off-the-shelf' CAR-T cell therapies (Table 1), whose production and potential technical hurdles will be readily alleviated through the flexibility of the CRISPR system. This technique will improve the current CAR therapeutics while generating universal, programmable and flexible CAR-T cells whose therapeutic effects are controllable. Embarking on such an approach will bring a paradigm shift in engineered universal CAR-T that can be directly infused in recipients without re-editing, albeit with multiple antigen target capabilities.

Engineering TCR T cells with CRISPR-Cas9

The CRISPR-Cas9 system's efficacy in generating CAR-based therapies targeted for $CD19^{+}$ haematological malignancies cannot be overemphasised. It also plays a role in constructing TCR T cells through its multiplex approach to generate efficient T cells. In terms of surface antigen, presentations of major histocompatibility complex (MHC) independent,

Table 1. The advantages of generating universal CAR-T versus autologous CAR-T

CAR-T types	Cost of production	Time of production	Quality control	Availability
Autologous	Very high with complex logistics	Long time, even longer in neonates and elderly	Difficulty in controlling parameters in the production process because of variable starting cell population	Difficulty in obtaining qualitative starting patient's cells could impact its production leading to failure to receive treatment
Universal	Relatively cheaper considering the number of recipients	Can be made in advance, with shorter, optimised production time, and made available to recipients on demand	Advanced production allows multiple rounds of quality control checks to ensure the product meets safety standard and quality	Stocks of pre-manufactured CAR-T products can be stored in a universal bank (similar to blood banks) and made available to recipients as when due

CAR-based therapies have been used successfully against relevant tumors; however, engineered TCR T cells can identify tumor cells via the MHC complex, the antigenic peptides present on their surface. Interestingly, they do this via the antigenic peptide fragment/ MHC combinations. According to a report, TCR T cells can infiltrate solid tumors more effectively than CAR-T cells.[68]

Studies have shown that tumor-specific TCRs targeting the intracellular proteome and/or metabolome can be generated.[69] Although some areas of concern have been identified, such as TCR mispairing – a condition of incorrect endogenous and recombinant TCR pairing, often resulting in reduced surface expression of therapeutic TCRs or sometimes autoreactivity.[70,71]

The use of endogenous rather than engineered TCRs has been suggested; however, one of the major pitfalls of such an approach is the low-affinity range of endogenous TCRs compared to engineered TCRs when targeting foreign pathogens, as most TAAs are self-derived.[72] Hence, therapeutic use of endogenous TCRs for cancer treatment can reduce efficacy with severe toxicity as these antigens also exist in normal cells. Despite the uncertainties and unintended consequences associated with the use of TCR T-cell, the use of CRISPR-Cas9 editing technique to induce endogenous knockout of TCRs has led to an increased surface expression of therapeutic TCRs, ultimately with improved sensitivity, specificity and cytotoxicity.[69]

Recently, a phase I trial, involving the transplantation of autologous T cells devoid of both endogenous TCR and PD-1, was shown to improve their biosafety.[73] Using the CRISPR-Cas9 system for genome editing of autologous T cells by knockout of specific genes has helped researchers and clinicians explore the optimal therapeutic conditions for engineered TCR T cells. The goal of such engineered T cells is to enhance their functions while reducing the risk of autoimmunity.[73] To this end, the CRISPR technique holds enormous possibilities for developing the next-level TCR T cells for immunotherapy and beyond. Interestingly, the CRISPR-Cas9 technology provides the avenue to do more basic research on TCR T cells to generate safe and better cell-based products for clinical use, accelerating bench to bedside treatment.

Strategies to augment natural killer (NK) cell antitumor activity and mitigate its exhaustion with CRISPR-Cas9

The immune system plays a critical function in preventing the onset and metastasis of cancer. In this regard, NK cells represent an essential effector lymphocyte of the innate immune cells, and their antitumor roles have been well recognised.[74–76] However, during tumor progression, NK cells are sometimes found exhausted within the TME. Numerous reports have demonstrated how the exhaustion of effector lymphocytes regulates and shapes the immune response to tumor progression and infections, limiting their antitumor potentials.

Since therapies targeted at activating and reinvigorating the immune effector functions can yield beneficial responses in patients with episodes of metastatic malignancies, this has led to long-lasting clinical responses, thus revolutionising oncology with dramatic benefits in both haematologic and solid tumors. Based on the success recorded for reinvigorating exhausted T cells and enhancing their antitumor functions, extending this approach beyond T-cell therapies is pertinent. Despite the documented success for T-cell therapies, a critical assessment of the tumors originating from patients who progress on anti-

PD-1 blockade showed an impaired antigen presentation and interferon signalling, leading to tumor evasion from T-cell response. Unlike T cells, NK cells can exert their cytotoxicity on tumor cells without prior sensitisation to antigens, particularly tumor cells with low or impaired antigen presentation machinery.[74] This makes approaches targeted towards preventing exhaustion of NK cells and reinvigorating their effector functions a laudable approach.

A critical understanding of the multiple mechanisms that might contribute to the anergy, exhaustion and senescence of NK cells, such as the presence of suppressive cytokines or soluble factors, regulatory immune cells and dysregulated receptor signals found within the TME, will guide to design modalities to augment NK-cell functions. Besides creating novel NK-cell-based antitumor therapies, a clear understanding of the above characteristics will enhance our knowledge of basic NK-cell biology and help overcome several hurdles limiting the clinical application of meaningful NK-cell-based therapies.

A review of the recent developments using the CRISPR system to augment NK-cell effector function against tumors regarding NK-cell immune checkpoints, cytokine therapy, NK-cell engagers and adoptive infusion of NK cells is discussed below.

Innovative NK cells engineering with CRISPR-Cas9

Natural killer (NK) cells represent one of the first lines of the host immune surveillance. They play vital antiviral and antitumor roles on stressed or transformed cells through numerous mechanisms (e.g. direct cytotoxicity, secretion of cytokines/chemokines and antibody-dependent cell-mediated cytotoxicity). Unlike T cells, NK cells lack antigen-specific recognition capability but play critical antitumor immunity roles.[77] The use of NK-cell immunotherapy is fascinating and represents a promising and dynamic strategy for cancer treatment, the antitumor effects of which require further improvement. In the past, attempts such as the use of antibodies, cytokines or gene-editing have been embarked upon to overcome tumor immune suppression and enhance tumor recognition in NK cell immunotherapy.[78,79] CRISPR-Cas9 genome-editing system offers flexibility in editing NK cells *ex vivo* for adoptive therapy. Alternatively, this technique allows tumors to be manipulated *in situ* to increase their susceptibility to *in vivo* NK surveillance.[68,80]

Recently, NK-cell cancer immunotherapy has been explored for hematopoietic malignancies. Like the CAR-T immunotherapy, CAR-engineered NK cells have shown tumor target specificity and cytotoxicity.[81,82] The current preclinical and clinical applications and research on engineered CAR-NK-cell-based immunotherapy targeted for different cancer types have been discussed.[68,83,84] The immunotherapeutic effect of the diverse engineered CAR molecules on NK cells to redirect the corresponding specific antigens in a cell-based approach has also been well discussed.[58,84,85]

The NK cell is a potent effector cell, and its use in CAR targeted immunotherapy has numerous advantages compared to the T cell. For example, allogeneic NK cells kill target cells antigen-independently, so they can be used for universal adoptive transfer, as they do not give rise to graft versus host diseases commonly seen in allogeneic T cells (HLA matching). Also, the inability of the CAR-NK cells to induce cytokine storm also makes them safer than CAR-T cells, and, finally, the abundance of sources for generating NK cells such as human peripheral blood (PBMC), umbilical cord blood (UCB), induced pluripotent stem cells (iPSCs), human embryonic stem cells (hESCs) and NK-92 cell lines helps overcome the trouble of obtaining the cells in abundance[58] (Figure 3).

Combining CRISPR-Cas9 with another gene-editing approach, Velasquez *et al*. reported a CAR-NK-based therapy bispecific T-cell engager (CD19-ENG) capable of targeting $CD22^{+}$ B cells leukaemia as well as also redirecting T cells to kill malignant $CD19^{+}$ B cells, hence preventing any immune escape by the tumor and improving its antitumor activity. For the first time, this study showed engineered CAR-NK cells specific for CD22 and augmented CD19 T cell targeting of B-cell malignancies.[86] Such combined cytolytic target killing of malignant cells opens a new window in gene editing of cancer immunotherapy with a significant improvement in current B-cell cell therapy and related malignancies.

These findings emphasise the enormous potentials of the CRISPR-Cas9-mediated gene editing of effector cells for clinical immunotherapies. Considering the strides already achieved in effector cell-mediated immunotherapy, CRISPR-based genetic manipulation has equipped scientists and

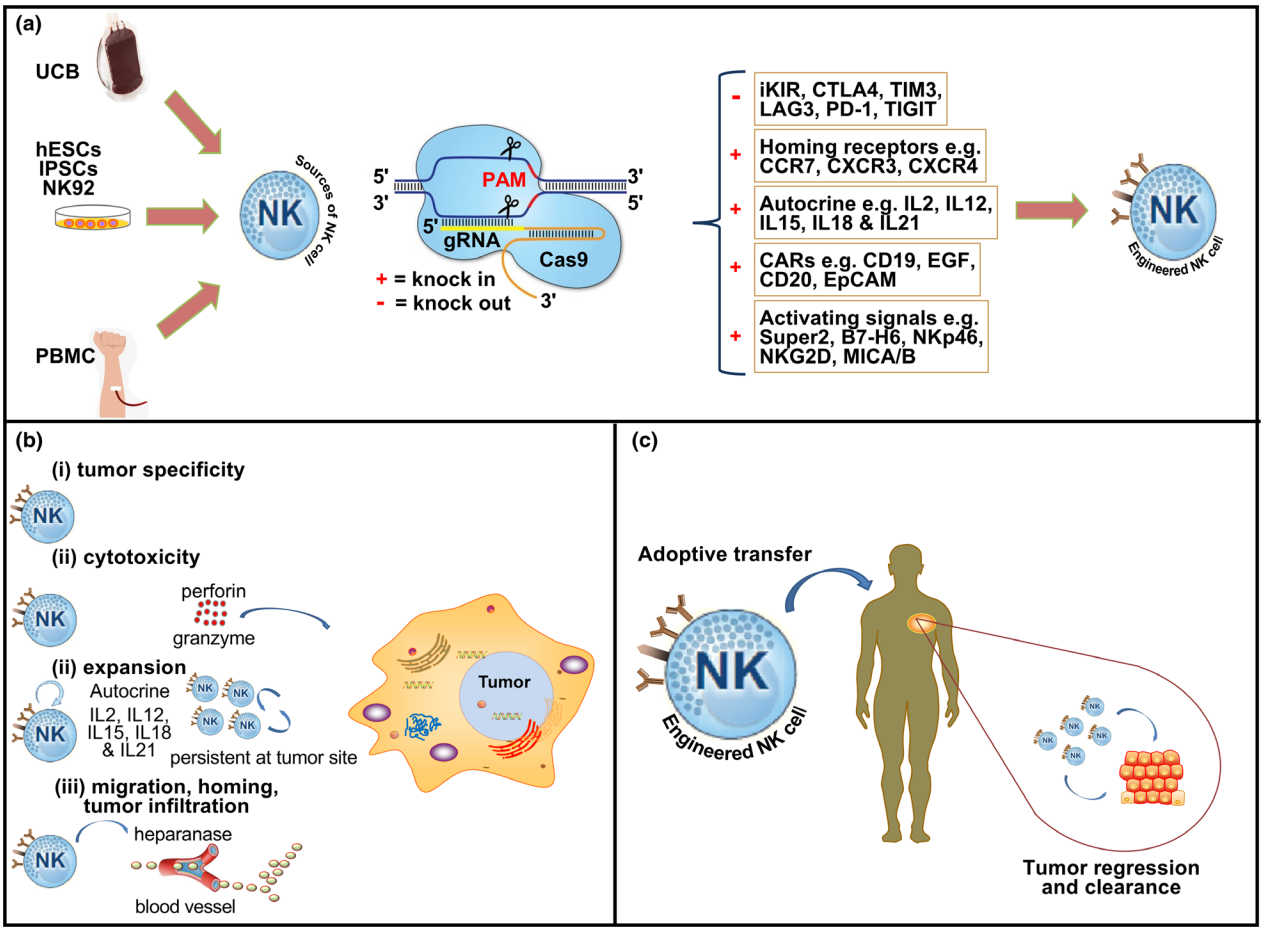

Figure 3. Overview of CRISPR-Cas9 genome-editing strategies for NK cell immunotherapy. **(a)** NK cell sources (UCB, umbilical cord blood; hESCs, hematopoietic embryonic stem cells; iPSCs, induced pluripotent stem cells; NK-92, NK-92 cell line; PBNK, peripheral blood mononuclear cells) and its manipulation via the multiplex capability of the CRISPR system. **(b)** Engineered NK cells with augmented antitumor capabilities such as tumor specificity, cytotoxicity, expansion and tumor infiltration. **(c)** Engineered NK cells adoptively transferred to confer tumor regression and clearance.

clinicians with the new treatment tool that can be used to win the battle against many cancer malignancies. To attain such a feat, specific improvements need to be made. First, in the CAR-NK design, the CAR molecules' introduction should be accomplished with the deletion of NK-cell inhibitory receptors such as NKG2A or TIM-3; this will confer sustained and intense cytotoxicity because of the lack of inhibitory signals usually encountered in the TME. Similarly, a multiplex TKO or DKO of inhibitory genes in NK cells as shown for CAR-T cell (TCR, HLA and PD-1/CTLA-4/PD-L1) should be given great attention.

A novel approach was suggested to overcome the immunosuppressive IL-4 cytokine, which involves the inversion of the cytokine receptor (ICR) by fusion of the IL-4 receptor exodomain with the IL-7 receptor endodomain to generate a 4/7 ICR that confer IL-4 immunosuppressive resistance to the CAR-T cell while improving its cytotoxicity.[87] Such an approach can be extended to engineered CAR-NK cells with varying potential ICR endodomain candidates (IL-15, IL-18 and IL-21) that still need to be fully established.

The targeted integration of the CAR genes at specific sites of the genome of effector cells is desirable compared to integration at a random site. The knock-in of CAR at the α constant locus of TCR improved T-cell antitumor activity.[64] Similarly, the integration of CAR into the TRAC locus prevented CAR signalling and immune cell exhaustion. These approaches can be employed by the CRISPR-Cas9 technique to generate CAR-NK cells with improved antitumor efficacy.

Furthermore, the use of small inhibitory molecules such as BX795 (which inhibits TBK1/IKK complex by acting downstream of RIG-I-like receptor and TCR) to enhance CRISPR-Cas9 material viral delivery can be explored[88]; this and other related non-toxic molecules can significantly improve the genetic editing of these effector cells (T and NK cells) for immunotherapy. As previously stated, NK cells are potent effector cells with natural cytolytic, antiviral and antitumor functions. The preferred choice of NK cells as alternative immunotherapy is partly because of their lack of TCRs that could cause graft versus host disease, potentially generating off-the-shelf cell therapy. Although NK cells have an effector potential, they are sometimes dysfunctional in the TME.[89] To this end, the CRISPR-Cas9 system allows for genetic modification of NK cells to reinvigorate their cytotoxic, antiviral and antitumor immunity through the following means.

Optimised innovative CAR molecules

The CRISPR-Cas9 system allows NK cells to be fortified with CARs that target various tumor antigens.[90–92] Loss of original tumor antigen is a concern for CAR-based immune cell therapy. NK cells can be armed with pan-specific CAR molecules to improve tumor recognition via multiple ligands, and hence elicit a superior antitumor response compared to a single ligand target. As proof of principle, NKG2D ligands, including (MHC class I chain-related protein A (MICA) and B (MICB), and human cytomegalovirus UL16-binding proteins, are poorly expressed in normal cells but highly expressed in virally transformed and tumor cells.[93] Incorporating full NKG2D protein on T or NK cells as part of the CAR design with the potential of multiple tumor ligand recognition showed an enhanced antitumor effect against NKG2D ligand-positive tumors.[94,95] Such pan-specific CAR-T or NK cells can also target NKG2D ligand-positive myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), hence overcoming the immunosuppressive TME.[94]

To achieve full activation of CAR-NK, the design of its intracellular domain should be different from CAR-T. Since DAP12 has been shown to play a predominant role in the transduction of activating signalling in NK cells,[96,97] it is crucial to optimise the intracellular domains with special consideration for DAP12 combination to enhance the cytotoxic signals for CAR-NK.

Stimulating activating pathways

NK-cell effector functions could be enhanced and sustained by activating receptors and cytokines (e.g. IL-2, IL-15, IL-18 and IL-21).[79] IL-2 and 15 have been established as essential for promoting NK-cell survival.[98] Additionally, IL-2 mutant form 'Super-2' reverses NK-cell exhaustion and promotes its proliferation.[99] The multiplex capability of the CRISPR-Cas system can be used to force express one or more cytokines such as 'Super-2', IL-15 or other cytokines in enhancing NK-cell survival and effector functions. The augmentation of the *in situ* expression of tumor-specific ligands for activating NK cell receptors is another laudable approach.[100,101] It can enhance NK cell antitumor responses via activating pathways made possible by the CRISPR-Cas9 system. For example, transcriptional activation of NKG2D ligands – MICA – has been done successfully using the CRISPR-Cas9 method.[102]

Enhancing NK cell infiltration

The homing and migratory ability of NK cells to the disease site, as well as its ability to infiltrate tumor tissues, is usually indicative of its success and good prognosis upon adoptive infusion during NK-cell immunotherapy.[103–105]

The surface expression of specific chemokine receptors on NK-cell-targeted towards tumor-specific ligands using the CRISPR-Cas9 technology holds much promise. The therapeutic benefits of the engineered chemokine receptor – CXCR2 on NK cells – have shown enhanced migratory potential towards a chemokine gradient CXCR2 ligands,[106] indicative of the enhancement of intratumoral infiltration of NK cells. Additionally, another separate report showed the increased migratory ability of NK cells genetically engineered with the chemokine receptor CCR7 towards its ligands (CCL-19 and CCL-21), offering tumor infiltration and homing.[107,108]

Since the TME is a mosaic of different components, including the stroma, thorough profiling and optimisation of chemokine receptors required for maximum tumor penetration will be required to overcome tumor-associated stroma impedance. To this end, engineered NK cells expressing the enzyme heparanase and other

modifications such as CAR expression hold the propensity to improve NK-cell tumor infiltration through the ability to degrade the extracellular matrix as this has been shown to be successful for CAR-T cells,[109] thus it can significantly improve NK-cell immunotherapy.

Overcoming NK cell inhibitory pathways

NK-cell activation involves a balance between activating and inhibitory signals on its surface.[110] Strikingly, tumor cells express ligands that prevent unwanted NK-cell activation as part of their immune escape mechanism.[100,101] Inimical signals from checkpoint receptors are implicated in causing tumoral NK-cell exhaustion.[89] Besides, several reports have shown that the blockade of checkpoint receptors related to NK cells (such as CD96, NKG2A, PD-1 or TIGIT) significantly improved its antitumor immunity.[111–113] There is a paucity of information on the role of LAG3 on NK cells. Recently, LAG3 has been implicated to play an inhibitory role and is expressed by activated NK cells.[114] Reports have demonstrated that the inhibitory signals received from LAG3 attenuate NK cell cytotoxicity, cytokine/chemokine release and its antitumor function.[114,115] Therefore, using the CRISPR-Cas9 system to genetically disrupt pathways associated with some of the checkpoint cell-surface receptors on NK cells might improve its effector functions.

CRISPR-Cas9 technology to improve immune checkpoint blockade

The increasing numbers of failed therapies targeted at cancer have brought about many novel cancer treatment strategies. In particular, ICB is one of the most successful cancer treatment options. The approach was pioneered with the application of monoclonal ICB antibodies: anti-PD-1/PDL1 antibodies and anti-CTLA-4. This was followed with drugs that explicitly target PD-L1, for example atezolizumab, durvalumab and avelumab; and despite their initial promise, unintended cytotoxicity and some clinical failures raised significant concerns.[116–118] One of the many ways to overcome this setback is to carefully elucidate the intrinsic expression of PD-L1 by cancer cells – which has been implicated as the most immune evasion mechanism.[119] Besides, since tumor expression of PD-L1 has been correlated to the efficacy of immune checkpoint inhibitors across different cancer types.[120] Therefore, it is logical and imperative to identify the mechanisms that regulate PD-L1 expression to augment existing treatment options to aid the development of novel strategies. To this end, the CRISPR system can be employed. As a proof of concept, genome-wide CRISPR screening has been used to identify an uncharacterised protein CKLF-like MARVEL transmembrane domain-containing protein 6 (CMTM6), which serves as a critical regulator for the surface expression of PD-L1 – whose increased expression also correlated with enhanced tumor cell clearance with ICBs.[121] Another closely similar CRISPR genome screening approach was employed to identify regulators of PD-L1 expression in H358 lung adenocarcinoma; the authors identified SMAD4 and uroporphyrinogen decarboxylase (UROD) in addition to CMTM6 as novel regulators of PD-L1 expression.[122] Another report showed using the CRISPR-based genome screening technique to identify another PD-L1 regulator in human lung cancer cells. A role of the translation initiation factor EIF5B was identified in lung adenocarcinomas, whose overexpression, however, correlates with poor prognosis and is sufficient to induce PD-L1.[122]

Palmer *et al.* used a CRISPR-based method to knock out the cytokine inducible SH2-containing (CISH) gene. In turn, CISH KO resulted in increased T-cell receptor (TCR) avidity, tumor cytolysis and neoantigen recognition. However, the CISH KO led to increased PD1 expression, whose adoptive transfer synergises with PD1 blockade, with durable tumor regression and survival benefits in the preclinical animal model. This research identified a new avenue that modulates the recognition of neoantigens and the expression of their activation/exhaustion markers that dictate the functionality in tumor-specific T cells.[123]

These findings and other similar CRISPR-based approaches can be employed to elucidate mechanisms governing the immune checkpoint regulation and identify novel therapeutic targets for improved immunotherapy. Besides the above described, the CRISPR genome screens offer many advantages that when it is applied *in vivo*, for example, it is possible to model the complex interaction and replicate the dynamic TME. Therefore, *in vivo* CRISPR-Cas9 genome screens

now identify regulators of immune evasion by cancer cells, including immune cell inhibitors.[124]

However, the *in vivo* CRISPR genome screening is somewhat similar to *in vitro* approaches in which sgRNA is used to modify and generate mutant tumor cells, which are then transplanted via different routes and allowed to develop. Harvested tumors are then compared with unmodified tumors from immune-competent mice to find any genetic hits that may play a role in the antitumor response.[124]

Several other studies have identified genes that could be targeted to promote tumor immunotherapy; for example, the loss of Ptpn2 and Adar1 was found to improve antigen presentation and tumor sensitisation to anti-PD-1 blockade to improve immunotherapy, respectively.[44,125] In a recent study, a novel CRISPR-Cas9 system was used to knock out the cyclin-dependent kinase 5 gene (CDK5), leading to the downregulation of PD-L1 expression on tumor cells while promoting the population of cytotoxic effector cells in the TME.[126]

The role of epigenetic modifiers in antitumor immune response has been well identified[127–129]; CRISPR genome screen using epigenetic sgRNA has identified genes that confer the efficacy of anti-PD-1 blockade.[130] Additionally, the histone chaperone Asf1a was reported to sensitise Kras/p53 tumor cells to anti-PD-1 therapy; and the loss of Asf1a also induced an inflammatory response, secretion of the cytokine – GMCSF, which modulates the polarisation of M1 macrophage and T-cell activation.[131] These reports reveal how the CRISPR system has been exploited to elucidate the various molecular mechanisms that govern the immune evasion of tumor cells. It is evident that CRISPR offers tremendous usefulness to identifying novel targets which may be explored to improve immune checkpoint therapy, particularly to overcome the recurrent resistance to immunotherapy.

THE FUTURE PROSPECTS FOR CRISPR-CAS9

The CRISPR-based technology has shown enormous potential in its routine clinical applications. Unlike the other gene-editing tools, CRISPR offers many advantages, particularly in terms of its ease of *in vivo* delivery and the design of novel therapies for cancers.

Current challenges and future perspectives for CRISPR technology in immunotherapy

One of the main concerns for the widespread use of CRISPR technology in adoptive immunotherapy is the CRISPR material's delivery vehicle. For example, viral vectors are usually employed to deliver gRNA and Cas9 to mammalian cells. There is a high chance of the immune response triggered by the delivery vehicle or the Cas9 protein. Viral vectors are sometimes known for their immunogenicity, and the Cas9 proteins (considering their microbial origin) could serve as a potential immunogen, thus limiting their use for gene therapy.[132] Although an increasing number of CRISPR-Cas9 enzymes have been discovered to date, it is interesting that only two class 2 enzymes (Cas9 and Cas12a) have gained popularity for their use in genome editing.

Also, it is necessary to carefully study and evaluate which of these variants is best suited to the workflow; for example, the different variants of the Cas9 enzymes have individual advantages and disadvantages that should be considered (Table 2). Further extensive research will discover more novel Cas protein variants alongside their unique functionality, which will open up further possibilities in genome engineering.

It is pertinent to develop a safe and efficient delivery system for the generally acceptable *in vivo* application of CRISPR-Cas9 because the insertion of mutagenesis could arise from the vector itself. Although the AAV-based vectors are currently the preferred mode of delivery on somatic cells, they can infect dividing and non-dividing cells, evoking a slight immune response.[133] One of the significant restrictions of the AAVs is their limited cargo capacity with restricted tissue tropism.

Other physical, non-viral methods (such as microinjection, electroporation) may be used to overcome these hurdles by introducing Cas9-encoding plasmids, Cas9-mRNA or a mixture of Cas9 protein and sgRNA directly into the immune cells and tissues of animals. For example, the use of electroporation to directly deliver CRISPR material to $CD4^+$ T cells, $CD34^+$ stem cells, cancer cells and embryonic stem cells has been shown.[134,135] Also, the direct delivery of Cas9–sgRNA ribonucleoproteins (RNPs) to the cell via a lipid complex or transfection may also be used. The RNP delivery system offers some advantages

Table 2. Variants of the Cas9 (type II) enzyme of the CRISPR system

Variant	Attributes	Reaction	Advantages
CRISPR-Cas9 WT	Cas9, sgRNA	Induces double-strand break at the target site	Highly versatile, stable, easy accessibility and effective
CRISPRa	dCas9, presence of activator peptide, sgRNA	Increase transcription	Has low toxicity
CRISPRi	dCas9, has a repressor peptide, sgRNA	Capable of blocking transcription elongation or knockdown of transcripts	Can be inducible, reversible, possesses low off-target effects
CRISPR-Cas9Nickase	Mutant Cas9 H840A or D10A, sgRNA	Induces a single-strand break	Convenient, highly robust, efficient, flexible, precise, can be scalable

compared to viral or non-viral approaches because it is delivered pre-assembled with a fast action when it complexes with target DNA. Its Cas9 nuclease also has a shorter duration, which may reduce off-target effects and increase its efficiency. Other delivery methods, such as hydrodynamic injections, have been highlighted. The introduction of Cas9 containing vectors through the tail vein of adult rodents for successful mutation and gene correction[23] shows other ways for the direct *in vivo* delivery of the CRISPR system for genetic manipulation.

However, the CRISPR-Cas9 system's off-target effects are still a major concern, particularly for CAR-T therapies. One smart way to protect normal tissues from tumor-specific T cells is by employing dual receptor circuits termed as the NOT and/or AND gates. In this approach, one CAR receptor targeted at tumor antigen and initialises the kill switch upon encounter with tumor cells can be engineered onto T cells. In this approach, one CAR receptor-targeted at tumor antigen that initiates the kill switch upon encounter with tumor cells and another inhibitory CAR molecule that expresses the inhibitory signal (such as CTLA-4 or PD-1) when in contact with antigens on normal tissue should be engineered onto T cells.[136] Similarly, another independent research has shown that it is possible upon the recognition of one antigen to drive the transcription of a CAR specific for a second antigen, allowing for a more-targeted CAR expression with accompanying reduced off-target toxicities.[137] For this approach, the CRISPR-Cas9 system can simultaneously express the 'NOT and/or AND gates' CAR receptor, particularly in overcoming antigens expressed on both normal and tumor tissues. Although this approach sounds exciting, there is a need for an extensive preclinical study to optimise CAR combination that fits well for maximum tumor impact.

The CRISPR genome-wide screen's concern is the conditional false-positive generated during the dropout screenings in cancers with aneuploidy. Also, the excessive DSBs – encountered in gene regions with multiple copy numbers, including those of non-expressed genes – can often result in DNA damage and ultimately apoptosis; therefore, excluding sgRNAs targeting non-expressed genes from the libraries will avert this. Lastly, since conventionally, sgRNAs are designed to target the 5' exon, false-negative results arising from initiation points of genes from other exons implying that the position of sgRNA is critical to the accuracy of the screening outcomes.[138]

Other concerns include the risks of neurological toxicity and cytokine release syndrome whenever CRISPR-Cas9 is used for any adoptive immunotherapy transfer (including CAR-T and CAR-NK). It is imperative to have clinicians who are well trained to manage any unintended adverse effects that may ensue. Another approach is to ensure a comprehensive and thorough study of the safety of these cell-based therapeutic, particularly at the preclinical level. This will allow the opportunity to evaluate the safety and efficacy of these cell-based therapies before human studies. It will also uncover unintended safety issues usually revealed in early-stage clinical trials.

The CRISPR system has revolutionised and championed novel ways of managing haematologic malignancies via CAR-T and CAR-NK. There remain many obstacles to broaden its application on solid tumors. The possibilities that can be achieved with the CRISPR system are endless. With the current advances made in immune cell gene editing, T and NK cell engineering, as well as optimised cell manufacturing protocols, have the potential to

broaden T and NK cell-based therapies to other cell types such as hematopoietic stem cells, induced pluripotent stem cells, including macrophages – which recently entered immunotherapy for treating solid tumors[139,140] – to foster the development of new cell-based therapies that are beyond oncology into other areas such as organ transplantation, infectious diseases and autoimmunity.

Overcoming challenges of immune suppression

To optimise immunotherapy regimes for complete tumor regression, the stimulation of robust antitumor response is required. However, overcoming the plethora of immunosuppressive mechanisms, particularly within the TME, remains a challenge. The use of CRISPR-Cas9 to develop highly effective tumor-infiltrating lymphocytes capable of penetrating the microenvironment and overcome the suppressive effects of immunosuppressive agents (such as cytokines and growth factors) synthesised by the tumor or stromal cells is desirable.

Transforming growth factor-beta (TGF-β) represents one of the pleiotropic immunosuppressive cytokines shown to inhibit T-cell proliferation, activation and differentiation[141]; similarly, its suppressive role on NK cells has also been well described.[142,143] In addition, its elevated serum level is often associated as a poor prognosis marker in several malignancies.[144] TGF-β has since been shown to exert immunosuppressive activity on cytotoxic lymphocytes by suppressing the expression of cytolytic products such as granzyme A and B, perforin, IFN-γ and FasL.

Therefore, approaches focused on using the CRISPR system to impair TGF-β signalling on immune effector cells will significantly enhance their antitumor capabilities.[145] Additionally, coadministration of anti-TGFβR2 monoclonal antibody together with small molecule drugs that disrupt TGF-β-mediated Smad 3 and 4 signalling is desirous.[146] By controlling the signalling axis of the various immune checkpoints with mAb or gene knockout using the CRISPR system, offers a vital strategy to overcome the immune-suppressive environment. Since Treg produces a high amount of TGF,[147,148] approaches such as endogenous knockout of TGF-β receptor II (TGFBR2) with CRISPR/Cas9 have been shown to significantly improve the efficacy of CAR-T cells and diminish the conversion of Treg;[149] hence, approaches that disrupt the suppressive effect of these regulatory cells including MDSCs will offer unprecedented success.

The presence of other cytokines, including IL-10, sialomucins and prostaglandin E2, which have been shown to protect tumor cells against T-cell cytotoxicity, should be investigated. Finally, knocking out diacylglycerol kinase (DGKζ) – an enzyme that converts diacylglycerol to phosphatidic acid – with CRISPR/Cas9 enhances CD3 signalling bolstering TCR signalling and T-cell functions.[150] Similarly, knockout of DGKζ has been shown to improve cytokine production, degranulation and effector function of NK cells.[151]

In addition to overcoming the immunosuppressive agents associated with the TME, the CRISPR/Cas9 system has also been used as a novel strategy to study the TME and device new treatment options in transgenic mice, offering the direct capability to induce specific genetic modifications in any working genetic background.[152] Therefore, employing the CRISPR system's multiplex advantages will offer the opportunity to create highly effective, next-generation T- and NK-cell CARs to improve immunotherapy.

The numerous immunosuppressive factors found at the tumor site must be overcome to successfully apply CAR-T and CAR-NK in solid tumors. Combination strategies such as immune checkpoint and CAR molecules have been reported to yield positive results in this regard.[153] Another approach is to incorporate additional transgenes so that CAR-T cells can secrete PD-1 blocking scFv or anti-PD-L1 antibodies at the tumor site simultaneously, enabling the full antitumor function of these tumor-infiltrating super CAR-T cells and other intratumoral T cells. In a similar vein, synthetic Notch 'synNotch' receptors have been implicated in driving both PD-1 and CTLA-4 inhibitors production.[154] Hence, the inclusion of fusion receptors such as interleukin (IL)-4–IL-7 chimeric cytokine receptors has the propensity to shift the inhibitory signals from IL-4 to IL-7 signalling – leading to proliferation and memory differentiation of T cells at the tumor site.[155] To achieve all the above-described innovative immunotherapy approaches, the CRISPR-Cas9 technology will be of immense benefit since its multiplex ability allows for the simultaneous knock-in and knockout of genes *in vitro* and *in vivo*. The future of personalised and highly sophisticated immune therapies may lie in fully exploiting this technology.

Besides identifying the mechanisms that regulate PD-L1 expression, other approaches contributing to immune evasion and acquired resistance to ICB, such as low MHC class I expression,[156,157] hold many potential. In a recent study, the genome-wide CRISPR screen was applied in K562 tumor cells (known for their low MHC-I expression) and cancer cell lines in which an evolutionarily conserved polycomb repressive complex 2 (PRC2) protein was identified and implicated in the transcriptional regulation of MHC-I antigen processing pathway (MHC-I APP), which highlights the tight epigenetic control of MHC-I expression in these tumor cells. This approach can explore the mechanisms that facilitate increased MHC-I levels for antigen presentation-licensing cytotoxic lymphocytes to kill tumor cells.[127] Other immune exhaustion markers such as CD39 and TOX, as well as those recently been identified (e.g. TIGIT, TIM-3, CTLA-4) and their respective ligands in tumors, can be screened to identify their regulation and how their expression pattern can be modulated to improve tumor-infiltrating lymphocytes activation in combination with ICB therapies.

Another major challenge for cancer immunotherapies is tumor relapse brought about by pre-existing heterogeneity or downregulation of target antigens reported in $CD19^{+}$ B-cell-derived malignancies such as acute lymphoblastic leukaemia.[158–160] To deal with this tumor escape arising from a single-antigen target, a pan-cancer antigen can be employed. It involves approaches such as the integration of multiple autonomous CARs using a single vector (e.g. bicistronic CAR),[161] coadministration of separately transduced CAR-T cells,[162] integration of two CARs to a single molecule (tandem CAR)[163] and co-transduction of multi-CAR vector on T cells are currently being tested.

Since T and NK cells are prone to exhaustion at tumor sites, switching their receptor extracellular domain using the CRISPR-Cas9 system can salvage this phenomenon. For example, fusing the extracellular PD-1 domain to an intracellular CD28 domain led to activated CAR-T being less susceptible to exhaustion with an enhanced *in vivo* antitumor activity.[164] CRISPR-Cas9 technology was also used to completely overcome the suppressive signalling from PD-1 through its deletion in CAR-T before its infusion.[165] Other CRISPR-Cas9 system-mediated clinical trial targeted towards melanoma, synovial sarcoma or MM is underway. TCR mispairing is also restricted by deleting endogenous TCR and PD-1 with a vector encoding the NY-ESO-1-specific HLA-A2.[166]

Other laudable approaches include using CAR-T cells capable of secreting cytokines such as IL-12,[167] or those with herpesvirus entry mediator,[168] and nanoparticles with adenosine receptor antagonists[169] or a IL-15 super-agonist[170] have all been shown to have potential to revolutionise the next-generation CAR molecules. Finally, synNotch receptors can deliver cytokines and bispecific antibodies to the tumors.[154] These innovative approaches offer the avenue to modulate the local TME while augmenting CAR-based therapies devoid of host systemic effects.

Although we are still far from harnessing the full potential of CRISPR-based technology, giant strides have been made in genomic research, gene editing and immune cell therapy. Many scientists can now manipulate biological samples (both *in vitro* and *in vivo*) to gain more insights, test hypotheses and answer fundamental scientific questions through the CRISPR technique. Clinicians are also expected to have more robust diagnostic and treatment options, as the much talked about personalised and precision medicine has been brought to the limelight through CRISPR-based technology. Since CRISPR-Cas9 has somewhat become the golden standard technology in genetic and biomolecular engineering, it is evident that unlocking the full capability of this technology for cancer research and therapy will improve lives.

ACKNOWLEDGMENTS

This work was sponsored by UCAS Scholarship for International Students.

CONFLICTS OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Lukman Olalekan Afolabi: Conceptualization; Writing-original draft; Writing-review & editing. **Mariam Olanrewaju Afolabi:** Conceptualization; Writing-original draft; Writing-review & editing. **Musbahu Muhammad Sani:** Conceptualization; Writing-original draft; Writing-review & editing. **Wahab Oluwanisola Okunowo:** Conceptualization; Writing-original draft; Writing-review & editing. **Liang Chen:** Validation; Writing-review & editing. **Dehong Yan:** Validation; Writing-review & editing. **Yaou Zhang:**

Supervision; Validation; Writing-review & editing. **Xiaochun Wan:** Funding acquisition; Supervision; Writing-review & editing.

FUNDING

This work was funded by the National Key R&D Program of China (2019YFA0906100), Key-Area Research and Development Program of Guangdong Province (2019B02 0201014).

REFERENCES

1. Cooper GM. The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. The Development and Causes of Cancer. *NCBI Bookshelf* 2000. Available from: https://www.ncbi.nlm.nih.gov/books/NBK9963/
2. Batool A, Malik F, Andrabi KI. Expansion of the CRISPR/Cas genome-sculpting toolbox: innovations, applications and challenges. *Mol Diagn Ther* 2021; **25**: 41–57.
3. Zhang B. CRISPR/Cas gene therapy. *J Cell Physiol* 2021; **236**: 2459–2481.
4. Naeem M, Majeed S, Hoque MZ *et al*. Latest developed strategies to minimize the off-target effects in CRISPR-Cas-mediated genome editing. *Cells* 2020; **9**: 1608.
5. Gruzdev A, Scott GJ, Hagler TB *et al*. CRISPR/Cas9-assisted genome editing in murine embryonic stem cells. *Methods Mol Biol* 2019; **1960**: 1–21.
6. Singh V, Gohil N, García RR *et al*. Recent advances of CRISPR-Cas9 genome editing technologies for biological and biomedical investigations. *J Cell Biochem* 2018; **119**: 81–94.
7. Zhang D, Hussain A, Manghwar H *et al*. Genome editing with the CRISPR-Cas system: an art, ethics and global regulatory perspective. *Plant Biotechnol J* 2020; **18**: 1651–1669.
8. Barrangou R, Fremaux C, Deveau H *et al*. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 2007; **315**: 1709–1712.
9. Lin J, Zhou Y, Liu J *et al*. Progress and application of CRISPR/Cas technology in biological and biomedical investigation. *J Cell Biochem* 2017; **118**: 3061–3071.
10. Xu Y, Li Z. CRISPR-Cas systems: Overview, innovations and applications in human disease research and gene therapy. *Comput Struct Biotechnol J* 2020; **18**: 2401–2415.
11. Bolotin A, Quinquis B, Sorokin A *et al*. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* 2005; **151**: 2551–2561.
12. Nuñez JK, Harrington LB, Doudna JA. Chemical and biophysical modulation of Cas9 for tunable genome engineering. *ACS Chem Biol* 2016; **11**: 681–688.
13. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 2014; **157**: 1262–1278.
14. Cao J, Wu L, Zhang S-M *et al*. An easy and efficient inducible CRISPR/Cas9 platform with improved specificity for multiple gene targeting. *Nucleic Acids Res* 2016; **44**: e149.
15. Jinek M, Chylinski K, Fonfara I *et al*. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012; **337**: 816–821.
16. Miyaoka Y, Berman JR, Cooper SB *et al*. Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing. *Sci Rep* 2016; **6**: 23549.
17. Ray U, Raghavan SC. Modulation of DNA double-strand break repair as a strategy to improve precise genome editing. *Oncogene* 2020; **39**: 6393–6405.
18. Gratz SJ, Ukken FP, Rubinstein CD *et al*. Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in Drosophila. *Genetics* 2014; **196**: 961–971.
19. McVey M, Lee SE. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends Genet* 2008; **24**: 529–538.
20. Sakuma T, Nakade S, Sakane Y *et al*. MMEJ-assisted gene knock-in using TALENs and CRISPR-Cas9 with the PITCh systems. *Nat Protoc* 2016; **11**: 118–133.
21. Gratz SJ, Cummings AM, Nguyen JN *et al*. Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. *Genetics* 2013; **194**: 1029–1035.
22. Wang H, Yang H, Shivalila CS *et al*. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 2013; **153**: 910–918.
23. Xue W, Chen S, Yin H *et al*. CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature* 2014; **514**: 380.
24. Platt RJ, Chen S, Zhou Y *et al*. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* 2014; **159**: 440–455.
25. Sachdeva M, Sachdeva N, Pal M *et al*. CRISPR/Cas9: molecular tool for gene therapy to target genome and epigenome in the treatment of lung cancer. *Cancer Gene Ther* 2015; **22**: 509.
26. Li J, Shou J, Guo Y *et al*. Efficient inversions and duplications of mammalian regulatory DNA elements and gene clusters by CRISPR/Cas9. *J Mol Cell Biol* 2015; **7**: 284–298.
27. Maddalo D, Manchado E, Concepcion CP *et al*. In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. *Nature* 2014; **516**: 423.
28. Liu Y, Zeng Y, Liu L *et al*. Synthesizing AND gate genetic circuits based on CRISPR-Cas9 for identification of bladder cancer cells. *Nat Commun* 2014; **5**: 5393.
29. Kasap C, Elemento O, Kapoor TM. DrugTargetSeqR: a genomics-and CRISPR-Cas9–based method to analyze drug targets. *Nat Chem Biol* 2014; **10**: 626.
30. Neggers JE, Vercruysse T, Jacquemyn M *et al*. Identifying drug-target selectivity of small-molecule CRM1/XPO1 inhibitors by CRISPR/Cas9 genome editing. *Chem Biol* 2015; **22**: 107–116.
31. Shi J, Wang E, Milazzo JP *et al*. Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. *Nat Biotechnol* 2015; **33**: 661–667.
32. Zhen S, Hua L, Takahashi Y *et al*. *In vitro* and *in vivo* growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR/Cas9. *Biochem Biophys Res Commun* 2014; **450**: 1422–1426.
33. Konermann S, Brigham MD, Trevino AE *et al*. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 2015; **517**: 583–588.

34. Gilbert LA, Larson MH, Morsut L *et al*. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 2013; **154**: 442–451.
35. Koike-Yusa H, Li Y, Tan EP *et al*. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol* 2014; **32**: 267–273.
36. Zhou Y, Zhu S, Cai C *et al*. High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. *Nature* 2014; **509**: 487–491.
37. Sun W, He B, Yang B *et al*. Genome-wide CRISPR screen reveals SGOL1 as a druggable target of sorafenib-treated hepatocellular carcinoma. *Lab Invest* 2018; **98**: 734–744.
38. Ouyang Q, Liu Y, Tan J *et al*. Loss of ZNF587B and SULF1 contributed to cisplatin resistance in ovarian cancer cell lines based on Genome-scale CRISPR/Cas9 screening. *Am J Cancer Res* 2019; **9**: 988–998.
39. Yang B, Kuang J, Wu C *et al*. Screening genes promoting exit from naive pluripotency based on genome-scale CRISPR-Cas9 knockout. *Stem Cells Int* 2020; **2020**: 8483035.
40. Du X, Shen X, Dai L *et al*. PSMD12 promotes breast cancer growth via inhibiting the expression of pro-apoptotic genes. *Biochem Biophys Res Commun* 2020; **526**: 368–374.
41. Zhou X, Li R, Jing R *et al*. Genome-wide CRISPR knockout screens identify ADAMTSL3 and PTEN genes as suppressors of HCC proliferation and metastasis, respectively. *J Cancer Res Clin Oncol* 2020; **146**: 1509–1521.
42. Tzelepis K, Koike-Yusa H, De Braekeleer E *et al*. A CRISPR dropout screen identifies genetic vulnerabilities and therapeutic targets in acute myeloid leukemia. *Cell Rep* 2016; **17**: 1193–1205.
43. Shi CX, Kortüm KM, Zhu YX *et al*. CRISPR genome-wide screening identifies dependence on the proteasome subunit PSMC6 for bortezomib sensitivity in multiple myeloma. *Mol Cancer Ther* 2017; **16**: 2862–2870.
44. Manguso RT, Pope HW, Zimmer MD *et al*. In vivo CRISPR screening identifies Ptpn2 as a cancer immunotherapy target. *Nature* 2017; **547**: 413–418.
45. Zhang JP, Song Z, Wang HB *et al*. A novel model of controlling PD-L1 expression in ALK^+ anaplastic large cell lymphoma revealed by CRISPR screening. *Blood* 2019; **134**: 171–185.
46. Liu J, Song T, Zhou W *et al*. A genome-scale CRISPR-Cas9 screening in myeloma cells identifies regulators of immunomodulatory drug sensitivity. *Leukemia* 2019; **33**: 171–180.
47. Doench JG. Am I ready for CRISPR? A user's guide to genetic screens. *Nat Rev Genet* 2018; **19**: 67–80.
48. Shifrut E, Carnevale J, Tobin V *et al*. Genome-wide CRISPR screens in primary human T cells reveal key regulators of immune function. *Cell* 2018; **175**: 1958–1971.
49. Singh N, Lee YG, Shestova O *et al*. Impaired death receptor signaling in leukemia causes antigen-independent resistance by inducing CAR T-cell dysfunction. *Cancer Discov* 2020; **10**: 552–567.
50. Dufva O, Koski J, Maliniemi P *et al*. Integrated drug profiling and CRISPR screening identify essential pathways for CAR T-cell cytotoxicity. *Blood* 2020; **135**: 597–609.
51. Wang D, Prager BC, Gimple RC *et al*. CRISPR Screening of CAR T Cells and Cancer Stem Cells Reveals Critical Dependencies for Cell-Based Therapies. *Cancer Discov* 2020; **11**: 1–20.
52. Roth TL, Li PJ, Blaeschke F *et al*. Pooled Knockin Targeting for Genome Engineering of Cellular Immunotherapies. *Cell* 2020; **181**: 728–744.
53. Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. *Science* 2013; **342**: 1432–1433.
54. Chevolet I, Speeckaert R, Schreuer M *et al*. Characterization of the in vivo immune network of IDO, tryptophan metabolism, PD-L1, and CTLA-4 in circulating immune cells in melanoma. *Oncoimmunology* 2015; **4**: e982382.
55. June CH, Sadelain M. Chimeric antigen receptor therapy. *N Engl J Med* 2018; **379**: 64–73.
56. Cho JH, Collins JJ, Wong WW. Universal chimeric antigen receptors for multiplexed and logical control of T cell responses. *Cell* 2018; **173**: 1426–1438.
57. Landgraf KE, Williams SR, Steiger D *et al*. convertible CARs: A chimeric antigen receptor system for flexible control of activity and antigen targeting. *Commun Biol* 2020; **3**: 1–13.
58. Hu Y, Tian Z-G, Zhang C. Chimeric antigen receptor (CAR)-transduced natural killer cells in tumor immunotherapy. *Acta Pharmacol Sin* 2018; **39**: 167–176.
59. Kochenderfer JN, Rosenberg SA. Treating B-cell cancer with T cells expressing anti-CD19 chimeric antigen receptors. *Nat Rev Clin Oncol* 2013; **10**: 267–276.
60. Liu X, Zhang Y, Cheng C *et al*. CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. *Cell Res* 2017; **27**: 154–157.
61. Torikai H, Reik A, Liu P-Q *et al*. A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood* 2012; **119**: 5697–5705.
62. Riolobos L, Hirata RK, Turtle CJ *et al*. HLA engineering of human pluripotent stem cells. *Mol Ther* 2013; **21**: 1232–1241.
63. Ren J, Liu X, Fang C *et al*. Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. *Clin Cancer Res* 2017; **23**: 2255–2266.
64. Eyquem J, Mansilla-Soto J, Giavridis T *et al*. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* 2017; **543**: 113–117.
65. Hubbard N, Hagin D, Sommer K *et al*. Targeted gene editing restores regulated CD40L function in X-linked hyper-IgM syndrome. *Blood* 2016; **127**: 2513–2522.
66. Lombardo A, Cesana D, Genovese P *et al*. Site-specific integration and tailoring of cassette design for sustainable gene transfer. *Nat Methods* 2011; **8**: 861–869.
67. Wang J, DeClercq J, Hayward S *et al*. Highly efficient homology-driven genome editing in human T cells by combining zinc-finger nuclease mRNA and AAV6 donor delivery. *Nucleic Acids Res* 2016; **44**: e30.
68. Afolabi LO, Adeshakin AO, Sani MM *et al*. Genetic reprogramming for NK cell cancer immunotherapy with CRISPR/Cas9. *Immunology* 2019; **158**: 63–69.
69. Legut M, Dolton G, Mian AA *et al*. CRISPR-mediated TCR replacement generates superior anticancer transgenic T cells. *Blood* 2018; **131**: 311–322.

70. Shao H, Zhang W, Hu Q *et al*. TCR mispairing in genetically modified T cells was detected by fluorescence resonance energy transfer. *Mol Biol Rep* 2010; **37**: 3951–3956.
71. van Loenen MM, de Boer R, Amir AL *et al*. Mixed T cell receptor dimers harbor potentially harmful neoreactivity. *Proc Natl Acad Sci USA* 2010; **107**: 10972–10977.
72. Liu X, Zhao Y. CRISPR/Cas9 genome editing: Fueling the revolution in cancer immunotherapy. *Curr Res Transl Med* 2018; **66**: 39–42.
73. First-in-human CRISPR trial. Nat Biotechnol, [updated 2016/08/09; cited 2021/01/10]. Available from: https://doi.org/10.1038/nbt0816-796a.
74. Stokic-Trtica V, Diefenbach A, Klose CSN. NK cell development in times of innate lymphoid cell diversity. *Front Immunol* 2020; **11**: 813.
75. Cózar B, Greppi M, Carpentier S *et al*. Tumor-infiltrating natural killer cells. *Cancer Discov* 2020; **11**: 34–44.
76. Bald T, Krummel MF, Smyth MJ *et al*. The NK cell–cancer cycle: advances and new challenges in NK cell–based immunotherapies. *Nat Immunol* 2020; **21**: 835–847.
77. Vivier E, Tomasello E, Baratin M *et al*. Functions of natural killer cells. *Nat Immunol* 2008; **9**: 503–510.
78. Vivier E, Ugolini S, Blaise D *et al*. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol* 2012; **12**: 239–252.
79. Fang F, Xiao W, Tian Z. NK cell-based immunotherapy for cancer. *Semin Immunol* 2017; **31**: 37–54.
80. Afolabi L, Bi J, Yang X *et al*. Suppression of the protein quality control system by TRIM30a sensitizes tumor to NK cell immune surveillance. *Eur J Immunol* 2019; **49**: 461–461.
81. Sivori S, Meazza R, Quintarelli C *et al*. NK Cell-Based Immunotherapy for Hematological Malignancies. *J Clin Med* 2019; **8**: 1702.
82. Imai C, Mihara K, Andreansky M *et al*. Chimeric receptors with 4–1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia* 2004; **18**: 676–684.
83. Liu D, Tian S, Zhang K *et al*. Chimeric antigen receptor (CAR)-modified natural killer cell-based immunotherapy and immunological synapse formation in cancer and HIV. *Protein Cell* 2017; **8**: 861–877.
84. Rezvani K, Rouce RH. The application of natural killer cell immunotherapy for the treatment of cancer. *Front Immunol* 2015; **6**: 578.
85. Hermanson DL, Kaufman DS. Utilizing chimeric antigen receptors to direct natural killer cell activity. *Front Immunol* 2015; **6**: 195.
86. Velasquez MP, Szoor A, Bonifant CL *et al*. Two-pronged cell therapy for B-cell malignancies: engineering NK cells to target CD22 and redirect bystander T cells to CD19. *Blood* 2016; **128**: 4560.
87. Mohammed S, Sukumaran S, Bajgain P *et al*. Improving chimeric antigen receptor-modified T cell function by reversing the immunosuppressive tumor microenvironment of pancreatic cancer. *Mol Ther* 2017; **25**: 249–258.
88. Li L, Gao Y, Srivastava R *et al*. Lentiviral delivery of combinatorial CAR/CRISPRi circuit into human primary T cells is enhanced by TBK1/IKKε complex inhibitor BX795. *J Transl Med* 2020; **18**: 1–12.
89. Bi J, Tian Z. NK cell exhaustion. *Front Immunol* 2017; **8**: 760.
90. Han J, Chu J, Keung Chan W *et al*. CAR-engineered NK cells targeting wild-type EGFR and EGFRvIII enhance killing of glioblastoma and patient-derived glioblastoma stem cells. *Sci Rep* 2015; **5**: 11483.
91. Kruschinski A, Moosmann A, Poschke I *et al*. Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. *Proc Natl Acad Sci USA* 2008; **105**: 17481–17486.
92. Chu J, Deng Y, Benson DM *et al*. CS1-specific chimeric antigen receptor (CAR)-engineered natural killer cells enhance *in vitro* and *in viv*o antitumor activity against human multiple myeloma. *Leukemia* 2014; **28**: 917–927.
93. Raulet DH, Gasser S, Gowen BG *et al*. Regulation of ligands for the NKG2D activating receptor. *Annu Rev Immunol* 2013; **31**: 413–441.
94. Zhang T, Lemoi BA, Sentman CL. Chimeric NK-receptor-bearing T cells mediate antitumor immunotherapy. *Blood* 2005; **106**: 1544–1551.
95. Chang YH, Connolly J, Shimasaki N *et al*. A chimeric receptor with NKG2D specificity enhances natural killer cell activation and killing of tumor cells. *Cancer Res* 2013; **73**: 1777–1786.
96. Müller N, Michen S, Tietze S *et al*. Engineering NK cells modified with an EGFRvIII-specific chimeric antigen receptor to overexpress CXCR4 improves immunotherapy of CXCL12/SDF-1α-secreting glioblastoma. *J Immunother* 2015; **38**: 197–210.
97. Töpfer K, Cartellieri M, Michen S *et al*. DAP12-based activating chimeric antigen receptor for NK cell tumor immunotherapy. *J Immunol* 2015; **194**: 3201–3212.
98. Liu E, Tong Y, Dotti G *et al*. Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity. *Leukemia* 2018; **32**: 520–531.
99. Levin AM, Bates DL, Ring AM *et al*. Exploiting a natural conformational switch to engineer an interleukin-2 'superkine'. *Nature* 2012; **484**: 529–533.
100. Lu Z, Bi J, Wan X. Artemisinin sensitizes tumor cells to NK cell-mediated cytolysis. *Biochem Biophys Res Commun* 2020; **524**: 418–423.
101. Afolabi LO, Bi J, Chen L *et al*. A natural product, Piperlongumine (PL), increases tumor cells sensitivity to NK cell killing. *Int Immunopharmacol* 2021; **96**: 107658.
102. Sekiba K, Yamagami M, Otsuka M *et al*. Transcriptional activation of the MICA gene with an engineered CRISPR-Cas9 system. *Biochem Biophys Res Commun* 2017; **486**: 521–525.
103. Villegas FR, Coca S, Villarrubia VG *et al*. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. *Lung Cancer* 2002; **35**: 23–28.
104. Peng LS, Zhang JY, Teng YS *et al*. Tumor-associated monocytes/macrophages impair NK-cell function via TGFβ1 in human gastric cancer. *Cancer Immunol Res* 2017; **5**: 248–256.
105. Jin S, Deng Y, Hao JW *et al*. NK cell phenotypic modulation in lung cancer environment. *PLoS One* 2014; **9**: e109976.

106. Kremer V, Ligtenberg MA, Zendehdel R *et al*. Genetic engineering of human NK cells to express CXCR2 improves migration to renal cell carcinoma. *J Immunother Cancer* 2017; **5**: 1–13.
107. Carlsten M, Levy E, Karambelkar A *et al*. Efficient mRNA-based genetic engineering of human NK cells with high-affinity CD16 and CCR7 augments rituximab-induced ADCC against lymphoma and targets NK cell migration toward the lymph node-associated chemokine CCL19. *Front Immunol* 2016; **7**: 105.
108. Levy ER, Carlsten M, Childs RW. mRNA transfection to improve NK cell homing to tumors. *Methods Mol Biol* 2016; **1441**: 231–240.
109. Putz EM, Mayfosh AJ, Kos K *et al*. NK cell heparanase controls tumor invasion and immune surveillance. *J Clin Invest* 2017; **127**: 2777–2788.
110. Lanier LL. NK cell recognition. *Annu Rev Immunol* 2005; **23**: 225–274.
111. André P, Denis C, Soulas C *et al*. Anti-NKG2A mAb is a checkpoint inhibitor that promotes anti-tumor immunity by unleashing both T and NK cells. *Cell* 2018; **175**: 1731–1743.
112. Zhang Q, Bi J, Zheng X *et al*. Blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent anti-tumor immunity. *Nat Immunol* 2018; **19**: 723–732.
113. Blake SJ, Stannard K, Liu J *et al*. Suppression of metastases using a new lymphocyte checkpoint target for cancer immunotherapy. *Cancer Discov* 2016; **6**: 446–459.
114. Narayanan S, Ahl PJ, Au VB *et al*. LAG3 is a Central Regulator of NK Cell Cytokine Production. bioRxiv 2020. https://doi.org/10.1101/2020.01.31.928200.
115. Cao Y, Wang X, Jin T *et al*. Immune checkpoint molecules in natural killer cells as potential targets for cancer immunotherapy. *Signal Transduct Target Ther* 2020; **5**: 1–19.
116. Brahmer JR, Lacchetti C, Schneider BJ *et al*. Management of immune-related adverse events in patients treated with immune checkpoint inhibitor therapy: American society of clinical oncology clinical practice guideline. *J Clin Oncol* 2018; **36**: 1714–1768.
117. Ratner L, Waldmann TA, Janakiram M *et al*. Rapid progression of adult T-cell leukemia-lymphoma after PD-1 inhibitor therapy. *N Engl J Med* 2018; **378**: 1947–1948.
118. Spain L, Diem S, Larkin J. Management of toxicities of immune checkpoint inhibitors. *Cancer Treat Rev* 2016; **44**: 51–60.
119. Daassi D, Mahoney KM, Freeman GJ. The importance of exosomal PDL1 in tumour immune evasion. *Nat Rev Immunol* 2020; **20**: 209–215.
120. Havel JJ, Chowell D, Chan TA. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nat Rev Cancer* 2019; **19**: 133–150.
121. Burr ML, Sparbier CE, Chan YC *et al*. CMTM6 maintains the expression of PD-L1 and regulates anti-tumour immunity. *Nature* 2017; **549**: 101–105.
122. Suresh S, Chen B, Zhu J *et al*. eIF5B drives integrated stress response-dependent translation of PD-L1 in lung cancer. *Nat Cancer* 2020; **1**: 533–545.
123. Palmer DC, Webber BR, Patel Y *et al*. Internal checkpoint regulates T cell neoantigen reactivity and susceptibility to PD1 blockade. bioRxiv 2020. https://doi.org/10.1101/2020.09.24.306571.
124. Potts MA, McDonald JA, Sutherland KD *et al*. Critical cancer vulnerabilities identified by unbiased CRISPR/Cas9 screens inform on efficient cancer Immunotherapy. *Eur J Immunol* 2020; **50**: 1871–1884.
125. Ishizuka JJ, Manguso RT, Cheruiyot CK *et al*. Loss of ADAR1 in tumours overcomes resistance to immune checkpoint blockade. *Nature* 2019; **565**: 43–48.
126. Deng H, Tan S, Gao X *et al*. Cdk5 knocking out mediated by CRISPR-Cas9 genome editing for PD-L1 attenuation and enhanced antitumor immunity. *Acta Pharm Sin B* 2020; **10**: 358–373.
127. Burr ML, Sparbier CE, Chan KL *et al*. An evolutionarily conserved function of polycomb silences the MHC class I antigen presentation pathway and enables immune evasion in cancer. *Cancer Cell* 2019; **36**: 385–401.
128. Peng D, Kryczek I, Nagarsheth N *et al*. Epigenetic silencing of TH1-type chemokines shapes tumour immunity and immunotherapy. *Nature* 2015; **527**: 249–253.
129. Adeegbe DO, Liu Y, Lizotte PH *et al*. Synergistic immunostimulatory effects and therapeutic benefit of combined histone deacetylase and bromodomain inhibition in non-small cell lung cancer. *Cancer Discov* 2017; **7**: 852–867.
130. Li X, Wang M, Xiang R. Clonal replacement of novel T cells: a new phenomenon in the tumor microenvironment following PD-1 blockade. *Signal Transduct Target Ther* 2019; **4**: 43.
131. Li XY, Moesta AK, Xiao C *et al*. Targeting CD39 in cancer reveals an extracellular ATP- and inflammasome-driven tumor immunity. *Cancer Discov* 2019; **9**: 1754–1773.
132. Bessis N, GarciaCozar F, Boissier M. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. *Gene Ther* 2004; **11**: S10–S17.
133. Dai W-J, Zhu L-Y, Yan Z-Y *et al*. CRISPR-Cas9 for in vivo gene therapy: promise and hurdles. *Mol Therapy Nucl Acids* 2016; **5**: e349.
134. Hou Z, Zhang Y, Propson NE *et al*. Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. *Proc Natl Acad Sci* 2013; **110**: 15644–15649.
135. Mandal PK, Ferreira LM, Collins R *et al*. Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. *Cell Stem Cell* 2014; **15**: 643–652.
136. Fedorov VD, Themeli M, Sadelain M. PD-1- and CTLA-4-based inhibitory chimeric antigen receptors (iCARs) divert off-target immunotherapy responses. *Sci Transl Med* 2013; **5**: 215ra172.
137. Roybal KT, Rupp LJ, Morsut L *et al*. Precision tumor recognition by T cells with combinatorial antigen-sensing circuits. *Cell* 2016; **164**: 770–779.
138. Munoz DM, Cassiani PJ, Li L *et al*. CRISPR screens provide a comprehensive assessment of cancer vulnerabilities but generate false-positive hits for highly amplified genomic regions. *Cancer Discov* 2016; **6**: 900–913.
139. Klichinsky M, Ruella M, Shestova O *et al*. Human chimeric antigen receptor macrophages for cancer immunotherapy. *Nat Biotechnol* 2020; **38**: 947–953.
140. Mukhopadhyay M. Macrophages enter CAR immunotherapy. *Nat Methods* 2020; **17**: 561.

141. Li MO, Wan YY, Sanjabi S *et al*. Transforming growth factor-β regulation of immune responses. *Annu Rev Immunol* 2006; **24**: 99–146.
142. Batlle E, Massagué J. Transforming growth factor-β signaling in immunity and cancer. *Immunity* 2019; **50**: 924–940.
143. Zaiatz-Bittencourt V, Finlay DK, Gardiner CM. Canonical TGF-β signaling pathway represses human NK cell metabolism. *J Immunol* 2018; **200**: 3934–3941.
144. Drake CG, Jaffee E, Pardoll DM. Mechanisms of immune evasion by tumors. *Adv Immunol* 2006; **90**: 51–81.
145. Thomas DA, Massagué J. TGF-β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* 2005; **8**: 369–380.
146. Kaklamani VG, Pasche B. Role of TGF-β in cancer and the potential for therapy and prevention. *Expert Rev Anticancer Ther* 2004; **4**: 649–661.
147. Rosenberg SA. IL-2: the first effective immunotherapy for human cancer. *J Immunol* 2014; **192**: 5451–5458.
148. Ghiringhelli F, Ménard C, Terme M *et al*. $CD4^+CD25^+$ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-β-dependent manner. *J Exp Med* 2005; **202**: 1075–1085.
149. Tang N, Cheng C, Zhang X *et al*. TGF-β inhibition via CRISPR promotes the long-term efficacy of CAR T cells against solid tumors. *JCI Insight* 2020; **5**: e133977.
150. Jung IY, Kim YY, Yu HS *et al*. CRISPR/Cas9-mediated knockout of DGK improves antitumor activities of human T cells. *Cancer Res* 2018; **78**: 4692–4703.
151. Yang E, Singh BK, Paustian AM *et al*. Diacylglycerol kinase ζ Is a target to enhance NK cell function. *J Immunol* 2016; **197**: 934–941.
152. Yamanaka Y. CRISPR/Cas9 genome editing as a strategy to study the tumor microenvironment in transgenic mice. *Methods Mol Biol* 2016; **1458**: 261–271.
153. Chong EA, Melenhorst JJ, Lacey SF *et al*. PD-1 blockade modulates chimeric antigen receptor (CAR)-modified T cells: refueling the CAR. *Blood* 2017; **129**: 1039–1041.
154. Roybal KT, Williams JZ, Morsut L *et al*. Engineering T cells with customized therapeutic response programs using synthetic notch receptors. *Cell* 2016; **167**: 419–432.
155. Leen AM, Sukumaran S, Watanabe N *et al*. Reversal of tumor immune inhibition using a chimeric cytokine receptor. *Mol Ther* 2014; **22**: 1211–1220.
156. Garrido F, Aptsiauri N, Doorduijn EM *et al*. The urgent need to recover MHC class I in cancers for effective immunotherapy. *Curr Opin Immunol* 2016; **39**: 44–51.
157. Lee JH, Shklovskaya E, Lim SY *et al*. Transcriptional downregulation of MHC class I and melanoma de-differentiation in resistance to PD-1 inhibition. *Nat Commun* 2020; **11**: 1897.
158. Maude SL, Laetsch TW, Buechner J *et al*. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med* 2018; **378**: 439–448.
159. Turtle CJ, Hanafi LA, Berger C *et al*. CD19 CAR-T cells of defined $CD4^+$:$CD8^+$ composition in adult B cell ALL patients. *J Clin Invest* 2016; **126**: 2123–2138.
160. Maude SL, Frey N, Shaw PA *et al*. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* 2014; **371**: 1507–1517.
161. Bielamowicz K, Fousek K, Byrd TT *et al*. Trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma. *Neuro Oncol* 2018; **20**: 506–518.
162. Huang L, Wang N, Li C *et al*. Sequential infusion of anti-CD22 and anti-CD19 chimeric antigen receptor T cells for adult patients with refractory/relapsed B-cell acute lymphoblastic leukemia. *Blood* 2017; **130**: 846.
163. Hegde M, Mukherjee M, Grada Z *et al*. Tandem CAR T cells targeting HER2 and IL13Rα2 mitigate tumor antigen escape. *J Clin Invest* 2016; **126**: 3036–3052.
164. Liu X, Ranganathan R, Jiang S *et al*. A chimeric switch-receptor targeting PD1 augments the efficacy of second-generation CAR T cells in advanced solid tumors. *Cancer Res* 2016; **76**: 1578–1590.
165. Rupp LJ, Schumann K, Roybal KT *et al*. CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. *Sci Rep* 2017; **7**: 737.
166. Stadtmauer EA, Fraietta JA, Davis MM *et al*. CRISPR-engineered T cells in patients with refractory cancer. *Science* 2020; **367**: eaba7365.
167. Pegram HJ, Lee JC, Hayman EG *et al*. Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning. *Blood* 2012; **119**: 4133–4141.
168. Boice M, Salloum D, Mourcin F *et al*. Loss of the HVEM tumor suppressor in lymphoma and restoration by modified CAR-T cells. *Cell* 2016; **167**: 405–418.
169. Siriwon N, Kim YJ, Siegler E *et al*. CAR-T cells surface-engineered with drug-encapsulated nanoparticles can ameliorate intratumoral T-cell hypofunction. *Cancer Immunol Res* 2018; **6**: 812–824.
170. Tang L, Zheng Y, Melo MB *et al*. Enhancing T cell therapy through TCR-signaling-responsive nanoparticle drug delivery. *Nat Biotechnol* 2018; **36**: 707–716.

Received: 5 May 2021 | Revised: 26 June 2021 | Accepted: 7 July 2021

DOI: 10.1002/bit.27882

REVIEW

BIOTECHNOLOGY AND BIOENGINEERING WILEY

Strengthening the CAR-T cell therapeutic application using CRISPR/Cas9 technology

Muhammad Sadeqi Nezhad[1] | Mahboubeh Yazdanifar[2] | Meghdad Abdollahpour-Alitappeh[3] | Arash Sattari[4] | Alexander Seifalian[5] | Nader Bagheri[6]

[1]Department of Clinical Laboratory Science, Young Researchers and Elites Club, Gorgan Branch, Islamic Azad University, Gorgan, Iran

[2]Stem Cell Transplantation and Regenerative Medicine, Department of Pediatrics, Stanford University School of Medicine, Palo Alto, CA, USA

[3]Cellular and Molecular Biology Research Center, Larestan University of Medical Sciences, Larestan, Iran

[4]Department of Clinical Laboratory Science, Gorgan Branch, Islamic Azad University, Gorgan, Iran

[5]Nanotechnology and Regenerative Medicine Commercialization Centre (Ltd), The London BioScience Innovation Centre, London, UK

[6]Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

Correspondence

Muhammad Sadeqi Nezhad, Department of Clinical Laboratory Science, Young Researchers and Elites Club, Gorgan Branch, Islamic Azad University, Gorgan 4919141558, Iran.
Email: labmsn9@gmail.com

Nader Bagheri, Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran.
Email: n.bagheri1985@gmail.com

Abstract

Adoptive cell immunotherapy with chimeric antigen receptor T (CAR-T) cell has brought a revolutionary means of treatment for aggressive diseases such as hematologic malignancies and solid tumors. Over the last decade, the United States Food and Drug Administration (FDA) approved five types of CAR-T cell therapies for hematologic malignancies, including Idecabtagene vicleucel (Abecma), Lisocabtagene maraleucel (Breyanzi), Brexucabtagene autoleucel (Tecartus), Tisagenlecleucel (Kymriah), and Axicabtagene ciloleucel (Yescarta). Despite outstanding results gained from different clinical trials, CAR-T cell therapy is not free from side effects and toxicities, and needs careful investigations and improvements. Gene-editing technology, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, has emerged as a promising tool to address some of the CAR-T therapy hurdles. Using CRISPR/Cas9 technology, CAR expression as well as other cellular pathways can be modified in various ways to enhance CAR-T cells antitumor function and persistence in immunosuppressive tumor microenvironment. CRISPR/Cas9 technology can also be used to decrease CAR-T cell toxicities and side effects. Hereby, we discussed the practical challenges and hurdles related to the accuracy, efficiency, efficacy, safety, and delivery of CRISPR/Cas9 technology to the genetically engineered-T cells. Combining of these two state-of-the-art technologies, CRISPR/Cas9 and CAR-T cells, the field of oncology has an extraordinary opportunity to enter a new era of immunotherapy, which offers novel therapeutic options for different types of tumors.

KEYWORDS

cancers, CAR-T cell, CRISPR/Cas9, immunotherapy, therapeutic

1 | INTRODUCTION

Immunotherapy, which uses the patient's immune system to target and kill cancer cells, has become a promising tool for cancer treatment (Koury et al., 2018). Adoptive T cell therapy is a type of immunotherapy involving the isolation and *in vitro* expansion of patient-derived T cells and reinfusion into the cancer patients. In this context, peripheral blood T cells are used to produce genetically modified-T cells expressing transgenic T cell receptor (TCR) and chimeric antigen receptor T (CAR-T) cells (Met et al., 2019; Morgan et al., 2010). CAR-T cell, a living drug, has been investigated for more than two decades. Cumulative research data have demonstrated the remarkable success of CAR-T cells in some

hematologic malignancies and solid tumors. In the beginning, CAR T-based therapy has been intensively used against hematologic malignancies, especially for patients with B-cell acute lymphoblastic leukemia (B-ALL) (Sadeqi Nezhad et al., 2020). Consequently, the United States Food and Drug Administration (FDA) approved five CAR-T products. (I) Idecabtagene vicleucel (Abecma) is an autologous B-cell maturation antigen (BCMA) CAR-T cell designed for patients with relapsed or refractory (R/R) myeloma (Munshi et al., 2021). (II) Lisocabtagene maraleucel (Breyanzi) is an autologous CD19 CAR-T cell specific for patients with R/R large B-cell lymphomas (Abramson et al., 2020). (III) Brexucabtagene autoleucel (Tecartus) is an autologous CD19 CAR-T cell designed for patients with R/R mantle-cell lymphoma (M. Wang et al., 2020). (IV) Tisagenlecleucel (Kymriah) is an autologous CD19 CAR-T cell specific for pediatric and young adult patients with CD19+ R/R B-cell ALL (Maude et al., 2018). (V) Axicabtagene ciloleucel (Yescarta) is an autologous CD19 CAR-T cell designed for patients with refractory large B-cell lymphoma (Neelapu et al., 2017). CAR-T cells have several limitations that stop them from performing successfully and efficiently. Despite the tremendous clinical success of CAR-T cell therapy in hematologic malignancies, there are multiple hurdles and barriers that restrict successful therapeutic outcomes. CAR-T cells were found to have a limited persistence, proliferation, and expansion in some individuals, especially patients with chronic lymphocytic leukemia (CLL) (Fraietta et al., 2018; Porter et al., 2015). Likewise, defects in intrinsic autologous T cells may prevent the success of CAR-T cells in patients (Fraietta et al., 2018). In some cancer types (e.g., ALL), the therapy may fail in patients diagnosed with rapid progressive disease who require an immediate treatment with CAR-T cells due to the long-time autologous CAR-T manufacturing process. Additionally, the adequate number of T cell collections from patients with hematologic malignancy is sometimes laborious and impracticable due to lymphopenia from recent or prior chemotherapy or underlying disease (Sadeqi Nezhad et al., 2021; Singh et al., 2016). Meanwhile, there are other problems associated with CAR-T cell therapy, including antigen escape, poor trafficking and tumor infiltration, low persistence, inhibition and resistance of T cells, and CAR-T associate clinical toxicities (Sterner & Sterner, 2021). Importantly, obstacles such as cost of treatment, gap between leukapheresis and manufacturing, and specific inclusion and exclusion criteria set by clinical trial restrict patients from getting the treatment (Xu et al., 2020). Furthermore, CAR-T therapy has also been used against solid tumors and showed promising therapeutic approaches; however, up to now FDA approved no CAR-T products for solid tumors. This signifies that the challenges in solid tumors are much more serious and require a thorough investigation. A major hurdle to the success of CAR-T cell therapy against solid tumors is tumor microenvironment and the lack of tumor-specific antigen (K. B. Long et al., 2018).

By the advent of genome editing technology, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, transcription activator-like effector nucleases (TALEN), and zinc finger nucleases (ZFNs), there is an opportunity to address many of these hindrances posed on CAR-T cell therapy (Berdien et al., 2014; Liu et al., 2017; J. Ren et al., 2017; Torikai et al., 2010). Genome editing refers to the delivery of an editing machinery system in cells of interest to modify their genome through either the replacement of faulty genes or insertion of new genes to treat diseases or boost the therapeutic outcomes (Gaj et al., 2016). CRISPR/Cas9 has surpassed the two other genome editing systems in the following ways: (a) CRISPR/Cas9 recognizes the DNA site through RNA–DNA interaction; (b) is easy designing; (c) results in higher specificity and efficiency; (d) provides an easy way to manipulate multiple target DNA, simultaneously (high-yield multiplexing); and (e) is a budget-friendly technology (H. Li et al., 2020).

In the following sections, we will provide an overview of the CRISPR/Cas9 technology, challenges and barriers posed on CAR-T cell therapy, and finally discuss methods by which the CRISPR/Cas9 system can potentially improve the success of CAR-T cell therapy.

1.1 | Overview of CRISPR/Cas9 technology: Mechanism of action as a genome editing platform

The immune system of many bacteria and almost all archaea harbor RNA-guided adaptive immune systems encoded by CRISPRs and CRISPR-associated (Cas) proteins to fight the invading bacteriophage or block the foreign plasmid transfer (Rath et al., 2015; Sorek et al., 2013). The short sequence of invading bacteriophage or plasmid DNA fragments stored in the CRISPR region is known as a protospacer sequence. The CRISPR RNA (crRNA) biogenesis process occurs upon the same entrance of pathogenic virus or plasmid in the future. The protospacer selected for transcription is based on protospacer adjacent motifs (PAMs) within the invading phage genomes and plasmids. These protospacer sequences, which serve as a genetic record of previously invaded viruses, are transcribed into a long precursor (pre-crRNA) and subsequently formed into mature crRNAs by endonucleolytic cleavage. Finally, the mature crRNA is combined with the Cas protein to generate a ribonucleoprotein complex structure that detects the target DNA by crRNA spacer, and degrades the viruses and plasmids (Garcia-Robledo et al., 2020; Ishino et al., 2018; Makarova et al., 2019).

There are different types of CRISPR systems. Among them, type II CRISPR locus recruits CRISPR-associated protein, Cas9, to produce double-stranded breaks (DSBs) in DNA of interest. Cas9 has a multitude of functions and is considered as a multifunctional protein. It possesses two distinct domains named HNH-nuclease and RuvC-like nuclease, which break the target DNA strand and the nontarget DNA strand, respectively (Makarova & Koonin, 2015; Wu et al., 2014). In genome engineering, the transactivating CRISPR RNA (tracrRNA) and crRNA are engineered as a single-guided RNA (sgRNA), which is a 17–20 nucleotide sequence corresponding to the target DNA. To further simplify, the CRISPR/Cas9 system is defined as sgRNA and Cas9 protein combination. sgRNA has a PAM sequence after the 3′-end of its sequence (5′-NGG-3′ for streptococcus pyogenes (Sp)-Cas9), which is essential for guiding the Cas9 protein to the target DNA where the complementary PAM sequence is present. Upon the interaction between the sgRNA and the target DNA, the Cas9 protein generates a DSB from three nucleotides upstream of the PAM sequence (Cao et al., 2018; Fonfara et al., 2014; Xiao-Jie et al., 2017).

Afterward, DSBs undergo two different mechanisms of repairs, homologous directed repair (HDR) and nonhomologous end joining (NHEJ). The former is used to knock-in a specific DNA which creates either an aberrant gene to develop a specific disease or repair a particular defective gene with homologous DNA. The latter is error-prone and a quick fix mechanism throughout the cell cycle (Allen et al., 2019; Jasin & Rothstein, 2013). This pathway requires no homologous sequence for ligation of DNA end and generates frameshift mutations through insertion and/or deletion (indel) mutations at the repair junctions. Once the DSB is induced, certain proteins, named Ku70 and Ku80, quickly bind to the DSB end and form the Ku heterodimer. The Ku complex forms a ring-shape to serve as a scaffold to recruit the NHEJ pathway molecules (Bischoff et al., 2020).

1.2 | Principle of CRISPR/Cas9 system gene delivery into T cells

The delivery of CRISPR/Cas9 to edit the genome of interest is defined into three distinct strategies. The first approach is to use plasmid DNA encoding the Cas9 protein and sgRNA from the same vector. The second format is to deliver the mixture of the Cas9 mRNA and the sgRNA. The last strategy is a ribonucleoprotein (RNP), the complex of Cas9 protein and sgRNA, which is considered advantageous compared with the two other systems (Luther et al., 2018). The RNP method has less off-target effects because it does not require the delivery of foreign DNA and the complex of Cas9-gRNA degrades over time. RNP-based delivery displays a fast, efficient and cost-effective method to modify the genome of the target. Another advantage of using RNP is the variety of methods that can be used to deliver the Cas9-gRNA complex, including electroporation (Gundry et al., 2016).

Although the first strategy of delivery, plasmid-based CRISPR-Cas9 system, is a simple and straightforward approach, it tends to cause off-target mutation in primary T cells (Kornete et al., 2018). The plasmid-based system encounters several challenges. Upon the entering of plasmid into the desired nucleus, it undergoes the transcription and translation processes to express the encoded proteins. These processes require more time to effectively target the gene of interest (Fujihara & Ikawa, 2014). More importantly, this format of delivery was found to result in an irreversible off-target cleavage site (Cradick, et al., 2013; Fu et al., 2013). The other negative aspect of the plasmid-based approach is its size limitation, as many current vectors have restrictions for large-sized genes. Moreover, transfection of plasmid DNA may activate the cyclic GMP-AMP synthase and, as a consequence, leads to host immunogenicity (Xu et al., 2019).

The second strategy is direct delivery of Cas9 mRNA and sgRNA into the target cells to form a Cas9/sgRNA complex inside the cells. One advantage of this approach is the use of mRNAs that can be translated in the cytoplasm, therefore requiring intracellular delivery which is much more convenient rather than delivery to the nucleus. Furthermore, the mRNA translation process reduces required time for genome editing. In addition, mRNA-based delivery demonstrated a low rate of off-target effects compared to the plasmid DNA strategy. However, this approach is limited because mRNA is fragile and may get degraded during the delivery or preparation process (Givens et al., 2018; Shen et al., 2014).

The last form of CRISPR/Cas9 delivery is RNP. This approach avoids the processes of transcription and translation, and provides the fastest means of gene editing compared to the two other methods (Schumann et al., 2015; Seki & Rutz, 2018). The delivery of RNP gives multiple advantages, including less off-target effects due to the fast degradation of Cas9 nuclease and no need for codon optimization and promoter selection (Hendel et al., 2015; Liang et al., 2015). RNP editing is very rapid, and indels can be measured after 3–24 hours. The Cas9 protein is rapidly degraded from cells within 24 hours, compared to the plasmid electroporation method that persists nearly 73 hours (Kim et al., 2014).

There are currently several nonviral nanovectors used for RNP delivery into the cells *in vitro*, including DNA nanoclews (the yarn-like DNA nanoparticles synthesized by rolling circle amplification), cationic lipid nanoparticles and lipoplexes (cationic liposomes, composed of nonviral [synthetic] lipid carriers of DNA), gold-based nanoparticles, and zeolitic imidazole frameworks (Wei et al., 2020; Xu et al., 2019).

The CRISPR/Cas9 system can be used either before the generation of CAR-T cells or after the production of CAR-T cells, as illustrated in Figure 1. Currently, the RNP delivery of CRISPR/Cas9 technology into the T cells represents as a promising approach compared to the other methods of delivery. T cells have been targeted by lentiviral and adenoviral vectors for delivery of CRISPR components. These deliveries seem to be ineffective due to low gene disruption efficiency, feeble site-specificity insert, and random disruption of unwanted genes (C. Li et al., 2015; W. Wang et al., 2014). The *in vivo* transfection of CRISPR/Cas9 model encounters different problems, including low disruption efficiency, insertional mutagenesis, off-target effects, toxicity and immunogenicity (Lino et al., 2018; Mout et al., 2017). These adverse effects will be explored later in the next section.

1.3 | The CRISPR/Cas9 system ability to generate off-the-shelf CAR-T cells

Lymphocytes used in genetically modified-T cell therapies are dominantly derived from patient's autologous T cells. This source of T cells has limitations including having insufficient number of T cells, time-consuming and laborious isolation process (Sharpe & Mount, 2015). These hurdles have brought the concept of universal or off-the-shelf T cells, where the allogeneic T cells derived from third party donors are genetically manipulated and can be used for different patients. The use of allogeneic T cells, as the main source of T cells in CAR-based therapy, is not simple. The infused allogeneic T cells expressing αβ TCR can recognize the recipient's cells as foreign tissues and destroy them, leading to a phenomenon known as graft versus host disease (GVHD) (Ju et al., 2005; Townsend et al., 2020).

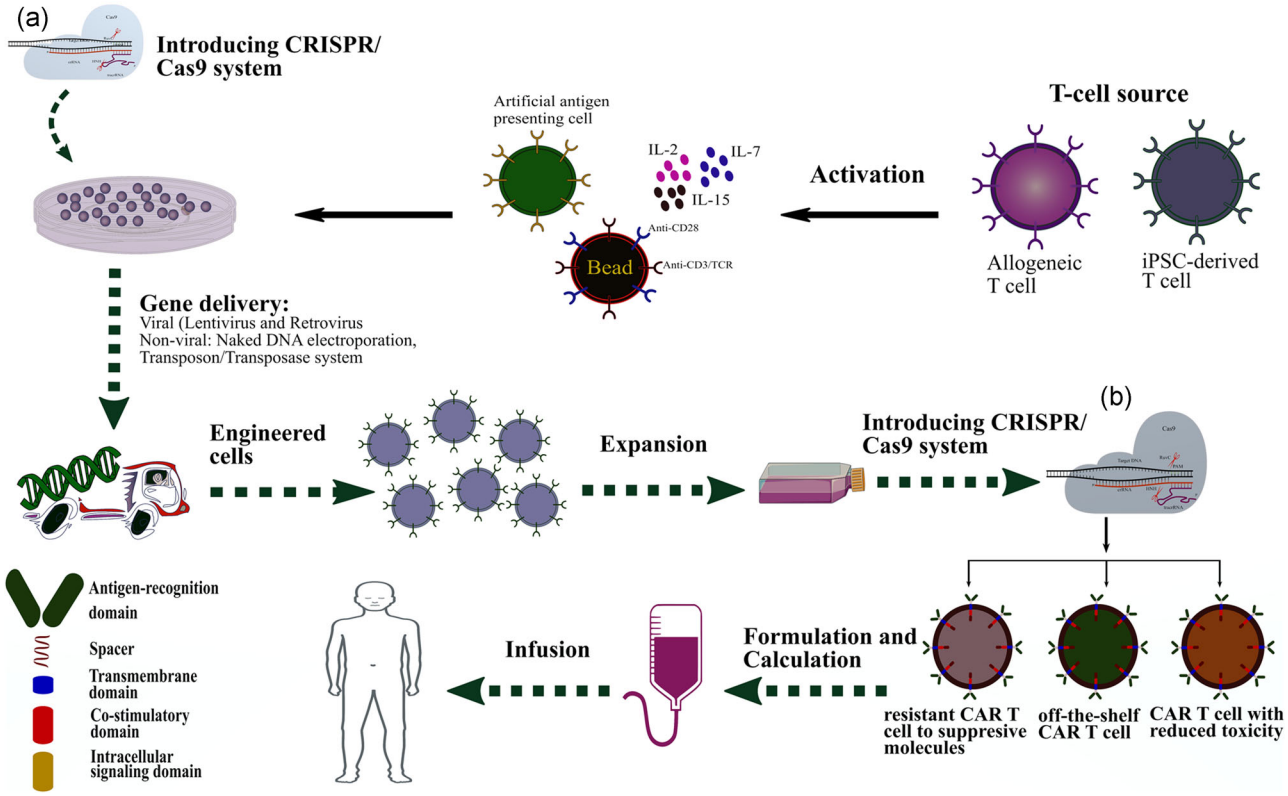

FIGURE 1 The general workflow for CRISPR Cas9-based CAR-T cell manufacturing. PBMCs obtain from the patient. Anticoagulants, red blood cells, and platelets contaminating the product would be removed in a washing step. Afterward, enrichment or depletion processes would be conducted for specific cell subsets. Next, T cells are activated by using different procedures, including monoclonal antibodies with interleukins (IL-2, IL-7, and IL-15), anti-CD3/CD28 antibody-coated magnetic beads, soluble CD3 antibody, artificial antigen-presenting cells (K562 cell lines), plate-bound antibody, and adhesion molecules (CD2). T cell activation pathways in cell culture media provide both the primary and costimulatory signals required for activation of the desired T cells. In this stage, the CAR transgene may be delivered into the activated T cells through different approaches, including viral (lentivirus and retrovirus) and nonviral (electroporation of naked DNA, mRNA, and transposon/transposase) methods, or CRISPR/Cas9 system may be applied first into the T cells to target the gene of interest. Subsequently, the activated T cells undergo an expansion process for a certain period (depending on the method of expansion, such as using static culture bags or dynamic culture vessels or rotating bioreactors). However, there are two options for CRISPR/Cas9 system introduction into the T cells. Option (a) is to deliver CRISPR/Cas9 system and then transfer the CAR transgene into the T cells; or (b) first develop CAR-T cells and then introduce the CRISPR/Cas9 system into the engineered-T cells. Nevertheless, there are different approaches to deliver CRISPR/Cas9: (I) transfection with DNA plasmid encoding both Cas9 protein and sgRNA, (II) the viral delivery using lentivirus and retrovirus, and nonintegrating viruses such as adenovirus and adenovirus-associated virus (AAV), (III) transfection with mRNA that encodes Cas9 or separate sgRNA, and (IV) CRISPR delivery via Cas9 protein with guide RNA (RNP complex). Finally, the prepared modified T-cells are calculated according to the patient's condition and type of cancer, then ready to introduce the engineered T cells to the patient through IV injection or intratumoral administration. CAR-T, chimeric antigen receptor T; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; IL, interleukin; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cell; RNP, ribonucleoprotein; sgRNA, single-guided RNA.

Several factors are found to promote the development of GVHD, including human leukocyte antigen class I (HLA-I) mismatched related donor or HLA matched unrelated donor. The most important factor is beta-2-microglobulin (β2M), a pivotal subunit of HLA-I protein that plays a key role in the removal of allogeneic cells expressing nonself HLA-I molecules in the recipient (Salas-Mckee et al., 2019; Torikai et al., 2013). Therefore, knocking out endogenous TCR and HLA (or β2M) as two crucial receptors of T cells may result in development of off-the-shelf CAR-T cells with no risk of GVHD.

Eyquem et al. disrupted T-cell receptor α constant (TRAC) locus through sgRNA targeting the 5'-end of the first exon of TCRα, and using adeno-associated virus (AAV) vector encoding the promoter-CAR-polyA cassette flanked by homology arms to knock in the CD19 CAR gene. Nearly 95% of transfused CAR^+ T cells were negative for TCR expression. NALM-6 mouse with pre-B acute lymphoblastic leukemia was introduced with 1×10^5 doses of CD19 TRAC-CAR-T cells, which successfully achieved tumor control, and just 2% of these cells expressed exhaustion or coinhibitory receptors such as PD1, LAG3, and TIM3, and maintained more effector memory phenotypes. The low expression of inhibitory receptors is mainly associated with greater *in vivo* antitumor activity and results in superior tumor eradication. These results underscore the pivotal role of TRAC in

regulation of CAR expression in two different ways. One is enhancing the optimal baseline expression, which participates in CAR internalization upon either the interaction with antigens or receiving signals. The other is the recovery of baseline CAR expression upon exposure to the antigen by controlling the transcriptional response. More importantly, targeting of the TCR locus may mitigate the probability of insertional oncogenesis and TCR-induced autoimmunity and alloreactivity, leading to safer modified-T cells and perpetual CAR expression. Hence, this study depicted that how by genome editing technology, a T cell-based therapy, can improve and yield a robust treatment (Eyquem et al., 2017).

Likewise, *Ren et al*. have knocked-out three different genomic loci, including TCR, β2M, and PD-1 simultaneously in human T cells via CRISPR/Cas9 system electroporation. They introduced the CAR transgene through lentiviral transduction and generated allogeneic CAR-T cells deprived of TCR, HLA-I, and PD-1, are known as universal CAR-T cells. The targeting efficiency of sgRNA yielded over 90%, and the disruption of HLA-I and TCR resulted in a low rapid rejection of CAR-T cells in allogeneic recipients. Importantly, it did not lead to GVHD in the *in vivo* model. The antitumor activity of CAR-T cells increased substantially by knocking out the PD-1 expression. One significant concern of this study is that triple loci-knocked-out CAR-T cells may trigger NK cell activation due to the absence of HLA-I in CAR-T cells. NK cell-specific antibody or NK cell depletion via chemotherapy may potentially avoid or mitigate NK-mediated rejection of transferred HLA-I negative CAR-T cells (Liu, et al., 2017).

Inconsistent with these data, *Georgiadis et al*. introduced human T cells with lentiviral vector encoding CD19 CAR and sgRNA targeting the TRAC region, and Cas9 mRNA was delivered by electroporation. A total of 5×10^5 of CD19-CAR TCR^- T-cells infused into the humanized murine model of Daudi B cell leukemia, resulting in significant clearance of tumors, with no GVHD and no evidence for overexpression of engineered-T cell exhaustion markers such as PD-1 (Georgiadis et al., 2018).

Similarly, CRISPR/Cas9 was used in CD19 CAR-T cells to ablate the constant TCR β-chain. CD19 CAR-T cells lacking TCR were highly functional and showed no alloreactivity in patient-derived xenografts of $CD19^+$ childhood ALL in a murine model (Stenger et al., 2020). These studies suggest that the CRISPR/Cas9 technology can be used as a promising tool for the development of off-the-shelf CAR-T cells by knocking out the TCR and β2M loci in allogeneic T cells, and further it can lead to remarkable GVHD reduction and alloreactivity by TCR disruption.

TCR and HLA knocked-out T cells present great potential for developing allogeneic third-party T cell products. However, there is controversy over benefits of TCR deletion. In a recent study, TCRβ knocked-out CD19 CAR-T cells have been compared to TCR intact CD19 CAR-T cells (Stenger et al., 2020). Although knocking out the endogenous TCR in CAR-T cells strongly eliminated alloreactivity compared to TCR-expressing CAR-T cells, coexpression of endogenous TCR plus CAR led to superior persistence of T cells and significantly extended the control of leukemia *in vivo*. This data highlights that despite the benefits of TCR knocked-out in developing off-the-shelf cell therapies, the presence of endogenous TCR might be better for long-term survival of T cells. Hence, a deeper understanding of T cell biology and TCR signaling provides useful insights for designing and engineering more effective CAR-T cells.

In conclusion, TCR^- CAR-T cells clearly control CD19 leukemia burden and improve survival. However, long-term *in vivo* persistence of TCR^- CAR-T cells seems to be lower than those expressing the endogenous TCR (Stenger et al., 2020). More studies are required to investigate the effect of TCR and CAR co-expression on T cell function, persistence, and antitumor response. Currently, there is no much evidence to shed light on this issue whether TCR knock-out leads to more robust activity or has negative consequence on T cells. Hence, if researchers preserve TCR on CAR-T cells, this CAR product cannot be used for third-party patients due to GVHD development. In addition, TCR removal appears to reduce the long-term persistence of T cells, which prevents CAR-T cells from long-term response *in vivo*. Novel strategies are needed to address the remaining challenges. One salient approach is that T cells express an inhibitory form of the TCR. The truncated CD3ζ known as TCR inhibitory molecule (TIM) prevents TCR mediated cytotoxic activity against the host (Gilham et al., 2018). The other approach would be to make a defect in TCR structure by knocking out a portion of TCR subunit or to target signaling pathways responsible for TCR engagement.

1.4 | Development of CAR-T cells that are resistant to suppressive molecules

The expression of inhibitory receptors such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte activation gene 3, and programmed cell death protein-1 (PD-1) on T cells controls and restricts T cell activities and responses (L. Long et al., 2018). These inhibitory molecules mitigate immune responses and cause exhaustion of T cells. The exhausted T cells alter the transcriptional program that distinguishes them from memory and prototypic effector T cell populations (Wherry & Kurachi, 2015). In addition, Fas receptor (CD95), a cell surface protein that belongs to the tumor necrosis factor α family of death receptors, contributes to the regulation of T cell activity. Interaction of the Fas molecule with its ligand (FasL) induces the T cell apoptosis cascades which may reduce the engineered T-cell response through induction of the activation-induced cell death (J. Ren et al., 2017).

Tumors can suppress immune responses and escape from immune cells by expressing negative regulatory pathways, known as immune checkpoints. One important key player of the immune checkpoints is PD-1, a type I transmembrane receptor inhibiting T cell proliferation and performance (Seliger, 2019). PD-1 is normally expressed on the surface of activated T cells, and its interaction with cognate ligands, PDL1 and PDL2, limits T cell activity and inhibits excessive stimulation, which leads to an immune escape for tumor cells (Zak et al., 2015). PD-1 expression by CAR-T cells has the same

deteriorating effect; therefore, disrupting PD-1 can boost T cell antitumor responses (Table 1).

The first CRISPR-edited T cell clinical trial was performed to test the safety and feasibility in patients with advanced and refractory cancer. Endogenous TCR subunit α and β and immune check point PD-1 were removed from T cells and transduced with synthetic TCR transgene. The highest editing efficiency was observed for TCR α and PD-1, and the lowest efficiency was seen for TCR β which indicates that this locus has a low response rate to genome editing and higher off-target mutations. In this study, CRISPR-edited T cells demonstrated a low level of clinical toxicities and persisted for approximately 9 months. However, during manufacturing process, chromosomal translocations were detected and decreased over time after infusion. Although this study provided evidence for feasibility and applicability of CRISPR-edited T cells for clinical purposes, concerns of off-target alterations and immunogenicity of Cas9 and unexpected large deletions and complex rearrangements into edited cells are required to be investigated meticulously with long-term follow-up (Stadtmauer et al., 2020).

Rupp et al. have transfected human T cells with Cas9 and sgRNA targeting PD-1 exon 1 through electroporation and subsequent introduction of lentiviral vector containing the CD19 CAR transgene. $CD19^+$ $PD\text{-}L1^+$ tumor xenograft models were injected with 4×10^6 PD-1-deficient CD19 CAR-T cells, resulting in clearance of tumors in all treated mice. This finding highlights the suppressive role of the PD-1/PD-L1 axis on CAR-T cells upon the engagement with the antigen of interest on tumor cells. Further, the study revealed the applicability of CRISPR-Cas9 genome editing as a viable tool for the enhancement of CAR-T cell performance (Rupp et al., 2017).

In contrast to the previous study where T cells were transfected at least twice using a combination of electroporation and lentiviral transduction, a new study transfected T cells using plasmids encoding Cas9, PD-1 targeting sgRNA, and the piggyBac transposon vector encoding CD133-CAR in one reaction via nucleofection process. This method led to 89.5%–95% insertions and deletions in PD-1 gene site. A total of 2×10^6 doses of PD-1-deficient CD133 CAR-T cells were infused to the orthotopic glioma xenografts in immunodeficient mice and led to outstanding outcomes. The modified T-cells demonstrated persistence and the survival of mice was enhanced. Besides, no sign of GVHD and CAR-T related side effects and aberrant proliferation of PD-1-deficient modified T-cells were detected due to the rapid elimination of these modified-T cells within 28 days, highlighting the role of PD-1 in the survival of CAR-T cells. Importantly, this study highlighted the use of plasmid DNA as a more efficient approach due to the low cost and easier preparation compared to the RNA, protein and virus delivery methods (B. Hu et al., 2019). Other studies are also consistent with these findings (W. Hu et al., 2019; Nakazawa et al., 2020).

More encouragingly, *Choi et al*. exerted the CRISPR-Cas9 system application against EGFRVIII CAR-T cells in which three different loci including PD-1, β2M, and TRAC regions were targeted to generate universal CAR modified-T cells resistant to PD-1 suppression. T cells were electroporated with the CRISPR/Cas9 complex targeting TRAC and β2M and Pdcd1 loci, and subsequently transduced with AAV encoding the EGFRVIII CAR. More than 80% of the T cell population was double knocked-out for surface expression of TCR and HLA-I. A total of 5×10^3 triple gene-deficient EGFRvIII CAR-T cells were administered through intravenous delivery or intraventricular infusion in murine models of human GBM. The former route of delivery did not significantly increase the survival rate of mice, while the latter means of infusion showed to be more efficacious against the GBM mice model. The triple gene-deficient EGFRvIII CAR-T cells also depicted highly antitumor response in preclinical glioma models (Choi et al., 2019).

Furthermore, the efficacy of CAR-T cell therapy towards solid tumors has been severely restricted by some physical and physiological barriers, such as tumor microenvironment (TME) hypoxia, acidic environment, nutritional deficiency, and the presence of immunosuppressive cells (regulatory T cells, myeloid-derived suppressor cells, tumor-associated macrophages, and neutrophils) (Yazdanifar et al., 2016). Solid tumors create a complex zone containing many cell types, tumor's vasculature, extracellular matrix components, connective tissues, and inflammatory mediators, which can impair T cell infiltration and function (Joyce & Fearon, 2015; Turley et al., 2015).

Other factors that hamper the efficacy of CAR-T cells include suppressive molecules (Indoleamine 2,3-dioxygenase(IDO), transforming growth factor-beta (TGF-b), PDL1, interleukin (IL)-10 and arginase-1), immunosuppressive inhibitor receptors, and soluble factors (prostaglandin E2 and indoleamine-2,3-dioxygenase) (Anderson et al., 2017; J. H. Chen et al., 2015; Koyama et al., 2016). To overcome the hostile TME, recently, a group of researchers has been successfully disrupted the endogenous TGF-ß receptor II (TGFBR2) gene in modified T-cells expressing mesothelin CAR, using the CRISPR/Cas9 technology. The modified-T cells diminished the induced Treg conversion and restrained the exhaustion. TGF-ß knockout mesothelin CAR-T cells completely eradicated the tumor cells by day 28 in pancreatic carcinoma patient-derived xenograft models expressing mesothelin and TGF-ß1 receptors. This study further shed light on the negative regulatory role of TGF-ß receptors in CAR-T cell cytotoxicity responses. It also highlighted that disruption of the TGFBR2 gene would enable modified-T cells to survive, proliferate effectively, and exert higher antitumor activity. Encouragingly, it was shown that knocking out of other immune checkpoints such as PD-1 simultaneously with TGF-ß1 may lead to a better therapeutic outcome in CAR-T cell therapy (Tang et al., 2020).

Diacylglycerol kinases (DGKs) are enzymes that phosphorylate diacylglycerol (DAG) signaling to encourage phosphatidic acid (PA) production. Both DAG and PA are bioactive molecules that regulate a multitude of intracellular signaling proteins involved in innate and adaptive immunity (S. S. Chen et al., 2016). Upon the interaction between TCR on T cells and antigen presenting cells, a cascade of signaling initiates by the activation of phospholipase Cγ1 (PLCγ1), which cleaves phosphatidylinositol-4,5-biphosphate (PIP_2) to form the second messengers DAG and inositol triphosphate (IP_3) (Baldanzi et al., 2016). DAG plays an important role in the activation of different downstream signaling pathways, such as AKT, NF-kB, and Ras pathways. The vital role of DGKs is to control DAG metabolism in T cells. Two DGK isoforms, DGKα and DGKζ, control DAG signaling in T cells.

TABLE 1 Application of the CRISPR/Cas9 system in CAR-T cell performances

Preclinical study	CRISPR target regions	CRISPR delivery method	CAR T product	CAR T target-antigen	CAR T delivery method	Overview	Year
Xenograft model of childhood ALL	TCR-β-chain	RNP Electroporation	TCR$^-$ CAR-T cells	CD19	Retroviral	TCR knockout CAR-T cells are a promising option for third party patients and showed no alloreactivity, despite their low persistency.	2020 Stenger et al. (2020)
Humanized murine model of glioma	TRAC, β2M, and PD-1	RNP Electroporation	Universal CAR-T cells resistant to PD-L1	EGFRvIII	AAV6 vector	CRISPR/Cas9 successfully generated universal CAR-T cells and enables them to resist against PD-L1. This CAR-T cells showed prolong survival and antitumor activity in mice.	2019 Choi et al. (2019)
Humanized murine model of leukemia	TRAC	Cas9 mRNA Electroporation and gRNA lentiviral delivery	TCR$^-$ CAR-T cells	CD19	Lentiviral coupling CAR and gRNA	Separated delivery of CRISPR components may bring several advantages, including time-saving and low immunogenicity. TCR$^-$ CAR-T cells mediated highly effective leukemic eradication with less evidence of exhaustion	2018 Georgiadis et al. (2018)
Orthotopic mouse glioma model	PD-1	Plasmid	PD1-deficient CD133 CAR-T cells	CD133	PiggyBac transposon system	PD1-deficient CD133 CAR-T cells showed enhanced cytotoxicity and proliferation but with appropriate cytokine secretion level. PD-1 destruction is associated with higher antitumor responses.	2018 B. Hu et al. (2019)
Mice with pre-B acute lymphoblastic leukemia	TRAC	Cas9 mRNA and gRNA Electroporation	TCR$^-$ CAR-T cells	CD19	AAV6 vector	Knocking out of TRAC locus led to CAR-T cells potency and delayed effector CAR T differentiation and exhaustion.	2017 Eyquem et al. (2017)
Xenograft tumor model of leukemia	PD-1	RNP Electroporation	PD1-deficient CD19 CAR-T cells	CD19	Lentiviral	This study shed light on PD-1 inhibitory roles in CAR-T cells. PD1-deficient CD19 CAR-T cells successfully eliminated tumor cells *in vivo*.	2017 Rupp et al. (2017)
Mice with B cell precursor leukemia (Nalm6 or Nalm6-PDL1 tumor cells)	TCR, β2M, and PD1	Cas9 and gRNA Electroporation	Universal CAR-T cells resistant to PD-L1	PSCA and CD19	Lentiviral	TCR and β2M knockout CAR-T cells can be considered as universal CAR-T cells. And PD1 knockout led to enhanced *in vivo* antitumor activity.	2016 Jiangtao Ren, Xiaojun Liu et al. (2017)

Abbreviations: β2M, β-2 microglobulin; AAV, adeno-associated virus; ALL, acute lymphoblastic leukemia; CART, chimeric antigen receptor T; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; PD-1, programmed cell death protein 1; TCR, T cell receptor; TRAC, T-cell receptor α constant.

DAG engages with pivotal proteins present in the CD3 signaling such as Ras activating protein (RasGRP1) and protein kinase C (PKC); thus, activation of DGK leads to downregulation of TCR distal molecules via metabolizing DAG at immune synapses (Riese et al., 2016). Accordingly, DGKs control T cell polarization and function during migration and activation, anergy, and response to tumor cells. The production of CARs with an appropriate signaling mechanism is an essential issue to boost the cellular activation, persistence, cytokine secretion, and cytotoxicity of CAR-T cells. Since DGKs participate in T cell signaling, a group of researchers used the CRISPR/Cas9 genome editing technology to disrupt DGK in CAR-T cells. Findings revealed that DGK-deficient CAR-T cells were significantly more resistant to soluble immunosuppressive components such as prostaglandin E2 and TGF-β in an *in vivo* model. DGK-deficient CAR-T cells increased effector functions *in vitro* and robust TCR signaling. This finding clearly suggested that DGKs can be considered as a potential therapeutic component to tackle suppressive factors present in solid tumors which prevent CAR-T cells activities (Jung et al., 2018).

The other major mediator of immunosuppression within the tumor microenvironment is adenosine, which restricts T cell antitumor activity through activation of the adenosine A2a receptor (A2aR). A2aR signaling suppresses innate and adaptive immune responses and its blockage in T cells seems to be beneficial (Cekic & Linden, 2014). Thus, the CRISPR/Cas9 system targeting A2aR was designed for CAR-T cells to evaluate the potential therapeutic activity of A2aR. Findings corroborated that A2aR knock-out CAR-T cells were significantly resistant to adenosine-mediated transcriptional changes, leading to increased secretion of interferon γ (INFγ) and tumor necrosis factor (TNF) and elevated expression of JAK-STAT signaling pathway (Giuffrida et al., 2021). However, the concerns of permanent A2aR deletion and its continuous immune responses need to be considered from the safety aspects.

Moreover, Casitas B-lineage lymphoma proto-oncogene-b (Cbl-b) regulates several signaling pathways and has a direct impact on T cell activation and tolerance in a negative way. Cbl-b is tightly expressed by lymphocytes such as $CD8^+$ and $CD4^+$ T cells and its expression status is controlled by CD28 and CTLA-4 stimulation and other inhibitory signals (Lutz-Nicoladoni et al., 2015). Cbl-b is believed to be associated with exhaustion of T cells; therefore, Cbl-b was knocked-out and its deficiency in T cells was studied. Interestingly, Cbl-b deletion suppressed CAR-T cell exhaustion and restored their effector function (Kumar et al., 2021). Despite the remarkable results obtained from Cbl-b deletion in T cells, more investigations are required to shed light on the mechanism of how Cbl-b deficiency leads to reduced levels of T cell exhaustion.

In conclusion, the CRISPR/Cas9 system is an essential tool for the development of high-performing CAR-T cells. Identification of negative regulatory molecules or proteins in T cells, which prevent them from successful therapeutic outcomes, are key factors for efficient development of CRISPR/Cas9. This system addressed significant challenges in the CAR-T field, including T cell exhaustion, persistence, survival, and toxicities. However, there are still many unanswered questions concerning the efficacy and safety of using CRISPR/Cas9 in immunotherapy and clinical use. Currently, the results obtained from preclinical studies are encouraging and pave the way for future clinical studies.

1.5 | The potential of CRISPR/Cas9 technology to reduce CAR-T associated toxicities

Although CAR-T cell therapy was demonstrated as a promising therapeutic option for different cancers, this novel treatment is not exempt from adverse events, and needs thorough consideration to tackle its limitations. One potential adverse event is on-target off-tumor effects, where CAR-T cells target the healthy tissues sharing the same epitope of antigens (Jung & Lee, 2018). The second adverse event is cytokine release syndrome (CRS), which mostly occurs by proinflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), TNF-α, IL-6, IFN-γ, and IL-2 (Maude et al., 2018; Neelapu et al., 2017; Park et al., 2018). One eminent strategy to tackle these obstacles is the use of the CRISPR/Cas9 gene-editing technology.

Sterner et al. used the CRISPR/Cas9 system armed with sgRNA targeting GM-CSF in CD19 CAR-T cells to control the CAR-T associated toxicities. These GM-CSF-deficient CD19 CAR-T cells reduced the GM-CSF secretion and enhanced the antitumor effect with low side effects of CRS and neurotoxicities in a leukemia NALM6 xenograft model (Sterner et al., 2019). This study clearly proposed that GM-CSF is associated with CRS; thus, future studies need to consider this gene as a potential target to reduce CAR-T related side effects.

CRISPR/Cas9 can be used to generate safer and more controllable CAR-T cells by adding inducible safety switches or suicide genes, which provide a tool for eliminating CAR-T cells in case of potential toxicities. An inducible Cas9-based suicide gene was incorporated in IL-15-expressing CD19 CAR-T cells by *Hoyos et al*. and their results confirmed that >95% of the CAR-T cells could be efficiently ablated within 24 hours upon pharmacologic activation of the suicide gene (Hoyos et al., 2010). Currently, there are three clinical trials (NCT02107963, NCT01822652, and NCT02439788) incorporating the Cas9-based suicide gene into CAR-T cell products to provide a means for eliminating the autologous CAR-T cells in case of unexpected off-target toxicity. Insertion of safety switches in the CAR construct is another approach to terminate the adverse effects without jeopardizing clinical responses. Inducible Cas9-based safety switch was tested in a CD19 CAR-T cells and results confirmed its feasibility for eliminating CAR-T cells in a dose-dependent manner in a humanized mouse model (Diaconu et al., 2017). This approach allows for both selective suppression of CAR-T cell activation in a case of CRS, and also complete depletion on demand.

As discussed here, many limitations of conventional CAR-T cells can be addressed using CRISPR/Cas9. However, there are concerns surrounding the safety of using these gene-edited cells in clinic and careful investigation must be applied. Several factors such as off-target effects, unintended mutations, and unwanted Cas9 activity can affect the safety of the CRISPR/Cas9 system (Kim et al., 2018). Besides, CRISPR/Cas9 might alter the function of gene-edited CAR-T

cells which could lead to the activation of unintended innate/adaptive immune responses (Kim et al., 2018). Although these events are rare, they can cause adverse effects in patients and need to be addressed in gene-edited T cells before clinical use.

1.6 | CRISPR/Cas9 in search of details in CAR-T cells: A new outlook

Since one of the key components of CAR-T based therapy is the type of T cells used in this therapy, choosing the qualified T cell population is an essential step in having effective CAR-T cells. Factors influencing the quality of T cells included but not limited to the mitochondria, genome, and other cellular components. These factors are in close association with persistence, expansion, homing, differentiation, and antitumor response of T cells that may alter the clinical outcome of CAR-T cell therapy (Besser et al., 2013; Fraietta et al., 2018; Goff et al., 2016; Rosenberg et al., 2011).

A novel approach was devised using CRISPR/Cas9-based genetic screening to identify molecular targets responsible for optimizing the overall antitumor qualities of T cells. Findings revealed that Mapk14 (p38) plays a central regulator of cell expansion, differentiation, oxidative, and genomic stresses of T cells. Several advantages were observed when p38 activity was inhibited. First, p38 inhibition leads to robust cellular expansion of T cells with limited oxidative and genomic stress and terminal differentiation. Second, p38 blockage can result in the generation of $CD62L^{+}$ $CD27^{high}$ T cells, decrease levels of reactive oxygen species, and increase cytokine secretion without mitigating cell expansion or TCR clonality. Third, inhibition of p38 significantly improves the T cell expansion process, and regulates T cell antioxidant metabolism. Finally, p38 inhibition improves the antitumor activities of T cells, a useful approach for CAR-T cell therapy (Gurusamy et al., 2020). These findings open a new avenue for immunotherapy and may solve the long-term challenges of CAR-T cell therapy. Inhibition of p38 can remarkably address the problems associated with low antitumor response rate, low level of cytokine productions, exhaustion, phenotype alterations, and expansion of T cells in clinical settings. Thus, exerting this approach for future CAR-T development would remarkably boost the therapeutic success of CAR-T cells in different types of cancer.

In addition, the CRISPR/Cas9 genome editing technology attempts to address challenges related to the safety of CAR-T cells concerning the on-target/off-tumor response (an antitumor response towards antigens expressed on healthy cells). In this case, the CRISPR/Cas9 system identified a bridge between receptor affinity and signaling, which enables CAR-T cells to be only activated upon tumor cells expressing high antigen levels and spare healthy cells. This novel method does not require to use low affinity antibodies for CAR-T cell construction to reduce on-target/off-tumor events. Instead, the CRISPR/Cas9 system and deep mutational scanning were used to generate a library of antigen-binding domain variants. This library was subjected to multiple rounds of selection based on either antigen binding or cell signaling. Hence, a green fluorescent protein reporter gene was integrated downstream of the endogenous IL-2 gene, serving as a reporter of CAR-T cell activation for high-throughput screening. This approach allows researchers to find suitable binding affinity of CAR-T cells through tuning the affinity of a CAR scFv domain using Cas9-mediated HDR, which enables them to generate a CAR-T cell sensitive to a particular antigen affinity (Di Roberto et al., 2020).

The CRISPR/Cas9 technology can also indirectly assist in developing more effective CAR-T cell therapies. Using large scale CRISPR screening libraries, we can discover novel antigens to be targeted by CAR-T cells. We can identify factors in T cells, tumor cells or TME which induce resistance to CAR-T cells. By genome-wide genetic perturbation/CRISPR screen, *Dufva et al*. investigated genes which their loss in cancer cells impaired the effector function of CAR-T cells and revealed the essentiality of death receptor signaling for CAR-T cell cytotoxicity. Similar studies provided a better understanding of mechanisms influencing CAR-T cell function and the potential for modulation using combination therapy or genetic engineering strategies (Dufva et al., 2020).

Lastly, annals of adoptive cell therapy revealed that scientists predominantly used genome editing technology to knock-out a specific gene of T cells to promote the quality of cell state. This view has changed and now researchers are trying to knock-in a specific gene to receive the optimal cells. However, a constructive method is needed to determine which knock-in gene constructs most significantly increase the cell functions *in vivo*. Indeed, a pooled knock-in platform, nonviral knock-in screening platform, was developed to provide the chance to screen, track, and barcode complex synthetic gene constructs in primary T cells. Pooled nonviral HDR templates precisely target the site of interest and integrate the desired gene within this area with relatively low rates of mis-assignment and without requiring viral packaging processes. Overall, this system offers the opportunity to explore the *in vitro* and *in vivo* functions of diverse sequence variants and discover the effects of gene's gain-of-function. Importantly, nonviral pooled knock-in screening will increase the discovery and development of synthetic DNA sequences to favorably manipulate the specificity and function of adoptive cellular therapies (Roth et al., 2020).

2 | CLINICAL TRANSLATION OF CRISPR-EDITED CAR-T CELLS

Currently, there are only a few clinical trials using the CRISPR/Cas9 technology in CAR-T cells. For example, *NCT04037566* is a first-in-human trial evaluating CD19 CAR-T cells with edited endogenous HPK1 in patients with R/R leukemia or lymphoma. *NCT04637763* is a phase I clinical trial investigating the effect and safety of CRISPR-edited allogenic CD19 CAR-T cells in patients with R/R B cell non-Hodgkin lymphoma. Finally, *NCT03545815* is a phase I clinical trial using CRISPR/Cas9 to knock-out PD-1 and TCR in CAR-T cells and directing them into patients with mesothelin positive multiple solid tumors. It is obvious that CRISPR-edited CAR-T cells are at preliminary

stages and more preclinical studies possibly under Good Laboratory Practice (GLP) are required to pave the way for clinical trials.

Translation of the CRISPR/Cas9 technology irrespective of CAR-T cells into clinical setting encounters some significant challenges which prevents this genome editing system from a successful therapeutic approach. These hurdles include but are not limited to the following issues.

One of the major obstacles that impeded translation of CRISPR/Cas9 into clinical use is its off-target alterations. In fact, sgRNA sometimes match with the region that is similar to those of target sequence and Cas9 consequently cleaves the off-target region. Attempts to maximize the specificity of CRISPR/Cas9 have increased by improving gRNA design, developing more robust delivery vehicle, and generating novel Cas9 nuclease (J. H. Hu et al., 2018; Kleinstiver et al., 2016; Shen et al., 2014). Interestingly, newly designed xCas9 and HypaCas9 variants seem to be more precisely with no reduction in target activity (J. S. Chen et al., 2017; J. H. Hu et al., 2018).

The other concern of using CRISPR/Cas9 is that it could introduce unexpected deletions and complex genomic rearrangements into edited cells, which would bring irrecoverable genotoxicity to clinical products (Shin et al., 2017). One approach to tackle this challenge would be the use of whole-genome sequence analysis, *in silico* off-target predictions, evaluating the risk of genotoxicity, and long-term patients follow up (Hsu et al., 2013; Zhang et al., 2015).

Furthermore, the immunogenicity of the Cas9 protein is another problem, which hinders the clinical translation of the CRISPR/Cas9 system. In this aspect, some individuals developed antibodies specific for the Cas9 protein and generate T cell immune memory for future encounter. The anti-Cas9 immune response mitigates the efficiency of editing process and could result in detrimental side effects. Finally, CRISPR/Cas9 can only exert its genome editing ability when the PAM sequence (NGG) is present. The conventional Cas9 protein only recognizes a few PAM sequences, restricting broad applications of this system. However, xCas9 is a smart version of Cas9 that can recognize more PAM sequences, which gives the CRISPR/Cas9 system to work on a broader range of application (J. H. Hu et al., 2018). Nevertheless, findings revealed that occasionally the HDR pathways for genomic insertion has low efficiency (Komor et al., 2017). This concern can be addressed by different constructive approaches, including using single-stranded DNA instead of double-stranded DNA, suppression of NHEJ pathway, and using nucleofection delivery method (Chu et al., 2015; Richardson et al., 2016).

3 | FUTURE PERSPECTIVE

Here, we reviewed studies highlighting the promising impact of new technologies in cancer immunotherapy (Figure 2). Editing CAR-T cells with CRISPR/Cas9 can overcome many challenges such as allogeneic reaction, tonic signaling, exhaustion, low performance in TME, and toxicity. In addition, the large-scale genetic screens using the CRISPR/Cas9 system provides scalable methods to interrogate thousands of genes in T cells with high efficiency and specificity. At present, preclinical studies concerning CRISPR-edited CAR-T cells demonstrated tangible and promising results that would lead to a new frontier in immunotherapy. However, still there is room to work further on new corresponding genes that have reverse effects on CAR-T cell therapy. Identifying such negative regulatory genes in CAR-T cells and target them by the CRISPR/Cas9 technology would definitely enhance the treatment. Although the CRISPR/Cas9 system is an emerging technique in cell therapy, it nowadays is a "go to" method for gene editing in many research labs in academic and pharmaceutical industry. With this fast advancement in the CRISPR/Cas9 application and emergence of new gene-editing

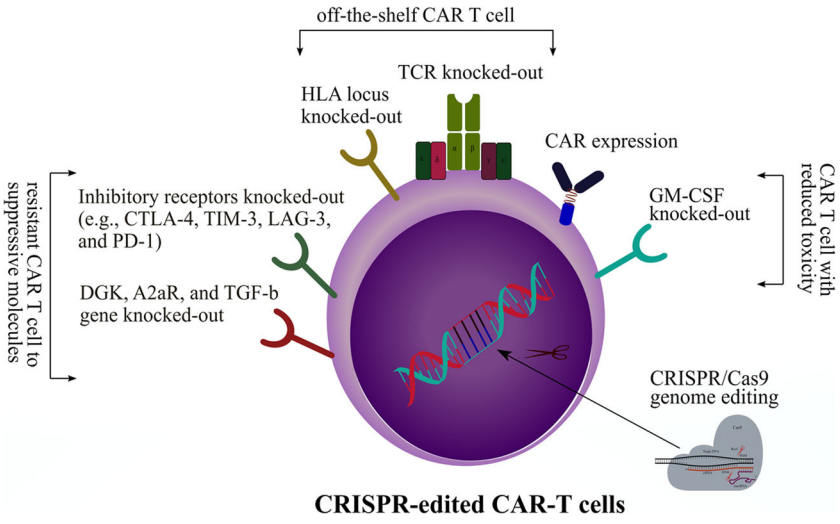

FIGURE 2 Representation of CRISPR-edited CAR-T cell. CRISPR/Cas9 genome editing technology improves CAR-T cell performance in three distinct ways, including the creation of off-the-shelf CAR-T products, development of resistant CAR-T cells to suppressive molecules, and generation of CAR-T cells with low side effects or toxicities. CAR-T, chimeric antigen receptor T; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; TCR, T cell receptor

technologies, we hope to soon witness the success of CAR-T cells in treating many refractory cancers including solid tumors.

4 | CONCLUSION

There are challenges associated with CAR-T cell therapy that can be addressed by genome editing technology. CRISPR/Cas9 has been a helpful tool for the success of CAR-T cell therapy against different tumor cells. This novel genome-editing technology addressed the problem that lies in the clinical use of allogeneic donor T cells. CRISPR/Cas9 can reduce the potential GVHD development caused by allogeneic CAR-T cells through eliminating TRAC and HLA loci. This approach would draw the concept of the development of off-the-shelf CAR-T cells, which could be used in different individuals regardless of HLA matches between donor and recipient. CAR-T cell performance has been improved by disrupting inhibitory molecules, such as PD-1, A2aR, Cbl-b, and TGF-β. Given the early positive outcome of the CRISPR/Cas9 edited CAR-T cells, there appears to be numerous opportunities for new cancer therapy. The annual market value for successful cancer therapy exceed billions of US dollars, and this encourages academic as well as the pharmaceutical industry to further investigate on this technology for the treatment of unmet clinical needs in many diseases.

ACKNOWLEDGMENTS

The authors would like to acknowledge the reviewers for their helpful and constructive comments on this manuscript.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. *Data acquisition*: Muhammad Sadeqi Nezhad, Meghdad Abdollahpour-Alitappeh, Alexander Seifalian, and Arash Sattari. *Quality control of data and algorithms*: Mahboubeh Yazdanifar, Nader Bagheri, Meghdad Abdollahpour-Alitappeh, and Muhammad Sadeqi Nezhad. *Data analysis and interpretation*: Muhammad Sadeqi Nezhad, Alexander Seifalian, Arash Sattari, and Meghdad Abdollahpour-Alitappeh. *Manuscript preparation*: Muhammad Sadeqi Nezhad, Mahboubeh Yazdanifar, Arash Sattari, Alexander Seifalian, and Meghdad Abdollahpour-Alitappeh. *Manuscript editing*: Muhammad Sadeqi Nezhad, Nader Bagheri, Arash Sattari, and Alexander Seifalian. *Manuscript review*: Muhammad Sadeqi Nezhad, Meghdad Abdollahpour-Alitappeh, Nader Bagheri, and Alexander Seifalian. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

ORCID

Muhammad Sadeqi Nezhad http://orcid.org/0000-0001-6244-385X

Meghdad Abdollahpour-Alitappeh http://orcid.org/0000-0002-5260-2527

Alexander Seifalian http://orcid.org/0000-0002-8334-9376

REFERENCES

Abramson, J. S., Palomba, M. L., Gordon, L. I., Lunning, M. A., Wang, M., Arnason, J., Mehta, A., Purev, E., Maloney, D. G., Andreadis, C., Sehgal, A., Solomon, S. R., Ghosh, N., Albertson, T. M., Garcia, J., Kostic, A., Mallaney, M., Ogasawara, K., Newhall, K., … Siddiqi, T. (2020). Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): A multicentre seamless design study. *Lancet*, *396*(10254), 839–852. https://doi.org/10.1016/s0140-6736(20)31366-0

Allen, F., Crepaldi, L., Alsinet, C., Strong, A. J., Kleshchevnikov, V., De Angeli, P., Páleníková, P., Khodak, A., Kiselev, V., Kosicki, M., Bassett, A. R., Harding, H., Galanty, Y., Muñoz-Martínez, F., Metzakopian, E., Jackson, S. P., & Parts, L. (2019). Predicting the mutations generated by repair of Cas9-induced double-strand breaks. *Nature Biotechnology*, *37*(1), 64–72.

Anderson, K. G., Stromnes, I. M., & Greenberg, P. D. (2017). Obstacles posed by the tumor microenvironment to T cell activity: A case for synergistic therapies. *Cancer Cell*, *31*(3), 311–325.

Baldanzi, G., Bettio, V., Malacarne, V., & Graziani, A. (2016). Diacylglycerol kinases: Shaping diacylglycerol and phosphatidic acid gradients to control cell polarity. *Frontiers in Cell and Developmental Biology*, *4*, 140. https://doi.org/10.3389/fcell.2016.00140

Berdien, B., Mock, U., Atanackovic, D., & Fehse, B. (2014). TALEN-mediated editing of endogenous T-cell receptors facilitates efficient reprogramming of T lymphocytes by lentiviral gene transfer. *Gene Therapy*, *21*(6), 539–548. https://doi.org/10.1038/gt.2014.26

Besser, M. J., Shapira-Frommer, R., Itzhaki, O., Treves, A. J., Zippel, D. B., Levy, D., Kubi, A., Shoshani, N., Zikich, D., Ohayon, Y., Ohayon, D., Shalmon, B., Markel, G., Yerushalmi, R., Apter, S., Ben-Nun, A., Ben-Ami, E., Shimoni, A., Nagler, A., & Schachter, J. (2013). Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies. *Clinical Cancer Research*, *19*(17), 4792–4800. https://doi.org/10.1158/1078-0432.Ccr-13-0380

Bischoff, N., Wimberger, S., Maresca, M., & Brakebusch, C. (2020). Improving precise CRISPR genome editing by small molecules: Is there a magic potion? *Cells*, *9*(5). https://doi.org/10.3390/cells9051318

Cao, J., Xiao, Q., & Yan, Q. (2018). The multiplexed CRISPR targeting platforms. *Drug Discovery Today: Technologies*, *28*, 53–61.

Cekic, C., & Linden, J. (2014). Adenosine A2A receptors intrinsically regulate CD8+ T cells in the tumor microenvironment. *Cancer Research*, *74*(24), 7239–7249. https://doi.org/10.1158/0008-5472.Can-13-3581

Chen, J. H., Perry, C. J., Tsui, Y.-C., Staron, M. M., Parish, I. A., Dominguez, C. X., Rosenberg, D. W., & Kaech, S. M. (2015). Prostaglandin E2 and programmed cell death 1 signaling coordinately impair CTL function and survival during chronic viral infection. *Nature Medicine*, *21*(4), 327–334.

Chen, J. S., Dagdas, Y. S., Kleinstiver, B. P., Welch, M. M., Sousa, A. A., Harrington, L. B., Sternberg, S. H., Joung, J. K., Yildiz, A., & Doudna, J. A. (2017). Enhanced proofreading governs CRISPR–Cas9 targeting accuracy. *Nature*, *550*(7676), 407–410. https://doi.org/10.1038/nature24268

Chen, S. S., Hu, Z., & Zhong, X. P. (2016). Diacylglycerol Kinases in T Cell Tolerance and Effector Function. *Frontiers in Cell and Developmental Biology*, *4*, 130. https://doi.org/10.3389/fcell.2016.00130

Choi, B. D., Yu, X., Castano, A. P., Darr, H., Henderson, D. B., Bouffard, A. A., Larson, R. C., Scarfò, I., Bailey, S. R., Gerhard, G. M., Frigault, M. J., Leick, M. B., Schmidts, A., Sagert, J. G., Curry, W. T., Carter, B. S., & Maus, M. V. (2019). CRISPR-Cas9 disruption of PD-1 enhances activity of universal EGFRvIII CAR T cells in a preclinical model of human glioblastoma. *Journal for Immunotherapy of Cancer*, *7*(1), 1–8.

Chu, V. T., Weber, T., Wefers, B., Wurst, W., Sander, S., Rajewsky, K., & Kühn, R. (2015). Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian

cells. *Nature Biotechnology*, *33*(5), 543–548. https://doi.org/10.1038/nbt.3198

Cradick, T. J., Fine, E. J., Antico, C. J., & Bao, G. (2013). CRISPR/Cas9 systems targeting β-globin and CCR5 genes have substantial off-target activity. *Nucleic Acids Research*, *41*(20), 9584–9592.

Di Roberto, R. B., Castellanos-Rueda, R., Frey, S., Egli, D., Vazquez-Lombardi, R., Kapetanovic, E., Kucharczyk, J., & Reddy, S. T. (2020). A functional screening strategy for engineering chimeric antigen receptors with reduced on-target, off-tumor activation. *Molecular Therapy*, *28*(12), 2564–2576. https://doi.org/10.1016/j.ymthe.2020.08.003

Diaconu, I., Ballard, B., Zhang, M., Chen, Y., West, J., Dotti, G., & Savoldo, B. (2017). Inducible caspase-9 selectively modulates the toxicities of CD19-specific chimeric antigen receptor-modified T cells. *Molecular Therapy*, *25*(3), 580–592. https://doi.org/10.1016/j.ymthe.2017.01.011

Dufva, O., Koski, J., Maliniemi, P., Ianevski, A., Klievink, J., Leitner, J., Pölönen, P., Hohtari, H., Saeed, K., Hannunen, T., Ellonen, P., Steinberger, P., Kankainen, M., Aittokallio, T., Keränen, M., Korhonen, M., & Mustjoki, S. (2020). Integrated drug profiling and CRISPR screening identify essential pathways for CAR-T cells cytotoxicity. *Blood*, *135*(9), 597–609. https://doi.org/10.1182/blood.2019002121

Eyquem, J., Mansilla-Soto, J., Giavridis, T., van der Stegen, S. J., Hamieh, M., Cunanan, K. M., Odak, A., Gönen, M., & Sadelain, M. (2017). Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature*, *543*(7643), 113–117.

Fonfara, I., Le Rhun, A., Chylinski, K., Makarova, K. S., Lécrivain, A.-L., Bzdrenga, J., Koonin, E. V., & Charpentier, E. (2014). Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems. *Nucleic Acids Research*, *42*(4), 2577–2590.

Fraietta, J. A., Lacey, S. F., Orlando, E. J., Pruteanu-Malinici, I., Gohil, M., Lundh, S., Boesteanu, A. C., Wang, Y., O'Connor, R. S., Hwang, W. T., Pequignot, E., Ambrose, D. E., Zhang, C., Wilcox, N., Bedoya, F., Dorfmeier, C., Chen, F., Tian, L., Parakandi, H., … Melenhorst, J. J. (2018). Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. *Nature Medicine*, *24*(5), 563–571. https://doi.org/10.1038/s41591-018-0010-1

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K., & Sander, J. D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnology*, *31*(9), 822–826.

Fujihara, Y., & Ikawa, M. (2014). CRISPR/Cas9-based genome editing in mice by single plasmid injection. *Methods in Enzymology*, *546*, 319–336. https://doi.org/10.1016/b978-0-12-801185-0.00015-5

Gaj, T., Sirk, S. J., Shui, S. L., & Liu, J. (2016). Genome-editing technologies: Principles and applications. *Cold Spring Harbor Perspectives in Biology*, *8*(12), https://doi.org/10.1101/cshperspect.a023754

Garcia-Robledo, J. E., Barrera, M. C., & Tobón, G. J. (2020). CRISPR/Cas: from adaptive immune system in prokaryotes to therapeutic weapon against immune-related diseases: CRISPR/Cas9 offers a simple and inexpensive method for disease modeling, genetic screening, and potentially for disease therapy. *International Reviews of Immunology*, *39*(1), 11–20.

Georgiadis, C., Preece, R., Nickolay, L., Etuk, A., Petrova, A., Ladon, D., Danyi, A., Humphryes-Kirilov, N., Ajetunmobi, A., Kim, D., Kim, J. S., & Qasim, W. (2018). Long terminal repeat CRISPR-CAR-coupled "universal" T cells mediate potent anti-leukemic effects. *Molecular Therapy*, *26*(5), 1215–1227.

Gilham, D. E., Michaux, A., Breman, E., Mauen, S., Bolsée, J., Huberty, F., Marijsse, J., Violle, B., Jacques-Hespel, C., Marchand, C., Ramelot, N., N'Guyen, T., Houssa, J., & Agaugue, S. (2018). TCR inhibitory molecule as a promising allogeneic NKG2D CAR-t cell approach In. *American Society of Clinical Oncology*, *36*, e15042.

Giuffrida, L., Sek, K., Henderson, M. A., Lai, J., Chen, A., Meyran, D., Todd, K. L., Petley, E. V., Mardiana, S., Mølck, C., Stewart, G. D., Solomon, B. J., Parish, I. A., Neeson, P. J., Harrison, S. J., Kats, L. M., House, I. G., Darcy, P. K., & Beavis, P. A. (2021). CRISPR/Cas9 mediated deletion of the adenosine A2A receptor enhances CAR T cell efficacy. *Nature Communications*, *12*(1), 3236. https://doi.org/10.1038/s41467-021-23331-5

Givens, B. E., Naguib, Y. W., Geary, S. M., Devor, E. J., & Salem, A. K. (2018). Nanoparticle-based delivery of CRISPR/Cas9 genome-editing therapeutics. *AAPS Journal*, *20*(6), 108. https://doi.org/10.1208/s12248-018-0267-9

Goff, S. L., Dudley, M. E., Citrin, D. E., Somerville, R. P., Wunderlich, J. R., Danforth, D. N., Zlott, D. A., Yang, J. C., Sherry, R. M., Kammula, U. S., Klebanoff, C. A., Hughes, M. S., Restifo, N. P., Langhan, M. M., Shelton, T. E., Lu, L., Kwong, M. L., Ilyas, S., … Rosenberg, S. A. (2016). Randomized, prospective evaluation comparing intensity of lymphodepletion before adoptive transfer of tumor-infiltrating lymphocytes for patients with metastatic melanoma. *Journal of Clinical Oncology*, *34*(20), 2389–2397. https://doi.org/10.1200/jco.2016.66.7220

Gundry, M. C., Brunetti, L., Lin, A., Mayle, A. E., Kitano, A., Wagner, D., Hsu, J. I., Hoegenauer, K. A., Rooney, C. M., Goodell, M. A., & Nakada, D. (2016). Highly efficient genome editing of murine and human hematopoietic progenitor cells by CRISPR/Cas9. *Cell Reports*, *17*(5), 1453–1461. https://doi.org/10.1016/j.celrep.2016.09.092

Gurusamy, D., Henning, A. N., Yamamoto, T. N., Yu, Z., Zacharakis, N., Krishna, S., Kishton, R. J., Vodnala, S. K., Eidizadeh, A., Jia, L., Kariya, C. M., Black, M. A., Eil, R., Palmer, D. C., Pan, J. H., Sukumar, M., Patel, S. J., & Restifo, N. P. (2020). Multi-phenotype CRISPR-Cas9 screen identifies p38 kinase as a target for adoptive immunotherapies. *Cancer Cell*, *37*(6), 818–833. https://doi.org/10.1016/j.ccell.2020.05.004

Hendel, A., Bak, R. O., Clark, J. T., Kennedy, A. B., Ryan, D. E., Roy, S., Steinfeld, I., Lunstad, B. D., Kaiser, R. J., Wilkens, A. B., Bacchetta, R., Tsalenko, A., Dellinger, D., Bruhn, L., & Porteus, M. H. (2015). Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nature Biotechnology*, *33*(9), nbt, 33, 3290–3989.

Hoyos, V., Savoldo, B., Quintarelli, C., Mahendravada, A., Zhang, M., Vera, J., Heslop, H. E., Rooney, C. M., Brenner, M. K., & Dotti, G. (2010). Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. *Leukemia*, *24*(6), 1160–1170. https://doi.org/10.1038/leu.2010.75

Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., Li, Y., Fine, E. J., Wu, X., Shalem, O., Cradick, T. J., Marraffini, L. A., Bao, G., & Zhang, F. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology*, *31*(9), 827–832. https://doi.org/10.1038/nbt.2647

Hu, B., Zou, Y., Zhang, L., Tang, J., Niedermann, G., Firat, E., Huang, X., & Zhu, X. (2019). Nucleofection with plasmid DNA for CRISPR/Cas9-mediated inactivation of programmed cell death protein 1 in CD133-specific CAR T cells. *Human Gene Therapy*, *30*(4), 446–458.

Hu, J. H., Miller, S. M., Geurts, M. H., Tang, W., Chen, L., Sun, N., Zeina, C. M., Gao, X., Rees, H. A., Lin, Z., & Liu, D. R. (2018). Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature*, *556*(7699), 57–63. https://doi.org/10.1038/nature26155

Hu, W., Zi, Z., Jin, Y., Li, G., Shao, K., Cai, Q., Ma, X., & Wei, F. (2019). CRISPR/Cas9-mediated PD-1 disruption enhances human mesothelin-targeted CAR T cell effector functions. *Cancer Immunology, Immunotherapy*, *68*(3), 365–377.

Ishino, Y., Krupovic, M., & Forterre, P. (2018). History of CRISPR-Cas from encounter with a mysterious repeated sequence to genome editing technology. *Journal of Bacteriology*, *200*(7).

Jasin, M., & Rothstein, R. (2013). Repair of strand breaks by homologous recombination. *Cold Spring Harbor Perspectives in Biology*, *5*(11), a012740.

Joyce, J. A., & Fearon, D. T. (2015). T cell exclusion, immune privilege, and the tumor microenvironment. *Science*, *348*(6230), 74–80.

Ju, X. P., Xu, B., Xiao, Z. P., Li, J. Y., Chen, L., Lu, S. Q., & Huang, Z. X. (2005). Cytokine expression during acute graft-versus-host disease after allogeneic peripheral stem cell transplantation. *Bone Marrow Transplantation*, *35*(12), 1179–1186. https://doi.org/10.1038/sj.bmt.1704972

Jung, I.-Y., Kim, Y.-Y., Yu, H.-S., Lee, M., Kim, S., & Lee, J. (2018). CRISPR/Cas9-mediated knockout of DGK improves antitumor activities of human T cells. *Cancer Research*, *78*(16), 4692–4703.

Jung, I.-Y., & Lee, J. (2018). Unleashing the therapeutic potential of CAR-T cell therapy using gene-editing technologies. *Molecules and Cells*, *41*(8), 717–723.

Kim, S., Kim, D., Cho, S. W., Kim, J., & Kim, J.-S. (2014). Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Research*, *24*(6), 1012–1019. https://doi.org/10.1101/gr.171322.113

Kim, S., Koo, T., Jee, H. G., Cho, H. Y., Lee, G., Lim, D. G., Shin, H. S., & Kim, J. S. (2018). CRISPR RNAs trigger innate immune responses in human cells. *Genome Research*, *28*(3), 367–373. https://doi.org/10.1101/gr.231936.117

Kleinstiver, B. P., Pattanayak, V., Prew, M. S., Tsai, S. Q., Nguyen, N. T., Zheng, Z., & Joung, J. K. (2016). High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*, *529*(7587), 490–495. https://doi.org/10.1038/nature16526

Komor, A. C., Badran, A. H., & Liu, D. R. (2017). CRISPR-based technologies for the manipulation of eukaryotic genomes. *Cell*, *168*(1), 20–36. https://doi.org/10.1016/j.cell.2016.10.044

Kornete, M., Marone, R., & Jeker, L. T. (2018). Highly efficient and versatile plasmid-based gene editing in primary T cells. *The Journal of Immunology*, *200*(7), 2489–2501.

Koury, J., Lucero, M., Cato, C., Chang, L., Geiger, J., Henry, D., Hernandez, J., Hung, F., Kaur, P., Teskey, G., & Tran, A. (2018). Immunotherapies: Exploiting the immune system for cancer treatment. *Journal of Immunology Research*, *2018*, 9585614. https://doi.org/10.1155/2018/9585614

Koyama, S., Akbay, E. A., Li, Y. Y., Herter-Sprie, G. S., Buczkowski, K. A., Richards, W. G., Gandhi, L., Redig, A. J., Rodig, S. J., Asahina, H., Jones, R. E., Kulkarni, M. M., Kuraguchi, M., Palakurthi, S., Fecci, P. E., Johnson, B. E., Janne, P. A., Engelman, J. A., Gangadharan, S. P., … Hammerman, P. S. (2016). Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nature Communications*, *7*(1), 1–9.

Kumar, J., Kumar, R., Kumar Singh, A., Tsakem, E. L., Kathania, M., Riese, M. J., Theiss, A. L., Davila, M. L., & Venuprasad, K. (2021). Deletion of Cbl-b inhibits $CD8^+$/T-cell exhaustion and promotes CAR-T cells function. *Journal for Immunotherapy of Cancer*, *9*(1), e001688. https://doi.org/10.1136/jitc-2020-001688

Li, C., Guan, X., Du, T., Jin, W., Wu, B., Liu, Y., Wang, P., Hu, B., Griffin, G. E., Shattock, R. J., & Hu, Q. (2015). Inhibition of HIV-1 infection of primary CD4+ T-cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9. *Journal of General Virology*, *96*(8), 2381–2393.

Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., & Zhao, X. (2020). Applications of genome editing technology in the targeted therapy of human diseases: Mechanisms, advances and prospects. *Signal Transduction and Targeted Therapy*, *5*(1), 1. https://doi.org/10.1038/s41392-019-0089-y

Liang, X., Potter, J., Kumar, S., Zou, Y., Quintanilla, R., Sridharan, M., Carte, J., Chen, W., Roark, N., Ranganathan, S., Ravinder, N., & Chesnut, J. D. (2015). Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *Journal of Biotechnology*, *208*, 44–53.

Lino, C. A., Harper, J. C., Carney, J. P., & Timlin, J. A. (2018). Delivering CRISPR: A review of the challenges and approaches. *Drug Delivery*, *25*(1), 1234–1257.

Liu, X., Zhang, Y., Cheng, C., Cheng, A. W., Zhang, X., Li, N., Xia, C., Wei, X., Liu, X., & Wang, H. (2017). CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. *Cell Research*, *27*(1), 154–157. https://doi.org/10.1038/cr.2016.142

Long, K. B., Young, R. M., Boesteanu, A. C., Davis, M. M., Melenhorst, J. J., Lacey, S. F., Degaramo, D. A., Levine, B. L., & Fraietta, J. A. (2018). CAR T cell therapy of non-hematopoietic malignancies: Detours on the road to clinical success. *Frontiers in immunology*, *9*, 2740. https://doi.org/10.3389/fimmu.2018.02740

Long, L., Zhang, X., Chen, F., Pan, Q., Phiphatwatchara, P., Zeng, Y., & Chen, H. (2018). The promising immune checkpoint LAG-3: From tumor microenvironment to cancer immunotherapy. *Genes & Cancer*, *9*(5-6), 176–189.

Luther, D. C., Lee, Y. W., Nagaraj, H., Scaletti, F., & Rotello, V. M. (2018). Delivery approaches for CRISPR/Cas9 therapeutics in vivo: Advances and challenges. *Expert Opinion on Drug Delivery*, *15*(9), 905–913. https://doi.org/10.1080/17425247.2018.1517746

Lutz-Nicoladoni, C., Wolf, D., & Sopper, S. (2015). Modulation of immune cell functions by the E3 ligase Cbl-b. *Frontiers in Oncology*, *5*(58), 58. https://doi.org/10.3389/fonc.2015.00058

Makarova, K. S., & Koonin, E. V. (2015). Annotation and classification of CRISPR-Cas systems, *CRISPR* (pp. 47–75). Springer.

Makarova, K. S., Wolf, Y. I., Iranzo, J., Shmakov, S. A., Alkhnbashi, O. S., Brouns, S. J., & Horvath, P. (2019). Evolutionary classification of CRISPR–Cas systems: A burst of class 2 and derived variants. *Nature Reviews Microbiology*, 1–17.

Maude, S. L., Laetsch, T. W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M. R., Stefanski, H. E., Myers, G. D., Qayed, M., De Moerloose, B., Hiramatsu, H., Schlis, K., Davis, K. L., Martin, P. L., Nemecek, E. R., Yanik, G. A., Peters, C., … Grupp, S. A. (2018). Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *New England Journal of Medicine*, *378*(5), 439–448.

Maude, S. L., Laetsch, T. W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M. R., Stefanski, H. E., Myers, G. D., Qayed, M., De Moerloose, B., Hiramatsu, H., Schlis, K., Davis, K. L., Martin, P. L., Nemecek, E. R., Yanik, G. A., Peters, C., … Grupp, S. A. (2018). Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *New England Journal of Medicine*, *378*(5), 439–448. https://doi.org/10.1056/NEJMoa1709866

Met, Ö., Jensen, K. M., Chamberlain, C. A., Donia, M., & Svane, I. M. (2019). Principles of adoptive T cell therapy in cancer. *Seminars in Immunopathology*, *41*(1), 49–58. https://doi.org/10.1007/s00281-018-0703-z

Morgan, R. A., Dudley, M. E., & Rosenberg, S. A. (2010). Adoptive cell therapy: Genetic modification to redirect effector cell specificity. *Cancer Journal*, *16*(4), 336–341. https://doi.org/10.1097/PPO.0b013e3181eb3879

Mout, R., Ray, M., Lee, Y.-W., Scaletti, F., & Rotello, V. M. (2017). In vivo delivery of CRISPR/Cas9 for therapeutic gene editing: progress and challenges. *Bioconjugate Chemistry*, *28*(4), 880–884.

Munshi, N. C., Anderson, L. D., Jr., Shah, N., Madduri, D., Berdeja, J., Lonial, S., Raje, N., Lin, Y., Siegel, D., Oriol, A., Moreau, P., Yakoub-Agha, I., Delforge, M., Cavo, M., Einsele, H., Goldschmidt, H., Weisel, K., Rambaldi, A., Reece, D., … San-Miguel, J. (2021). Idecabtagene vicleucel in relapsed and refractory multiple myeloma. *New England Journal of Medicine*, *384*(8), 705–716. https://doi.org/10.1056/NEJMoa2024850

Nakazawa, T., Natsume, A., Nishimura, F., Morimoto, T., Matsuda, R., Nakamura, M., & Park, Y.-S. (2020). Effect of CRISPR/Cas9-Mediated PD-1-disrupted primary human third-generation CAR-T cells targeting EGFRvIII on in vitro human glioblastoma cell growth. *Cells*, *9*(4), 998.

Neelapu, S. S., Locke, F. L., Bartlett, N. L., Lekakis, L. J., Miklos, D. B., Jacobson, C. A., Braunschweig, I., Oluwole, O. O., Siddiqi, T., Lin, Y., Timmerman, J. M., Stiff, P. J., Friedberg, J. W., Flinn, I. W., Goy, A.,

Hill, B. T., Smith, M. R., Deol, A., Farooq, U., … Go, W. Y. (2017). Axicabtagene ciloleucel CAR-T cells therapy in refractory large B-cell lymphoma. *New England Journal of Medicine*, *377*(26), 2531–2544. https://doi.org/10.1056/NEJMoa1707447

Park, J. H., Romero, F. A., Taur, Y., Sadelain, M., Brentjens, R. J., Hohl, T. M., & Seo, S. K. (2018). Cytokine release syndrome grade as a predictive marker for infections in patients with relapsed or refractory B-cell acute lymphoblastic leukemia treated with chimeric antigen receptor T cells. *Clinical Infectious Diseases*, *67*(4), 533–540.

Porter, D. L., Hwang, W. T., Frey, N. V., Lacey, S. F., Shaw, P. A., Loren, A. W., Bagg, A., Marcucci, K. T., Shen, A., Gonzalez, V., Ambrose, D., Grupp, S. A., Chew, A., Zheng, Z., Milone, M. C., Levine, B. L., Melenhorst, J. J., & June, C. H. (2015). Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Science Translational Medicine*, *7*(303), 303ra139. https://doi.org/10.1126/scitranslmed.aac5415

Rath, D., Amlinger, L., Rath, A., & Lundgren, M. (2015). The CRISPR-Cas immune system: Biology, mechanisms and applications. *Biochimie*, *117*, 119–128.

Ren, J., Liu, X., Fang, C., Jiang, S., June, C. H., & Zhao, Y. (2017). Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. *Clinical Cancer Research*, *23*(9), 2255–2266.

Ren, J., Zhang, X., Liu, X., Fang, C., Jiang, S., June, C. H., & Zhao, Y. (2017). A versatile system for rapid multiplex genome-edited CAR T cell generation. *Oncotarget*, *8*(10), 17002–17011.

Richardson, C. D., Ray, G. J., DeWitt, M. A., Curie, G. L., & Corn, J. E. (2016). Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nature Biotechnology*, *34*(3), 339–344. https://doi.org/10.1038/nbt.3481

Riese, M. J., Moon, E. K., Johnson, B. D., & Albelda, S. M. (2016). Diacylglycerol kinases (DGKs): Novel targets for improving T cell activity in cancer. *Frontiers in Cell and Developmental Biology*, *4*, 108. https://doi.org/10.3389/fcell.2016.00108

Rosenberg, S. A., Yang, J. C., Sherry, R. M., Kammula, U. S., Hughes, M. S., Phan, G. Q., Citrin, D. E., Restifo, N. P., Robbins, P. F., Wunderlich, J. R., Morton, K. E., Laurencot, C. M., Steinberg, S. M., White, D. E., & Dudley, M. E. (2011). Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clinical Cancer Research*, *17*(13), 4550–4557. https://doi.org/10.1158/1078-0432.Ccr-11-0116

Roth, T. L., Li, P. J., Blaeschke, F., Nies, J. F., Apathy, R., Mowery, C., Yu, R., Nguyen, M., Lee, Y., Truong, A., Hiatt, J., Wu, D., Nguyen, D. N., Goodman, D., Bluestone, J. A., Ye, C. J., Roybal, K., Shifrut, E., & Marson, A. (2020). Pooled knockin targeting for genome engineering of cellular immunotherapies. *Cell*, *181*(3), 728–744. https://doi.org/10.1016/j.cell.2020.03.039

Rupp, L. J., Schumann, K., Roybal, K. T., Gate, R. E., Chun, J. Y., Lim, W. A., & Marson, A. (2017). CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. *Scientific Reports*, *7*(1), 1–10.

Sadeqi Nezhad, M., Abdollahpour-Alitappeh, M., Rezaei, B., Yazdanifar, M., & Seifalian, A. M. (2021). Induced pluripotent stem cells (iPSCs) provide a potentially unlimited T cell source for CAR-T cell development and off-the-shelf products. *Pharmaceutical Research*, *38*, 931–945. https://doi.org/10.1007/s11095-021-03067-z

Sadeqi Nezhad, M., Seifalian, A., Bagheri, N., Yaghoubi, S., Karimi, M. H., & Adbollahpour-Alitappeh, M. (2020). Chimeric antigen receptor based therapy as a potential approach in autoimmune diseases: How close are we to the treatment? *Frontiers in Immunology*, *11*, 603237. https://doi.org/10.3389/fimmu.2020.603237

Salas-Mckee, J., Kong, W., Gladney, W. L., Jadlowsky, J. K., Plesa, G., Davis, M. M., & Fraietta, J. A. (2019). CRISPR/Cas9-based genome editing in the era of CAR T cell immunotherapy. *Human Vaccines & Immunotherapeutics*, *15*(5), 1126–1132.

Schumann, K., Lin, S., Boyer, E., Simeonov, D. R., Subramaniam, M., Gate, R. E., Haliburton, G. E., Ye, C. J., Bluestone, J. A., Doudna, J. A., & Marson, A. (2015). Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(33), 10437–10442. https://doi.org/10.1073/pnas.1512503112

Seki, A., & Rutz, S. (2018). Optimized RNP transfection for highly efficient CRISPR/Cas9-mediated gene knockout in primary T cells. *Journal of Experimental Medicine*, *215*(3), 985–997. https://doi.org/10.1084/jem.20171626

Seliger, B. (2019). Basis of PD1/PD-L1 therapies. *Journal of Clinical Medicine*, *8*(12), 2168.

Sharpe, M., & Mount, N. (2015). Genetically modified T cells in cancer therapy: opportunities and challenges. *Disease Models & Mechanisms*, *8*(4), 337–350.

Shen, B., Zhang, W., Zhang, J., Zhou, J., Wang, J., Chen, L., Wang, L., Hodgkins, A., Iyer, V., Huang, X., & Skarnes, W. C. (2014). Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nature Methods*, *11*(4), 399–402.

Shen, B., Zhang, W., Zhang, J., Zhou, J., Wang, J., Chen, L., Wang, L., Hodgkins, A., Iyer, V., Huang, X., & Skarnes, W. C. (2014). Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nature Methods*, *11*(4), 399–402. https://doi.org/10.1038/nmeth.2857

Shin, H. Y., Wang, C., Lee, H. K., Yoo, K. H., Zeng, X., Kuhns, T., Yang, C. M., Mohr, T., Liu, C., & Hennighausen, L. (2017). CRISPR/Cas9 targeting events cause complex deletions and insertions at 17 sites in the mouse genome. *Nature Communications*, *8*, 15464, 15464. https://doi.org/10.1038/ncomms15464

Singh, N., Perazzelli, J., Grupp, S. A., & Barrett, D. M. (2016). Early memory phenotypes drive T cell proliferation in patients with pediatric malignancies. *Science Translational Medicine*, *8*(320), 320–323. https://doi.org/10.1126/scitranslmed.aad5222

Sorek, R., Lawrence, C. M., & Wiedenheft, B. (2013). CRISPR-mediated adaptive immune systems in bacteria and archaea. *Annual Review of Biochemistry*, *82*, 237–266.

Stadtmauer, E. A., Fraietta, J. A., Davis, M. M., Cohen, A. D., Weber, K. L., Lancaster, E., Mangan, P. A., Kulikovskaya, I., Gupta, M., Chen, F., Tian, L., Gonzalez, V. E., Xu, J., Jung, I. Y., Melenhorst, J. J., Plesa, G., Shea, J., Matlawski, T., Cervini, A., … June, C. H. (2020). CRISPR-engineered T cells in patients with refractory cancer. *Science*, *367*(6481), https://doi.org/10.1126/science.aba7365

Stenger, D., Stief, T. A., Kaeuferle, T., Willier, S., Rataj, F., Schober, K., Vick, B., Lotfi, R., Wagner, B., Grünewald, T., Kobold, S., Busch, D. H., Jeremias, I., Blaeschke, F., & Feuchtinger, T. (2020). Endogenous TCR promotes in vivo persistence of CD19-CAR-T cells compared to a CRISPR/Cas9-mediated TCR knockout CAR. *Blood*, *136*(12), 1407–1418. https://doi.org/10.1182/blood.2020005185

Stenger, D., Stief, T. A., Kaeuferle, T., Willier, S., Rataj, F., Schober, K., Vick, B., Lotfi, R., Wagner, B., Grünewald, T., Kobold, S., Busch, D. H., Jeremias, I., Blaeschke, F., & Feuchtinger, T. (2020). Endogenous TCR promotes in vivo persistence of CD19-CAR-T cells compared to a CRISPR/Cas9-mediated TCR knockout CAR. *Blood*, *136*, 1407–1418.

Sterner, R. C., & Sterner, R. M. (2021). CAR-T cell therapy: Current limitations and potential strategies. *Blood Cancer Journal*, *11*(4), 69. https://doi.org/10.1038/s41408-021-00459-7

Sterner, R. M., Sakemura, R., Cox, M. J., Yang, N., Khadka, R. H., Forsman, C. L., Hansen, M. J., Jin, F., Ayasoufi, K., Hefazi, M., Schick, K. J., Walters, D. K., Ahmed, O., Chappell, D., Sahmoud, T., Durrant, C., Nevala, W. K., Patnaik, M. M., Pease, L. R., … Kenderian, S. S. (2019). GM-CSF inhibition reduces cytokine release syndrome and neuroinflammation but enhances CAR-T cell function

in xenografts. *Blood: The Journal of the American Society of Hematology*, *133*(7), 697–709.

Tang, N., Cheng, C., Zhang, X., Qiao, M., Li, N., Mu, W., Wei, X. F., Han, W., & Wang, H. (2020). TGF-β inhibition via CRISPR promotes the long-term efficacy of CAR T cells against solid tumors. *JCI Insight*, *5*(4).

Torikai, H., Reik, A., Soldner, F., Warren, E. H., Yuen, C., Zhou, Y., Crossland, D. L., Huls, H., Littman, N., Zhang, Z., Tykodi, S. S., Kebriaei, P., Lee, D. A., Miller, J. C., Rebar, E. J., Holmes, M. C., Jaenisch, R., Champlin, R. E., Gregory, P. D., & Cooper, L. J. (2013). Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. *Blood*, *122*(8), 1341–1349. https://doi.org/10.1182/blood-2013-03-478255

Townsend, M. H., Bennion, K., Robison, R. A., & O'Neill, K. L. (2020). Paving the way towards universal treatment with allogenic T cells. *Immunologic Research*, *68*, 1–8.

Turley, S. J., Cremasco, V., & Astarita, J. L. (2015). Immunological hallmarks of stromal cells in the tumour microenvironment. *Nature Reviews Immunology*, *15*(11), 669–682.

Wang, M., Munoz, J., Goy, A., Locke, F. L., Jacobson, C. A., Hill, B. T., Timmerman, J. M., Holmes, H., Jaglowski, S., Flinn, I. W., McSweeney, P. A., Miklos, D. B., Pagel, J. M., Kersten, M. J., Milpied, N., Fung, H., Topp, M. S., Houot, R., … Reagan, P. M. (2020). KTE-X19 CAR-T cells therapy in relapsed or refractory mantle-cell lymphoma. *New England Journal of Medicine*, *382*(14), 1331–1342. https://doi.org/10.1056/NEJMoa1914347

Wang, W., Ye, C., Liu, J., Zhang, D., Kimata, J. T., & Zhou, P. (2014). CCR5 gene disruption via lentiviral vectors expressing Cas9 and single guided RNA renders cells resistant to HIV-1 infection. *PLOS One*, *9*(12), e115987.

Wei, T., Cheng, Q., Min, Y.-L., Olson, E. N., & Siegwart, D. J. (2020). Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing. *Nature Communications*, *11*(1), 3232. https://doi.org/10.1038/s41467-020-17029-3

Wherry, E. J., & Kurachi, M. (2015). Molecular and cellular insights into T cell exhaustion. *Nature Reviews Immunology*, *15*(8), 486–499. https://doi.org/10.1038/nri3862

Wu, X., Kriz, A. J., & Sharp, P. A. (2014). Target specificity of the CRISPR-Cas9 system. *Quantitative biology*, *2*(2), 59–70.

Xiao-Jie, G. E. L., Li-Juan, J., Koo, T., & Kim, J.-S. (2017). Therapeutic applications of CRISPR RNA-guided genome editing. *Briefings in Functional Genomics*, *16*(1), 38–45.

Xu, X., Huang, S., Xiao, X., Sun, Q., Liang, X., Chen, S., Zhao, Z., Huo, Z., Tu, S., & Li, Y. (2020). Challenges and clinical strategies of CAR-T cells therapy for acute lymphoblastic leukemia: Overview and developments. *Frontiers in immunology*, *11*, 569117. https://doi.org/10.3389/fimmu.2020.569117

Xu, X., Wan, T., Xin, H., Li, D., Pan, H., Wu, J., & Ping, Y. (2019). Delivery of CRISPR/Cas9 for therapeutic genome editing. *The Journal of Gene Medicine*, *21*(7), e3107.

Yazdanifar, M., Zhou, R., & Mukherjee, P. (2016). Emerging immunotherapeutics in adenocarcinomas: A focus on CAR-T cells. *Current Trends in Immunology*, *17*, 95–115.

Zak, K. M., Kitel, R., Przetocka, S., Golik, P., Guzik, K., Musielak, B., Dömling, A., Dubin, G., & Holak, T. A. (2015). Structure of the complex of human programmed death 1, PD-1, and its ligand PD-L1. *Structure*, *23*(12), 2341–2348.

Zhang, X.-H., Tee, L. Y., Wang, X.-G., Huang, Q.-S., & Yang, S.-H. (2015). Off-target effects in CRISPR/Cas9-mediated genome engineering. *Molecular Therapy—Nucleic Acids*, *4*, e264. https://doi.org/10.1038/mtna.2015.37

Received: 13 August 2018 | Accepted: 19 November 2018

DOI: 10.1002/jcp.27972

REVIEW ARTICLE

WILEY Journal of Cellular Physiology

CRISPR/Cas9 technology as a potent molecular tool for gene therapy

Ansar Karimian[1,2,3] | Khalil Azizian[4] | Hadi Parsian[1] | Sona Rafieian[5] | Vahid Shafiei-Irannejad[6] | Maryam Kheyrollah[7] | Mehdi Yousefi[8,9] | Maryam Majidinia[10] | Bahman Yousefi[11,12]

[1]Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran

[2]Cancer & Immunology Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran

[3]Student Research Committee, Babol University of Medical Sciences, Babol, Iran

[4]Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Science, Tabriz, Iran

[5]Department of Oral and Maxillofacial Pathology, School of Dentistry, Zanjan University of Medical Sciences, Zanjan, Iran

[6]Cellular and Molecular Research Center, Urmia University of Medical Sciences, Urmia, Iran

[7]Department of Molecular Medicine, National Institue of Genetic Engeneering and Biotechnology, Tehran, Iran

[8]Molecular Medicine Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[9]Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[10]Tumor Research Center, Urmia University of Medical Sciences, Urmia, Iran

[11]Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

[12]Department of Biochemistry and Clinical Laboratories, Faculty of Medicine, Tabriz University of Medical Science, Tabriz, Iran

Correspondence
Maryam Majidinia, Solid Tumor Research Center, Urmia University of Medical Sciences, Orjhans Street, Resalat Blvd, Urmia 571478334, Iran.
Email: majidinia25@gmail.com
Bahman Yousefi, Department of Biochemistry and Clinical Laboratories, Faculty of Medicine, Tabriz University of Medical Science, University Street, Tabriz 5166614766, Iran.
Email: yousefib@tbzmed.ac.ir

Abstract

Clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9 (CRISPR-Cas9) is an RNA-guided gene editing tool which offers several advantageous characteristics in comparison with the conventional methods (e.g., zinc finger nucleases and transcription activator-like effector nucleases) such as cost-effectiveness, flexibility, and being easy-to-use. Despite some limitations such as efficient delivery and safety, CRISPR-Cas9 is still the most convenient tool for gene editing purposes. Due to the potential capability of the CRISPR-Cas9 system in genome editing and correction of casual mutations, it can be considered as a possible therapeutic system in the treatment of disorders associated with the genome mutations and in particular cancer treatment. In this review, we will discuss CRISPR-Cas-based gene editing along with its classifications and mechanism of action. Furthermore, the therapeutic application of the CRISPR-Cas9 system in mutational disorders, delivery systems, as well as its advantages and limitations with a special emphasis on cancer treatment will be discussed.

KEYWORDS

cancer treatment, CRISPR-Cas9, gene therapy, genome editing

Maryam Majidinia and Bahman Yousefi contributed equally to this work.

1 | INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPR) is described as RNA mediated adaptive immune system defense which is detected in bacteria and archaea. This system prevents the invasion of viruses and plasmids to these organisms (Jinek et al., 2012). Cas9, belonging to the Type II CRISPR system, has attracted the interest of many scientists. Cas9 encodes a guide RNA (gRNA), forms a direct binding to target DNA with the Watson–Crick base pairing and promotes its cleavage. The host cell responds to this double-strand break with two different mechanisms: (a) nonhomologues end joining (NHEJ) and (b) homology-directed repair (HDR) which lead, respectively to insertion/deletion and frameshift mutation in target DNA and HDR that offers a donor DNA as template for homologous recombination (Gasiunas, Barrangou, Horvath, & Siksnys, 2012; Guernet & Grumolato, 2017; Zhan, Rindtorff, Betge, Ebert, & Boutros, 2018). Cas9 has many applications in genetic engineering such as gene editing, gene expression, and gene functional studies. On the basis of these characteristics, Cas9 has attracted much attention in the treatment of many diseases caused by mutations. Thus, it appears that Cas9 has made a revolution in the treatment of diseases like cancer (Hsu, Lander, & Zhang, 2014; Jia et al., 2018).

In this review, we will discuss how CRISPR-Cas9 opens a new avenue in gene editing in addition to its application as a tool for gene therapy. We will also explain CRISPR-Cas classification and its general function mechanism for gene editing. Moreover, a critical comparison of system delivery for CRISPR and possible challenges will be considered and finally, the potential role of CRISPR-Cas9 in cancer treatment will be discussed.

2 | CRISPR-Cas SYSTEM CLASSIFICATION

Generally, the CRISPR-Cas system is composed of a clustered set of CRISPR-associated (Cas) genes and CRISPR array (repeated sequences and unique spacer sequences; Hsu et al., 2014). Diversity in Cas genes and their placement is the basis of CRISPR-Cas classification (Figure 1; Makarova, Wolf et al., 2015). The Cas genes are responsible for coding functional proteins known as effector complexes. CRISPR-Cas systems are divided into two classes and each class has several types and subtypes (Makarova, Wolf et al., 2015). Class 1 is found in bacteria and archaea (hyperthermophiles), while Class 2 is detected only in bacteria (not hyperthermophiles; Chylinski, Makarova, Charpentier, & Koonin, 2014). This part of the review illustrates the important types of different classes of the CRISPR-Cas system. Class 1 of CRISPR-Cas contains Type I, Type III, and Type IV; however, Type II and Type V are categorized in Class 2 (Chylinski et al., 2014). Figure 1 demonstrates the schematic structure of different classes and types of the CRISPR-Cas system. Generally, the structure of functional proteins is simpler in Class 2 in comparison with Class 1. Thus, the act of functional proteins (Cas proteins) in Type II and Type V is carried out by Cas9 and Cpf1, respectively. Cas9 and Cpf1 are single and large proteins. However, the functional proteins in Class 1 are multisubunit and consist of several proteins (CASCADE complex for Type I; Cmr or Csm RAMP complexes for Type III; Makarova, Wolf et al., 2015).

Both the Cas1 and Cas2 genes are observed in all types except Type IV (Makarova, Wolf et al., 2015). Different Cas proteins have various roles in the CRISPR-Cas system. The Cas1 protein is a wellknown integrase enzyme which is required for specific breaking of a CRISPR array to insert a newly identified spacer (Nuñez, Lee,

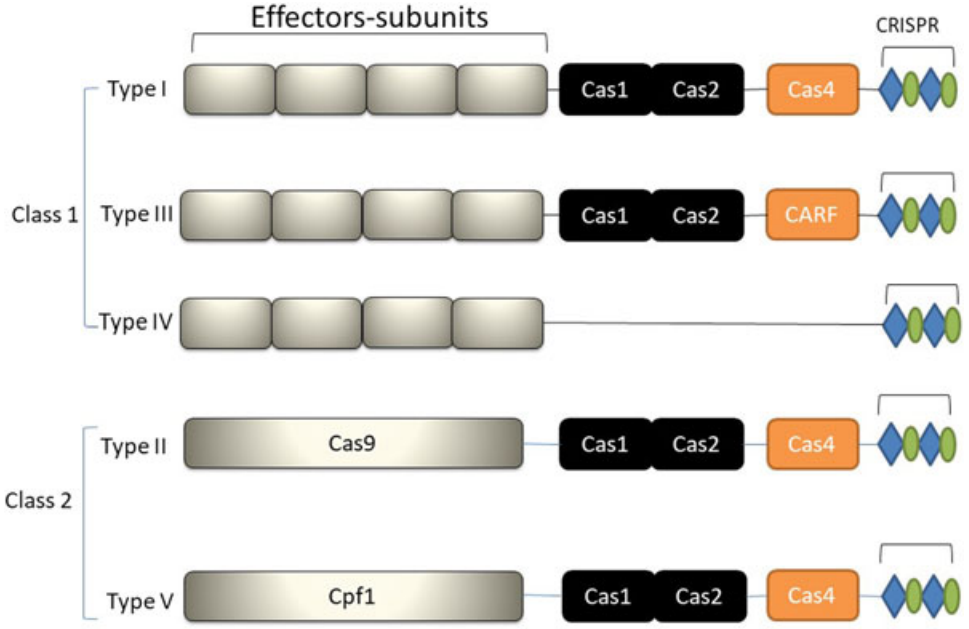

FIGURE 1 The schematic illustration of the CRISPR-Cas system classification. Blue lozenge: repeated units; green circle: spacer sequences; gray rectangle: effector module complex. CRISPR-Cas: clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease [Color figure can be viewed at wileyonlinelibrary.com]

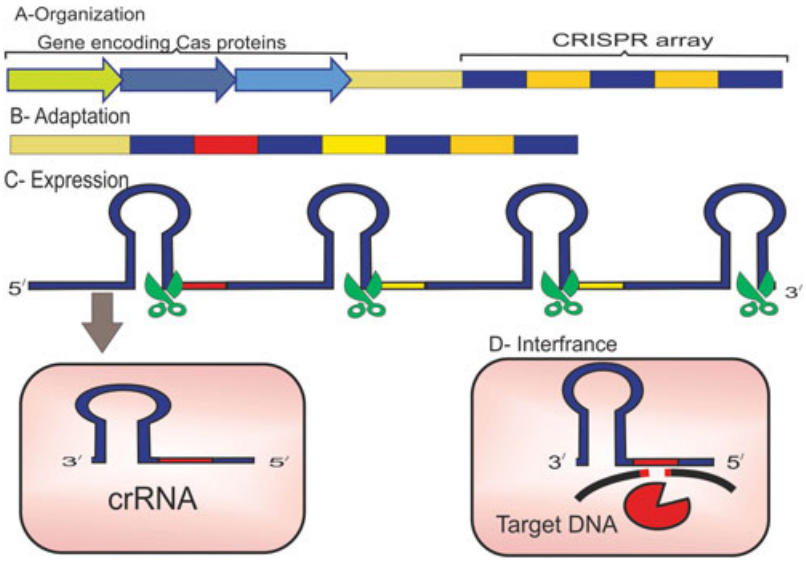

FIGURE 2 The schematic diagram of the CRISPR-Cas function. A-Organization: genes encoding the Cas protein and CRISPR array including repeated sequences and spacer sequences. B-Adaptation: insertion of the new spacer into CRISPR array. C-Expression: transcript of CRISPR array to precrRNA and process to mature crRNA. Assemble crRNA with Cas9 protein. D-Degradation of target DNA by Cas9. CRISPR: clustered regularly interspaced short palindromic repeats; crRNA: CRISPR-RNA [Color figure can be viewed at wileyonlinelibrary.com]

Engelman, & Doudna, 2015; Wiedenheft et al., 2009). The role of the Cas2 protein is unclear; however, this protein has RNase and DNase activities and is needed for the adaptation phase in *Escherichia.coli* (see below; Makarova, Wolf et al., 2015; Nam et al., 2012).

3 | CRISPR-Cas MECHANISMS

The function of the CRISPR-Cas against foreign DNA is illustrated in three phases including adaptation (spacer acquisition), expression (the process of CRISPR-RNA [crRNA], and interference (destruction and cleavage of invader DNA or RNA; Figure 2). Although subunits involved in the adaptation phase (Cas1 and Cas2) are highly conserved, the proteins involved in the expression and interference phases have main diversity among different types and organisms (Al-Attar, Westra, van der Oost, & Brouns, 2011; Barrangou et al., 2007; Makarova, Haft et al., 2011).

During the adaptation phase, a short segment of invader DNA should be integrated into the host chromosome (in CRISPR loci; Barrangou et al., 2007; Makarova, Haft et al., 2011). In this phase, a special segment (protospacer) is separated from invader DNA (bacteriophage or plasmid) and then is inserted and integrated into the 5′ end of a CRISPR array by the Cas1–Cas2 protein complex. This inserted segment makes a new and special spacer unit. The Cas1–Cas2 protein complex is composed of two units of Cas1 and one unit of Cas2 (Amitai & Sorek, 2016).

In the second stage, crRNA will be generated. To this end, in the expression stage, a CRISPR array (repeats and spacers) is transcripted to long RNA (known as precrRNA) and then is cleaved and processed into short RNAs (crRNAs or mature crRNAs). The processing of precrRNA to mature crRNAs is performed by using the multisubunit crRNA-effector complex (Cas6 in Type I and Type III; Staals et al., 2013) or Cas9 (RNase in Type II; Makarova, Wolf et al., 2015; Rouillon et al., 2013). The 5′ end of crRNA is single strand and determines the site of binding to target sequences. Moreover, the 3′ end of crRNA is duplex and requires to be assembled with the Cas9 protein.

In the interference phase, the crRNA–Cas complex (in the Type I and Type III systems) and crRNA–Cas9 (in the Type II system) are capable of cleaving invader/target DNA or RNA. The protospacer-adjacent motif located in the downstream of target sequences is an important element to recognize targets by Cas9 or the Cas complex. After identifying target sequences and base pairing between the invader target and crRNA, nuclease activity is initiated. The nuclease portion is varied among different types and performed by the HD endonuclease domain of Cas3 in Type I, (Beloglazova et al., 2011; Huo et al., 2014; Makarova, Wolf et al., 2015), Cas7 and Cas10 in Type III (Ramia et al., 2014; van Duijn et al., 2012), and Cas9 in Type II (Jinek et al., 2012). In the Type II CRISPR-Cas system, Cas9 contains two nuclease domains, RuvC and HNH domains. These nuclease portions lead to DNA and RNA breaking.

4 | APPLICATION OF CRISPR-Cas9 IN GENE THERAPY

Recently, genetic engineering (manipulating of DNA or RNA) has been used to prevent or treat human and animal diseases (Darband et al., 2018). This manipulation involves insertion, replacement or deletion of the related genes to special genetic disorders. The most common tools for gene editing in a site-specific manner are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the most novel tool, the CRISPR-Cas system. ZFNs and TALENs are meganuclease proteins that recognize specific DNA sequences and are known as protein guided tools (the DNA-binding protein; H. Kim & Kim, 2014; Li, Suzuki, Kim, Liu, & Izpisua Belmonte, 2014). However, in CRISPR-Cas systems, the target sequences bind to single guide RNA (sgRNA), and thus this tool is known as the RNA-guided system (Cong et al., 2013; Mali et al., 2013). Clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9 (CRISPR-Cas9) is more applicable than ZFN or TALEN-based tools and has several important advantages. For example, for the CRISPR-Cas9 system application, Cas9

(nuclease protein) is the same in all cases and only requires designation of 20-base pair sgRNA; however, meganuclease should be synthesized for each case separately (in ZFN- or TALEN-based tools; Xiao-Jie, Hui-Ying, Zun-Ping, Jin-Lian, & Li-Juan, 2015). Moreover, ZFN and TALEN are more labor-intensive and expensive in comparison with the CRISPR-Cas9 technology (Doudna & Charpentier, 2014; Schmidt & Grimm, 2015). Furthermore, CRISPR-Cas has the potential for simultaneous multiplexed editing of genes, in contrast to ZFN and TALEN (Cong et al., 2013; Schmidt & Grimm, 2015). In recent years, Cas9 has been used for different genetic editing both in vitro and in vivo (Platt et al., 2014). CRISPR-Cas was first used for cancer therapy purposes (see below). However, by the emergence of a new era, this tool has been used for protective and therapeutic aims (Xiao-Jie et al., 2015); one of the important aims is CRISPR-Cas application against infectious diseases. For example, some studies have demonstrated a significant reduction in expression of human immunodeficiency virus (HIV) due to the impact of CRISPR-Cas on the long terminal repeat sequence of HIV-1 (Ebina, Misawa, Kanemura, & Koyanagi, 2013; W. Hu, Kaminski, et al., 2014, Z. Hu, Yu, et al., 2014). Similar results were observed by Seeger and Sohn (2014) who reported the decreased expression of core antigen in hepatitis B virus (HBV; Seeger & Sohn, 2014). Another CRISPR-Cas9-mediated gene therapy is related to diseases caused by single gene defects known as monogenetic disorders. In mouse models, cataracts, and Duchenne muscular dystrophy caused by a defect in a single gene can be corrected by CRISPR-Cas application (Wu, Liang et al., 2013, Wu, Zhou et al., 2015).

5 | STRATEGIES FOR GENOME EDITING

We have three main strategies for genome editing using Cas9 (Table 1). The first strategy is using a plasmid encoding Cas9 protein and sgRNA known as the plasmid-based CRISPR-Cas9 strategy. In this strategy, both the Cas9 gene and sgRNA are assembled in the same plasmid; thus, applying this strategy prevents multiple transfections (Ran et al., 2013). However, in this system, plasmid requires to be transferred into the nucleus of target cells, which is the most important challenge in the plasmid-based CRISPR-Cas9 system (Liu, Zhang, Liu, & Cheng, 2017). The second strategy is the direct transfer or delivery of the Cas9 messenger RNA (mRNA) and sgRNA combination into host cells. The most important characteristic in this strategy is the poor stability of mRNA (Liu et al., 2017) that causes transient expression and short duration of genome editing. The last strategy is the direct delivery of the Cas9 protein and sgRNA combination. This strategy has several advantages including fast action, high stability, and poor inducing antigenicity responses.

6 | DELIVERY SYSTEMS

Two important delivery systems were introduced for the CRISPR-Cas9 protein including viral and nonviral delivery systems (the physical delivery system). In comparison with viral vectors, nonviral vectors are safer and simpler. By contrast, viral vectors have high delivery efficiency (Liu et al., 2017).

6.1 | Viral delivery

Generally, five classes of viral vectors are utilized for the delivery system. Some classes of these vectors such as retroviruses and lentiviruses are able to integrate into the host genome whereas the other classes, adeno-associated viruses (AAVs), adenoviruses, and herpes viruses, are known as nonintegrating vectors. Among viral delivery systems, two recombinant viruses (AAVs and Lentivirus) are more popular (Thomas, Ehrhardt, & Kay, 2003).

6.1.1 | Adeno-associated viruses

The AAV belongs to the parvovirus family which is dependent on herpes or adenovirus (helper virus) for infection and replication in

TABLE 1 Summary information of different strategies for CRISPR-Cas9 delivery

Strategies	Delivery systems	Advantages	Disadvantages
Plasmid-based CRISPR-Cas9 system	▪ Electroporation ▪ Hydrodynamic injection ▪ Lipid nanoparticles ▪ AAV ▪ Lentivirus	▪ Simple ▪ Comfortable ▪ More stable than Cas9 mRNA with the sgRNA ▪ Avoiding multitransfection	▪ Difficulty in plasmid transfer to nucleus ▪ Slow effect in gene editing ▪ Unwanted random integration in host genome
Cas9 mRNA and sgRNA	▪ Electroporation ▪ Lipid nanoparticles	▪ Low cytotoxicity ▪ Transient expression ▪ Fast effect	▪ Multitransfection ▪ High degradability of mRNA
Cas9 protein and sgRNA	▪ Electroporation ▪ Lipid nanoparticles ▪ Gold nanoparticles.	▪ Fastest effect ▪ High efficiency ▪ Avoiding unwanted integration ▪ Low antigenicity ▪ Avoid multitransfection	-

Note. AAV: adeno-associated viruses; CRISPR-Cas9: clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9; mRNA: messenger RNA; sgRNA: single guide RNA.

host cells (Wu, Yang, & Colosi, 2010). AAVs are small (20–25 nm) and nonenveloped viruses capable of binding to different receptors. For example, AAV2, AAV3, and AAV6 bind to heparin whereas acid sialic is the primary receptor for AAV4 and AAV5 (Wu, Asokan, & Samulski, 2006). Application of the AAV as a vector to the CRISPR-Cas9 system has several advantages such as nonpathogenicity for human, low immunogenicity, broad cell tropism, and high infection efficiency (Liu et al., 2017; Wu, Yang, & Colosi, 2010). The AAV has a small single-stranded DNA genome (4.7 kb; Sonntag, Schmidt, & Kleinschmidt, 2010). The genome size is one of the limitations in use of AAV vectors (LaFountaine, Fathe, & Smyth, 2015; Liu et al., 2017). The genome encoding for Cas9 and a sgRNA recovered from the *Streptococcus pyogenes* (SpCas9) is about 4.3 kb and along with other required sequences including promoter and polyadenylation signal, genome length increases to 4.7 kb (Ding et al., 2014). Scientists proposed several methods to solve this challenge such as replacement of typical Cas9 (SpCas9) by truncated SpCas9 or *Staphylococcus aureus* Cas9 (SaCas9 i.e., 1 kb shorter). These truncated SpCas9 and SaCas9 demonstrated similar activity and gene editing efficiency (Cong et al., 2013; Ran et al., 2013). Another solution was the use of the dual-AAV system, in which Cas9-encoding DNA and sgRNA were packed in two AAV vectors separately (Yang et al., 2016). Yet another solution was the use of AAV8, which overcame the packaging limitation (Mashiko et al., 2014). However, the results of AAV8 packaging are unclear (Hruscha & Schmid, 2015).

6.1.2 | Lentivirus

Lentivirus is another important viral vector for delivery of the CRISPR-Cas9 system (Liu et al., 2017). Lentivirus belongs to the retroviridae family which is able to integrate into the host genome (Thomas et al., 2003). This enveloped virus is larger than the AAV and its genome is composed of single-stranded RNA, which is capable of packaging genome about 9 kb in length (100 nm; Waehler, Russell, & Curiel, 2007). The lentivirus vector has numerous advantages such as high infection efficiency, low-level immunogenicity, long expression and large packing size (Liu et al., 2017; Zufferey et al., 1998). Furthermore, some studies have demonstrated that lentivirus vectors are useful tools for the elimination of viral infections (Kaminski et al., 2016; Wang & Quake, 2014).

6.2 | The nonviral delivery system

Several approaches have been illustrated for nonviral delivery systems. Among them, electroporation, hydrodynamic delivery, lipid transfection, and gold nanoparticles are the most favorable tools.

6.2.1 | Electroporation

Production of a competent cell (increased permeability of cell membrane) by electrical current is known as electroporation. The temporarily increased cell membrane permeability allows RNA, DNA, and proteins to enter into target cells (Gori et al., 2015). Recently, electroporation has been defined as a suitable tool for delivery of CRISPR-Cas9 systems. Several studies have utilized electroporation as a delivery mechanism for genome editing. The electroporation is utilized for all the three strategies including the plasmid-based CRISPR-Cas9 system (Hou et al., 2013; Mandal et al., 2014; Nakamura, Katahira, Sato, Watanabe, & Funahashi, 2004), Cas9 mRNA and sgRNA (Qin et al., 2015), and Cas9 protein and sgRNA complex (RNP; Schumann et al., 2015). A previous study demonstrated that electroporation was more efficient for RNP than the other two strategies (Liang et al., 2015). Other advantages were defined for electroporation such as high efficiency and applicability in vivo and in vitro. However, the induction of cell death is the major disadvantage in electroporation.

6.2.2 | Hydrodynamic delivery

In this method, a large amount of solution containing DNA (10% of blood weight) is swiftly injected in mouse veins. Subsequently, this injected nucleic acid is absorbed by liver cells. Recently demonstrated, hydrodynamic delivery is an applicable tool for delivery of the CRISPR-Cas9 system in the liver of preclinical models (Yin et al., 2014). This method is fast, simple, and cost-effective, and can be used in all strategies of CRISPR-Cas9 systems. However, it can only be used in small and preclinical models (mice), efficiency is limited to liver tissue and has adverse effects on heart and liver of host (Suda & Liu, 2007).

6.2.3 | Lipid transfection

Lipid nanoparticles or complexes are popular methods for DNA and/or RNA delivery into target cells. Different charges between nucleic acids (negative charge) and lipids (positive charge) allow suitable complex establishment for entering the cell (endocytosis and macropinocytosis; Gori et al., 2015). Moreover, this encapsulation protects nucleic acids from the host nucleases. First, this method was utilized in small interfering RNA (siRNA) therapy (Fitzgerald et al., 2014) but now is applied for CRISPR-Cas9 delivery to cells. Although this method has low efficiency it is simple, safe, and applicable to all strategies of CRISPR-Cas9 systems.

6.2.4 | Gold nanoparticles

This new delivery method is defined for RNPs in vitro. Uptake of these nanoparticles to target cells is mediated by the cholesterol-dependent membrane fusion process (not cellular endocytosis; Mout et al., 2017). High delivery efficiency is probably related to different entering mechanisms. However, the toxicity of these nanoparticles at high concentration is a limiting factor in vivo.

7 | CHALLENGES IN CRISPR-Cas9 APPLICATIONS

The ultimate and ideal goal of using CRISPR-Cas9 is to treat cancer by removing malignant mutations and replacing them with normal

DNA sequences. To achieve this goal in the future, which will be confronted with many problems, in the first step, it is necessary to obtain a profound knowledge about the pathophysiology and biology of cancers. Moreover, little progress has been made in research on some tumors including solid tumors, especially heterogeneous tumors (breast cancer), due to many challenges. The most important obstacles are the delivery of Cas9 to in vivo models and the safety aspect of studies for human trials (Ewelina, Cai, Lin, Kingston, & Cai, 2017). In addition to the benefits that have been introduced to CRISPR-Cas9 in the genome editing process, there are a number of challenges, including off-target effects, gRNA production, and efficient delivery, which should be considered (Zhang, Wen, & Guo, 2014).

7.1 | Off-target effects

Of the cases mentioned, off-target effects are serious challenges in this system, especially when the genomic medicine discussion is concerned (Mahfouz, Piatek, & Stewart, 2014). Compared to the two systems for genome editing, zinc finger nucleases (ZFNs) and TALENs, CRISPR-Cas9 shows a high risk of off-target effects in human cells (Zhang et al., 2014). Off-target effects cause genomic toxicity, carcinogenesis, genome instability, gene functional disruptions, epigenetic alterations (Wen, Yuan, Ma, Xu, & Yuan, 2016), cell death and cell transformation (Xiao-Jie et al., 2015; Zhang et al., 2014; Figure 2a). Therefore, off-target effects should be identified and controlled, and their levels should be nearly zero, especially for therapeutic purposes. To prevent these adverse events and ensure the specificity of the CRISPR-Cas9 performance, it is better to select target sequences with the lowest homology with off-target locations (Figure 2b). In addition, the dosage of CRISPR-Cas9 is another factor affecting off-target effects, which should be carefully controlled (Zhang et al., 2014). The structure and composition of gRNA affect the level of off-target effects. The use of truncated sgRNAs at 5′ end by two to three nucleotides that are sensitive to mismatches reduces off-target effects (Wen et al., 2016). According to early reports, Cas9 tolerates the mismatches between sgRNA and the target sequence depending on the position of the mismatches, their numbers, and the identity of their nucleotides. In later studies, they used empirical mismatches to select sufficient sgRNA with a minimum off-target activity (Shalem, Sanjana, & Zhang, 2015; Figure 2c). Because CRISPR-Cas9 applies changes permanently, off-target effects must be carefully controlled (Xiao-Jie et al., 2015).

7.2 | Unwanted deletions and insertions

In on-target DNA repair after Cas9 cutting, small insertions and deletions (<20 bp) have been observed to rarely occur. However, in some studies, very large and unwanted deletions (up to 600 bp or up to 1.5 kb) have occurred which may lead to pathological impairment in these cases that also causes problems in the normal tissue or cells (Kosicki, Tomberg, & Bradley, 2018).

7.3 | Production of gRNA

Production of gRNA is another challenge in the CRISPR-Cas9 system. Due to the posttranscriptional processes and modification of mRNA by RNA polymerase II, it is difficult to apply RNA polymerase II to produce gRNA. Recently, Gao & Zhao, (2014) have designed an artificial gene RGR which produces adult gRNA successfully (Zhang et al., 2014).

7.4 | Efficient delivery system

One of the important challenges ahead is the issue of targeted delivery of CRISPR-Cas9 to cancer cells. Delivery methods include viral methods by adenovirus or lentivirus vectors and nonviral physical methods (White & Khalili, 2016). One of the nonviral physical methods is the DNA or RNA injection system for delivery of CRISPR-Cas9. The efficiency of a delivery method depends on the types of target cells and target tissues (Zhang et al., 2014). Furthermore, immune responses to viral vectors that are involved in the delivery process are important challenges of the CRISPR-Cas9 system (Wen et al., 2016).

Given the challenges described for CRISPR-Cas9 systems and given the importance of gene therapy systems, especially in cancer therapy, high efficiency in genome editing and the delivery process is very important in the long run. To achieve the best gene therapy technology, we need to design more effective delivery tools as well as stronger and more powerful sgRNAs (Xiao-Jie et al., 2015). Ultimately, with all these descriptions, the CRISPR-Cas9 system can be upgraded with the help of protein engineering and directed evolutions (Mahfouz et al., 2014).

8 | COMPARISONS BETWEEN THE USE OF THE RNA INTERFERENCE AND CRISPR-Cas9 SYSTEM IN MUTATIONS OF TUMOR SUPPRESSOR GENES AND ONCOGENES

After completion of the human genome project, the main goal of studies has been to evaluate the functional characteristics of important genetic elements in normal conditions and disorders (Shalem et al., 2014). The mechanism of gene silencing (such as oncogenes) based on the RNA interference (RNAi) functions by small RNAs including siRNA and micro RNAs (D. H. Kim & Rossi, 2007). Inactivation approaches of genes based on the RNAi are limited by an uncertain degree of gene inhibition stability and gene silencing (Azimi et al., 2018). Although this is not problematic for some purposes, to achieve correct results for many purposes, the inactivation of genes must be complete and permanent (Davidson & McCray, 2011; Sánchez-Rivera & Jacks, 2015). In addition, RNAi function is limited to gene transcript (mRNA); however, the CRISPR-Cas9 system can target all elements throughout the genome including promoters, enhancers, introns, and intergenic regions (Shalem et al., 2014). By targeting mRNA, RNAi decreases the expression of target proteins whereas, in the CRISPR-Cas9 system, due to a mutation that results in loss-of-function in genomic DNA, the function of these

genes becomes knockout (Shalem et al., 2014). Given the comparison between the RNAi and CRISPR-Cas9 system according to Table 1, as well as the benefits identified by the advancement of technology for the CRISPR-Cas9 system, this system is used as an easy tool for the purpose of editing various genes (tumor suppressor gene mutations and oncogenes). Hence, in studies, the CRISPR-Cas9 system has provided a suitable platform for applied research related to cancer therapy (Xiao-Jie et al., 2015; Zhen, Lu et al., 2016).

9 | POTENTIAL THERAPEUTIC APPLICATION OF CRISPR-CAS9 IN CANCER

The targeted genomic engineering by the CRISPR-Cas9 system plays an important role in the study of biological processes and treatment of diseases such as cancer (Zhen, Hua et al., 2014). Furthermore, the simple designability and multiplexing of the CRISPR-Cas9 system are important factors in cancer treatment research (Wen et al., 2016). In 2008, 16.1% of cancer incidence was due to pathogen infections whereas 95% of them were associated with *Helicobacter pylori*, hepatitis C virus, HBV, and human papillomavirus (HPV; Wen et al., 2016). To date, many studies have used the siRNA system to dispose HPV16-E7 mRNA that causes pRb regeneration (a type of tumor suppressor inducing apoptosis). The limitation of siRNA is that its effect is induced in the long run, and cannot be inherited to the next generation (W. Hu, Kaminski et al., 2014, Z. Hu, Yu et al., 2014).

9.1 | Genome editing

With these descriptions, the E7-specific gRNAs/Cas9 system was designed to eliminate the viral oncogene E7 at DNA level (W. Hu, Kaminski et al., 2014, Z. Hu, Yu et al., 2014). E6 and E7 are HPV-encoded oncogenes that play an important role in the development of malignancy in cervical cancer. Binding of E6 to p53 (tumor suppressor protein) and E7 to pRb (tumor suppressor protein belonging to the retinoblastoma family; Zhen, Lu et al., 2016), p21 and p27 (as well as cyclin-dependent kinase inhibitors; Zhen, Hua et al., 2014) stimulates cell divisions and ultimately leads to cell proliferation (Zhen, Lu et al., 2016). The CRISPR-Cas9 system specifically targets the transcription of E6 and E7 oncogenes and consequently leads to the accumulation of p53 and p21 proteins, which ultimately reduces the proliferation of cervical cancer cells in vitro (White & Khalili, 2016; Zhen, Hua et al., 2014, Zhen, Lu et al.,2016). The xenograft tumors in mice model were also suppressed by the CRISPR-Cas9 system (Zhen, Hua et al., 2014). The CRISPR-Cas9/gRNA system makes the genome editing process in two ways: (a) NHEJ: Cas9 cut double-strand DNA and creates a double-strand break. Double strand breaks (DBSs) are repaired by NHEJ (Karimaian, Majidinia, Bannazadeh Baghi, & Yousefi, 2017; Majidinia & Yousefi, 2016a, 2016b; Majidinia et al., 2017). The NHEJ may have an error in the repair process and, at the DBS site, an/a insertion/deletion occurs that causes frameshifts or premature stop codons, which ultimately leads to the destruction of the open reading frame of the target gene. This method does not lead to the production of the target gene. (b) homologous recombination DNA repair (HR): by using a homologous donor DNA, the target gene is restored and produces the correct product (Majidinia & Yousefi, 2017aa, 2017b; Figure 3; Figure 4).

9.2 | Cotreatment with CRISPR-Cas9/HPV16 E6/E7 and chemotherapy agents

Tumor cells sometimes resist against growth suppressive signals and apoptosis, and E6 and E7 oncogenes increase this kind of resistance (Majidinia & Yousefi, 2016a, 2016b; Majidinia & Yousefi, 2017aa, 2017b; Yousefi, Zarghami, Samadi, & Majidinia, 2016; Majidinia, Alizadeh, Yousefi, Akbarzadeh, & Zarghami, 2016; Yousefi, Samadi et al., 2015, Yousefi, Azimi et al., 2017). *cis*-Diaminedichloroplatinum II (CDDP) is one of the most commonly used chemotherapeutic agents for various cancers, which inhibits the expression of E6 and E7 oncogenes and causes p53 accumulation in the nucleus of cancer cells, leading to apoptosis induction. Severe side effects and development of resistance in cancer cells are the main obstacles against successful cancer chemotherapy with this compound. Developing a new therapeutic strategy in which to either sensitize cancer cells to CDDP or reduce side effects of CDDP, is likely to be useful in cancer therapy. To determine whether CRISPR-Cas9/HPV16 E6/E7 could sensitize cancer cells to CDDP for the treatment of cervical cancer, a study was conducted. The results showed that CRISPR-Cas9/HPV16 E6/E7 in combination with CDDP significantly inhibited the growth of cancer cells. It was suggested that CRISPR-Cas9 targeting E6 and E7 oncogenes could act as a CDDP-sensitizer (Zhen, Lu et al., 2016).

9.3 | Epigenetic editing and transcriptome modulation with CRISPR-Cas9

So far, only the genome editing function has been used by the CRISPR-Cas9 system in cancer modeling and cancer treatment goals. Given the importance of epigenetic and transcriptomal aberrations in tumorigenesis, epigenetic, and transcriptome manipulation with a help of the CRISPR-Cas9 system is a promising strategy in the treatment of cancer. For example, fusing epigenetic modifiers to the CRISPR-Cas9 system can lead to the desired location, modify the conditions of methylation or histone on the location and apply cancer therapeutic function. Finally, the CRISPR-Cas9 system can review the genome editing (wt-Cas9 and nCas9), epigenetic regulations (dCas9), and transcriptome modulations (RCas9 and dRCas9) in cancer therapy studies on an average basis (Wen et al., 2016).

9.4 | Drug development with the CRISPR-Cas9 system

One of the functions of the CRISPR-Cas9 system for the treatment of cancer is the development of anticancer drugs. XPO1 is a nuclear-cytoplasmic transport protein, which is inhibited by Selinexor (a treatment agent for prostate cancer and myeloma),

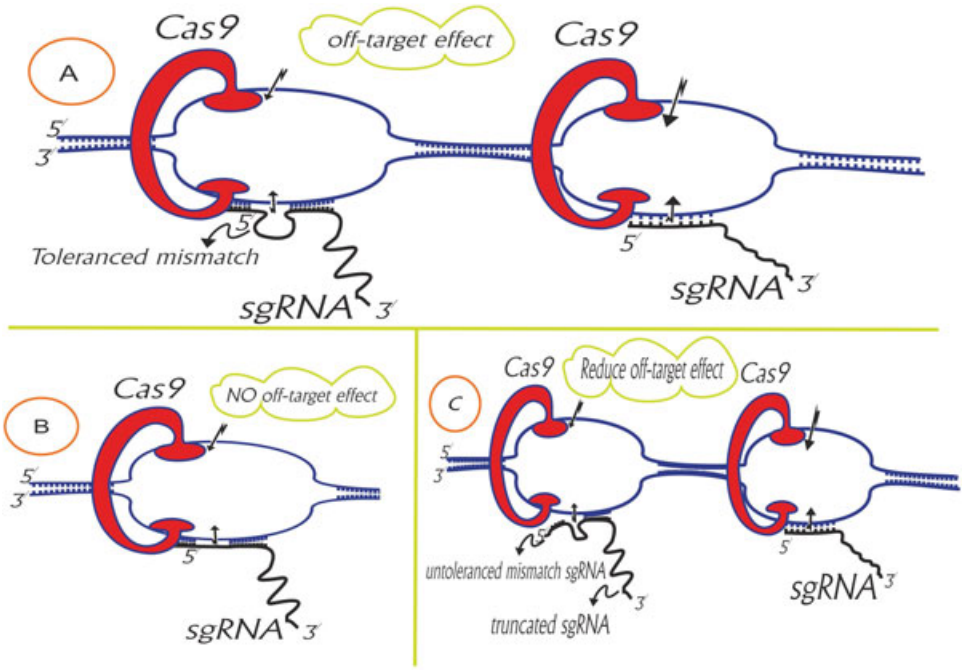

FIGURE 3 The schematic illustration of the off-target effects and their destruction. (a) Large genomes have sequences that are exactly or highly homologous to the DNA of the target sequence. On this basis, Cas9 cleaves the homologous sequences in addition to the target sequence, which causes off-target mutations. In this case, if there is a mismatch between the sgRNA and the target sequence, it will be tolerated and will not cause any problem. (b) To prevent off-target effects, a target sequence must be selected that has the lowest homology in the genome sequence. Therefore, only the target sequence is cleaved by Cas9, and off-target effects are either reduced or absent. (c) Another way to prevent off-target effects is the use of sgRNAs that are truncated at 5′ end by 2–3 nucleotides and are sensitive to mismatches that cannot tolerate the mismatch. Therefore, it leaves the target sequence from 5′ end, leading to reduced off-target effects. sgRNA: single guide RNA [Color figure can be viewed at wileyonlinelibrary.com]

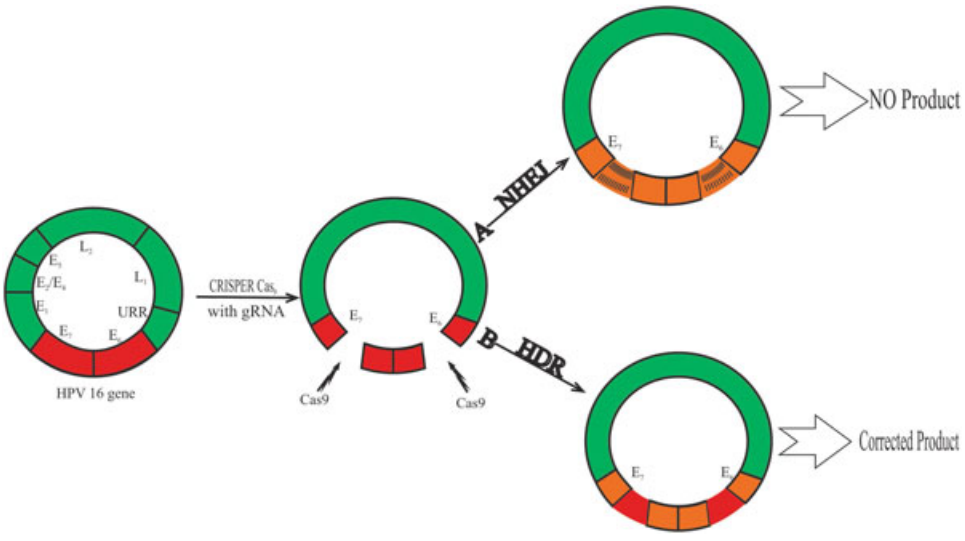

FIGURE 4 The schematic description of the site-specific editing of HPV16 genome by CRISPR-Cas9/gRNA. gRNA guides Cas9 to the target sequences (E6 and E7). With the endonuclease properties of Cas9, the target sequence is cleaved and produces DSBs. There are two genome editing pathways for the CRISPR-Cas9/gRNA system. A, NHEJ: it may often have an error in the repair process and creates insertion/deletion mutations leading to the production of frameshifts or premature stop codons at a DBS site, which ultimately leads to the destruction of the ORF of the target genes. Finally, in this pathway, the target genes have no product. B, HDR: by using a homologous donor DNA which creates new sequences into the target genes, the target genes restore and produce the correct product. CRISPR-Cas9: clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9; DSB: double-strand break; gRNA: guide RNA; HDR: homology-directed repair; HPV: human papillomavirus; NHEJ: nonhomologues end joining; ORF: open reading frame [Color figure can be viewed at wileyonlinelibrary.com]

due to the connection to the 528 cysteine residue of XPO1, leading to the nuclear accumulation of oncosuppressors and as a result the cell cycle arrest and induction of apoptosis. To validate this drug-target interaction, transfected Cas9/sgRNA and a single strand oligodeoxynucleotide bearing desired point mutations (capable of substituting XPO1 528 cysteine residue with serine) into leukemic cancer cells. As expected, this mutation caused the abrogated Selinexor-meditated functional inhibition of XPO1 by blocking XPO1-Selinexor binding, indicating that XPO1 528 cysteine residue is the main goal of Selinexor (Wen et al., 2016).

10 | CONCLUSION

Among different types of CRISPR-Cas systems, CRISPR-Cas9 is the most popular type to apply in genome editing. CRISPR-Cas9 is a robust tool in genome editing and also has the therapeutic potential. Despite numerous advantages, some challenges for CRISPR-Cas9 such as safety and efficient delivery were described. The CRISPR-Cas9 is more applicable in comparison to other gene editing tools such as ZFN and TALENs because it is a cost-effective and flexible tool. Fast action and poor inducing antigenicity responses are the most important advantages of the RNP strategy, which make this strategy the best strategy. To the best of our knowledge, no disadvantage has been so far mentioned for this strategy.

According to the features introduced for the CRISPR-Cas9 system and many limitations mentioned for RNAi, the importance of the CRISPR-Cas9 system has been clearly proven; this system is used as an easy tool for the purpose of editing various genes (tumor suppressor gene mutations and oncogenes). Finally, the CRISPR-Cas9 system has provided a suitable platform for applied research related to cancer therapy in in vivo and in vitro conditions.

ACKNOWLEDGMENTS

The authors would like to thank Clinical Research Development Unit, Shohada Hospital, Tabriz University of Medical Sciences for kind supports.

CONFLICTS OF INTEREST

The authors declare that there are no conflict of interest.

ORCID

Mehdi Yousefi http://orcid.org/0000-0001-9823-5454
Maryam Majidinia http://orcid.org/0000-0001-9776-5816

REFERENCES

Al-Attar, S., Westra, E. R., van der Oost, J., & Brouns, S. J. J. (2011). Clustered regularly interspaced short palindromic repeats (CRISPRs): The hallmark of an ingenious antiviral defense mechanism in prokaryotes. *Biological Chemistry*, *392*(4), 277–289.

Amitai, G., & Sorek, R. (2016). CRISPR–Cas adaptation: Insights into the mechanism of action. *Nature Reviews Microbiology*, *14*(2), 67–76.

Azimi, A., Majidinia, M., Shafiei-Irannejad, V., Jahanban-Esfahlan, R., Ahmadi, Y., Karimian, A., … Yousefi, B. (2018). Suppression of p53R2 gene expression with specific siRNA sensitizes HepG2 cells to doxorubicin. *Gene*, *642*, 249–255.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., … Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science*, *315*(5819), 1709–1712.

Beloglazova, N., Petit, P., Flick, R., Brown, G., Savchenko, A., & Yakunin, A. F. (2011). Structure and activity of the Cas3 HD nuclease MJ0384, an effector enzyme of the CRISPR interference. *The EMBO Journal*, *30*(22), 4616–4627.

Chylinski, K., Makarova, K. S., Charpentier, E., & Koonin, E. V. (2014). Classification and evolution of Type II CRISPR-Cas systems. *Nucleic Acids Research*, *42*(10), 6091–6105.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., … Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science*, *339*, 1231143.

Darband, S. G., Mirza-Aghazadeh-Attari, M., Kaviani, M., Mihanfar, A., Sadighparvar, S., Yousefi, B., & Majidinia, M. (2018). Exosomes: Natural nanoparticles as bio shuttles for RNAi delivery. *Journal of Controlled Release*, *289*, 158–170.

Davidson, B. L., & McCray, P. B. (2011). Current prospects for RNA interference-based therapies. *Nature Reviews Genetics*, *12*(5), 329–340.

Ding, Q., Strong, A., Patel, K. M., Ng, S.-L., Gosis, B. S., Regan, S. N., … Musunuru, K. (2014). Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing novelty and significance. *Circulation Research*, *115*(5), 488–492.

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, *346*(6213), 1258096–1258096.

Ebina, H., Misawa, N., Kanemura, Y., & Koyanagi, Y. (2013). Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. *Scientific Reports*, *3*, 2510.

Ewelina, B., Cai, L., Lin, X., Kingston, C., & Cai, F. (2017). Application of clustered regularly interspersed short palindromic repeats/cas9 editing technologies in breast cancer research. *Translational Surgery*, *2*(2), 44.

Fitzgerald, K., Frank-Kamenetsky, M., Shulga-Morskaya, S., Liebow, A., Bettencourt, B. R., Sutherland, J. E., … Simon, A. (2014). Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin Type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: A randomised, single-blind, placebo-controlled, phase 1 trial. *The Lancet.*, *383*(9911), 60–68.

Gao, Y., & Zhao, Y. (2014). Self-processing of ribozyme-flanked RNAs into guide RNAs in vitro and in vivo for CRISPR-mediated genome editing. *Journal of Integrative Plant Biology*, *56*(4), 343–349.

Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences*, *109*(39), E2579–E2586.

Gori, J. L., Hsu, P. D., Maeder, M. L., Shen, S., Welstead, G. G., & Bumcrot, D. (2015). Delivery and specificity of CRISPR/Cas9 genome editing technologies for human gene therapy. *Human Gene Therapy*, *26*(7), 443–451.

Guernet, A., & Grumolato, L. (2017). CRISPR/Cas9 editing of the genome for cancer modeling. *Methods*, *121*, 130–137.

Hou, Z., Zhang, Y., Propson, N. E., Howden, S. E., Chu, L.-F., Sontheimer, E. J., & Thomson, J. A. (2013). Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. *Proceedings of the National Academy of Sciences*, *110*(39), 15644–15649.

Hruscha, A., & Schmid, B. (2015). Generation of zebrafish models by CRISPR/Cas9 genome editing . *Neuronal Cell Death*, *1254*, 341–350.

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, *157*(6), 1262–1278.

Hu, W., Kaminski, R., Yang, F., Zhang, Y., Cosentino, L., Li, F., … Khalili, K. (2014). RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. *Proceedings of the National Academy of Sciences*, *111*(31), 11461–11466.

Hu, Z., Yu, L., Zhu, D., Ding, W., Wang, X., Zhang, C., … Wang, H. (2014). Disruption of HPV16-E7 by CRISPR/Cas system induces apoptosis and growth inhibition in HPV16 positive human cervical cancer cells. *BioMed Research International*, *2014*, 1–9.

Huo, Y., Nam, K. H., Ding, F., Lee, H., Wu, L., Xiao, Y., … Ke, A. (2014). Structures of CRISPR Cas3 offer mechanistic insights into cascade-activated DNA unwinding and degradation. *Nature Structural and Molecular Biology*, *21*(9), 771–777.

Jia, C., Huai, C., Ding, J., Hu, L., Su, B., Chen, H., & Lu, D. (2018). New applications of CRISPR/Cas9 system on mutant DNA detection. *Gene*, *641*, 55–62.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*, 1225829.

Kaminski, R., Chen, Y., Fischer, T., Tedaldi, E., Napoli, A., Zhang, Y., … Khalili, K. (2016). Elimination of HIV-1 genomes from human T-lymphoid cells by CRISPR/Cas9 gene editing. *Scientific Reports*, *6*, 22555.

Karimaian, A., Majidinia, M., Bannazadeh Baghi, H., & Yousefi, B. (2017). The crosstalk between Wnt/β-catenin signaling pathway with DNA damage response and oxidative stress: Implications in cancer therapy. *DNA Repair*, *51*, 14–19.

Kim, D. H., & Rossi, J. J. (2007). Strategies for silencing human disease using RNA interference. *Nature Reviews Genetics*, *8*(3), 173–184.

Kim, H., & Kim, J.-S. (2014). A guide to genome engineering with programmable nucleases. *Nature Reviews Genetics*, *15*(5), 321–334.

Kosicki, M., Tomberg, K., & Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. *Nature Biotechnology*, *36*(8), 765–771.

LaFountaine, J. S., Fathe, K., & Smyth, H. D. C. (2015). Delivery and therapeutic applications of gene editing technologies ZFNs, TALENs, and CRISPR/Cas9. *International Journal of Pharmaceutics*, *494*(1), 180–194.

Li, M., Suzuki, K., Kim, N. Y., Liu, G.-H., & Izpisua Belmonte, J. C. (2014). A cut above the rest: Targeted genome editing technologies in human pluripotent stem cells. *Journal of Biological Chemistry*, *289*(8), 4594–4599.

Liang, X., Potter, J., Kumar, S., Zou, Y., Quintanilla, R., Sridharan, M., … Chesnut, J. D. (2015). Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *Journal of Biotechnology*, *208*, 44–53.

Liu, C., Zhang, L., Liu, H., & Cheng, K. (2017). Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. *Journal of Controlled Release*, *266*, 17–26.

Mahfouz, M. M., Piatek, A., & Stewart, C. N. (2014). Genome engineering via TALENs and CRISPR/Cas9 systems: Challenges and perspectives. *Plant Biotechnology Journal*, *12*(8), 1006–1014.

Majidinia, M., & Yousefi, B. (2016a). Long non-coding RNAs in cancer drug resistance development. *DNA Repair*, *45*, 25–33.

Majidinia, M., & Yousefi, B. (2016b). DNA damage response regulation by microRNAs as a therapeutic target in cancer. *DNA Repair*, *47*, 1–11.

Majidinia, M., & Yousefi, B. (2017a). Breast tumor stroma: A driving force in the development of resistance to therapies. *Chemical Biology & Drug Design*, *89*(3), 309–318.

Majidinia, M., & Yousefi, B. (2017b). DNA repair and damage pathways in breast cancer development and therapy. *DNA Repair*, *54*, 22–29.

Majidinia, M., Alizadeh, E., Yousefi, B., Akbarzadeh, M., & Zarghami, N. (2016). Downregulation of Notch signaling pathway as an effective chemosensitizer for cancer treatment. *Drug Research*, *66*(11), 571–579.

Majidinia, M., Sadeghpour, A., Mehrzadi, S., Reiter, R. J., Khatami, N., & Yousefi, B. (2017). Melatonin: A pleiotropic molecule that modulates DNA damage response and repair pathways. *Journal of Pineal Research*, *63*(1), e12416.

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J. J., Charpentier, E., Horvath, P., … Koonin, E. V. (2011). Evolution and classification of the CRISPR–Cas systems. *Nature Reviews Microbiology*, *9*(6), 467–477.

Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., … Koonin, E. V. (2015). An updated evolutionary classification of CRISPR–Cas systems. *Nature Reviews Microbiology*, *13*(11), 722–736.

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., … Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science*, *339*(6121), 823–826.

Mandal, P. K., Ferreira, L. M. R., Collins, R., Meissner, T. B., Boutwell, C. L., Friesen, M., … Cowan, C. A. (2014). Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. *Cell Stem Cell*, *15*(5), 643–652.

Mashiko, D., Young, S. A., Muto, M., Kato, H., Nozawa, K., Ogawa, M., … Ikawa, M. (2014). Feasibility for a large scale mouse mutagenesis by injecting CRISPR/Cas plasmid into zygotes. *Development, Growth & Differentiation*, *56*(1), 122–129.

Mout, R., Ray, M., Yesilbag Tonga, G., Lee, Y.-W., Tay, T., Sasaki, K., & Rotello, V. M. (2017). Direct cytosolic delivery of CRISPR/Cas9-ribonucleoprotein for efficient gene editing. *ACS Nano*, *11*(3), 2452–2458.

Nakamura, H., Katahira, T., Sato, T., Watanabe, Y., & Funahashi, J. (2004). Gain- and loss-of-function in chick embryos by electroporation. *Mechanisms of Development*, *121*(9), 1137–1143.

Nam, K. H., Ding, F., Haitjema, C., Huang, Q., DeLisa, M. P., & Ke, A. (2012). Double-stranded endonuclease activity in *Bacillus halodurans* clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas2 protein. *Journal of Biological Chemistry*, *287*(43), 35943–35952.

Nuñez, J. K., Lee, A. S. Y., Engelman, A., & Doudna, J. A. (2015). Integrase-mediated spacer acquisition during CRISPR–Cas adaptive immunity. *Nature*, *519*(7542), 193–198.

Platt, R. J., Chen, S., Zhou, Y., Yim, M. J., Swiech, L., Kempton, H. R., … Zhang, F. (2014). CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell*, *159*(2), 440–455.

Qin, W., Dion, S. L., Kutny, P. M., Zhang, Y., Cheng, A. W., Jillette, N. L., … Wang, H. (2015). Efficient CRISPR/Cas9-mediated genome editing in mice by zygote electroporation of nuclease. *Genetics*, *200*(2), 423–430.

Ramia, N. F., Spilman, M., Tang, L., Shao, Y., Elmore, J., Hale, C., … Stagg, S. M. (2014). Essential structural and functional roles of the Cmr4 subunit in RNA cleavage by the Cmr CRISPR-Cas complex. *Cell Reports*, *9*(5), 1610–1617.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, *8*(11), 2281–2308.

Rouillon, C., Zhou, M., Zhang, J., Politis, A., Beilsten-Edmands, V., Cannone, G., … White, M. F. (2013). Structure of the CRISPR interference complex CSM reveals key similarities with cascade. *Molecular Cell*, *52*(1), 124–134.

Sánchez-Rivera, F. J., & Jacks, T. (2015). Applications of the CRISPR–Cas9 system in cancer biology. *Nature Reviews Cancer*, *15*(7), 387–393.

Schmidt, F., & Grimm, D. (2015). CRISPR genome engineering and viral gene delivery: A case of mutual attraction. *Biotechnology Journal*, *10*(2), 258–272.

Schumann, K., Lin, S., Boyer, E., Simeonov, D. R., Subramaniam, M., Gate, R. E., … Marson, A. (2015). Generation of knock-in primary human T cells

using Cas9 ribonucleoproteins. *Proceedings of the National Academy of Sciences*, *112*(33), 10437–10442.

Seeger, C., & Sohn, J. A. (2014). Targeting hepatitis B virus with CRISPR/Cas9. Molecular *Therapy-Nucleic Acids*, *1*(3), 1-7.

Shalem, O., Sanjana, N. E., & Zhang, F. (2015). High-throughput functional genomics using CRISPR–Cas9. *Nature Reviews Genetics*, *16*(5), 299–311.

Shalem, O., Sanjana, N. E., Hartenian, E., Shi, X., Scott, D. A., Mikkelsen, T. S., … Zhang, F. (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*, *343*(6166), 84–87.

Sonntag, F., Schmidt, K., & Kleinschmidt, J. A. (2010). A viral assembly factor promotes AAV2 capsid formation in the nucleolus. *Proceedings of the National Academy of Sciences*, *107*(22), 10220–10225.

Staals, R. H. J., Agari, Y., Maki-Yonekura, S., Zhu, Y., Taylor, D. W., Van duijn, E., … Shinkai, A. (2013). Structure and activity of the RNA-targeting Type III-B CRISPR-Cas complex of *Thermus thermophilus*. *Molecular Cell*, *52*(1), 135–145.

Suda, T., & Liu, D. (2007). Hydrodynamic gene delivery: Its principles and applications. *Molecular Therapy*, *15*(12), 2063–2069.

Thomas, C. E., Ehrhardt, A., & Kay, M. A. (2003). Progress and problems with the use of viral vectors for gene therapy. *Nature Reviews Genetics*, *4*(5), 346–358.

van Duijn, E., Barbu, I. M., Barendregt, A., Jore, M. M., Wiedenheft, B., Lundgren, M., … Heck, A. J. (2012). Native tandem and ion mobility mass spectrometry highlight structural and modular similarities in clustered-regularly-interspaced shot-palindromic-repeats (CRISPR)-associated protein complexes from *Escherichia coli* and *Pseudomonas aeruginosa*. *Molecular & Cellular Proteomics*, *11*(11), 1430–1441.

Waehler, R., Russell, S. J., & Curiel, D. T. (2007). Engineering targeted viral vectors for gene therapy. *Nature Reviews Genetics*, *8*(8), 573–587.

Wang, J., & Quake, S. R. (2014). RNA-guided endonuclease provides a therapeutic strategy to cure latent herpesviridae infection. *Proceedings of the National Academy of Sciences*, *111*(36), 13157–13162.

Wen, W. S., Yuan, Z. M., Ma, S. J., Xu, J., & Yuan, D. T. (2016). CRISPR-Cas9 systems: Versatile cancer modelling platforms and promising therapeutic strategies. *International Journal of Cancer*, *138*(6), 1328–1336.

White, M. K., & Khalili, K. (2016). CRISPR/Cas9 and cancer targets: Future possibilities and present challenges. *Oncotarget*, *7*(11), 12305.

Wiedenheft, B., Zhou, K., Jinek, M., Coyle, S. M., Ma, W., & Doudna, J. A. (2009). Structural basis for DNase activity of a conserved protein implicated in CRISPR-mediated genome defense. *Structure*, *17*(6), 904–912.

Wu, Y., Liang, D., Wang, Y., Bai, M., Tang, W., Bao, S., … Li, J. (2013). Correction of a genetic disease in mouse via use of CRISPR-Cas9. *Cell Stem Cell*, *13*(6), 659–662.

Wu, Y., Zhou, H., Fan, X., Zhang, Y., Zhang, M., Wang, Y., … Li, J. (2015). Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. *Cell Research*, *25*(1), 67–79.

Wu, Z., Asokan, A., & Samulski, R. J. (2006). Adeno-associated virus serotypes: Vector toolkit for human gene therapy. *Molecular Therapy*, *14*(3), 316–327.

Wu, Z., Yang, H., & Colosi, P. (2010). Effect of genome size on AAV vector packaging. *Molecular Therapy*, *18*(1), 80–86.

Xiao-Jie, L., Hui-Ying, X., Zun-Ping, K., Jin-Lian, C., & Li-Juan, J. (2015). CRISPR-Cas9: A new and promising player in gene therapy. *Journal of Medical Genetics*. jmedgenet-2014-102968

Yang, Y., Wang, L., Bell, P., McMenamin, D., He, Z., White, J., … Wilson, J. M. (2016). A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice. *Nature Biotechnology*, *34*(3), 334–338.

Yin, H., Xue, W., Chen, S., Bogorad, R. L., Benedetti, E., Grompe, M., … Anderson, D. G. (2014). Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nature Biotechnology*, *32*(6), 551–553.

Yousefi, B., Zarghami, N., Samadi, N., & Majidinia, M. (2016). Peroxisome proliferator-activated receptors and their ligands in cancer drug-resistance: Opportunity or challenge. *Anti-Cancer Agents in Medicinal Chemistry*, *16*(12), 1541–1548.

Yousefi, B., Samadi, N., Baradaran, B., Rameshknia, V., Shafiei-Irannejad, V., Majidinia, M., … Zarghami, N. (2015). Differential effects of peroxisome proliferator-activated receptor agonists on doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells. *Cellular and Molecular Biology (Noisy-le-grand)*, *61*(8), 118–122.

Yousefi, B., Azimi, A., Majidinia, M., Shafiei-Irannejad, V., Badalzadeh, R., Baradaran, B., … Samadi, N. (2017). Balaglitazone reverses P-glycoprotein-mediated multidrug resistance via upregulation of PTEN in a PPARγ-dependent manner in leukemia cells. *Tumor Biology*, *39*(10), 1010428317716501.

Zhang, F., Wen, Y., & Guo, X. (2014). CRISPR/Cas9 for genome editing: Progress, implications, and challenges. *Human Molecular Genetics*, *23*(R1), R40–R46.

Zhen, S., Hua, L., Takahashi, Y., Narita, S., Liu, Y.-H., & Li, Y. (2014). In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR/Cas9. *Biochemical and Biophysical Research Communications*, *450*(4), 1422–1426.

Zhen, S., Lu, J.-J., Wang, L.-J., Sun, X.-M., Zhang, J.-Q., Li, X., … Zhao, L. (2016). In vitro and in vivo synergistic therapeutic effect of cisplatin with human papillomavirus16 E6/E7 CRISPR/Cas9 on cervical cancer cell line. *Translational Oncology*, *9*(6), 498–504.

Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., & Trono, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *Journal of Virology*, *72*(12), 9873–9880.

Zhan, T., Rindtorff, N., Betge, J., Ebert, M. P., & Boutros, M. (2018). CRISPR/Cas9 for cancer research and therapy. *Seminars in Cancer Biology*, *55*, 106–119.

How to monitor and minimize off-target events during genome editing

The CRISPR-Cas9 genome editing system has seen exponential growth in adoption with broad applications from basic research to therapeutics. The system is composed of Cas9 nuclease and a target-specific guide RNA (gRNA) that as a complex induces a double-stranded DNA break at a desired location. CRISPR-mediated genome editing is an extremely powerful tool that enables researchers to create different cellular models by removing, adding, or altering sections of a DNA sequence in the genome in a wide range of different cell types and gene loci. While it can achieve high editing efficiencies, the CRISPR-Cas9 system can also cleave the target DNA at unintended locations (known as off-target events) that can result in undesired phenotypes or loss of functional gene activity, which is especially detrimental for therapeutic applications.

To minimize the occurrence of off-target events in applications of the CRISPR-Cas9 system, several factors must be evaluated including: (1) delivery format of the CRISPR-Cas9 system—the purified protein format of Cas9 nuclease offers the fastest clearance time upon delivery, decreasing the time for off-target edits to occur; (2) optimization of gRNA design—leveraging an *in silico* predictive tool to design and select gRNA with a high score and less predicted off-target events further decreases the potential for unintended edits; (3) the specificity of Cas9 enzyme—using a high-fidelity Cas9 enzyme with improved specificity further minimizes off-target events. While optimizing any one of these factors will help decrease the chance of off-target events, the best possible outcome is achieved when all three factors are considered.

Even with the optimization of each of these factors, off-target events cannot be completely eliminated, and thus still pose risks for the genome editing project. As a result, accurate detection and monitoring of off-target events is an important step in any genome editing project, especially if the end goal is a therapeutic application.

Here we discuss strategies to optimize the various factors that cause off-target events and describe an unbiased analysis system, TEG-seq, to help detect off-target events. We also describe the use of this system to screen for a high-fidelity Cas9 mutant with improved specificity.

Method to monitor and detect off-target events

To optimize the various factors that result in unintended edits, a robust analytical method is required to reproducibly measure off-target events. Over the years, several off-target analysis systems have been developed, most as *in vitro* methods [1-4] where the genome editing reaction is performed in tubes without cellular context (such as histones and other DNA-binding proteins), which may influence the off-target potential of a system. Several *in cellulo* detection methods have also been developed including GUIDE-seq (genome-wide, unbiased identification of double-stranded breaks (DSBs) enabled by sequencing), which is currently the most widely used method for off-target detection [5-6]. Unlike *in vitro* methods, the editing reaction in *in cellulo* methods is done directly in the cells where specific cellular context is represented. However, most of the currently available *in cellulo* detection methods are not sensitive enough to detect low-frequency off-target events.

To address the sensitivity limitation of GUIDE-seq, we developed a more targeted genome-wide off-target screening method: TEG-seq (tag-enriched GUIDE-seq) [7]. In this method, specific 5′ phosphorylated primers are used for PCR amplification and differential marking of amplicons containing a double-stranded DNA tag (dsTag) inserted in DSB sites (Figure 1). With this alternative *in cellulo* method, only amplicons with a dsTag are phosphorylated at the 5′ end and can be ligated to a barcoded adaptor (BC-A) for further amplification and enrichment. These improvements significantly reduce nonspecific amplification and improve sensitivity of DSB detection. TEG-seq was also applied in experiments to detect and predict off-target events in engineered rats and mice embryos, which showed it is better than other methods [8].

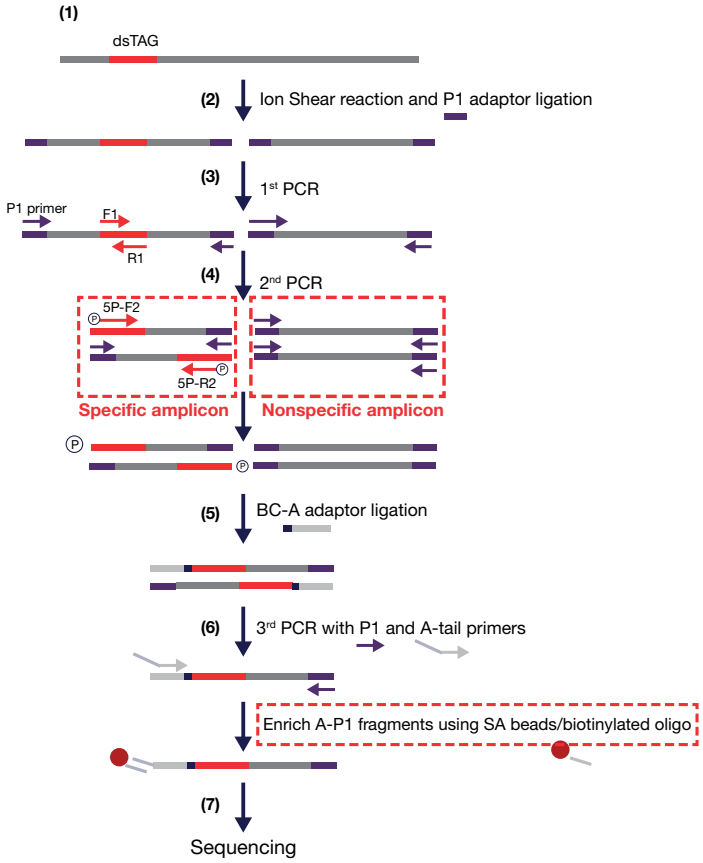

Figure 1. The TEG-seq workflow. (1) A double-stranded DNA tag (dsTag) is cotransfected with Cas9–gRNA ribonucleoprotein (RNP) or plasmid that expresses Cas9 and gRNA. The dsTag integrates at any site containing a DSB. (2) The genomic DNA is extracted and fragmented to 200–600 bp using enzyme-based Ion Shear™ chemistry. The P1 adaptor is ligated to the fragmented genomic DNA. (3) The first PCR is performed in separate tubes using P1/F1 for the forward primers and P1/R1 for the reverse primers. (4) In the second (nested) PCR, 5′ phosphorylated primers (5P-F2 and 5P-R2) are used that generate PCR products containing a 5′ phosphate only in the 5P-F2/P1 and 5P-R2/P1 products, but not the P1/P1 product. (5) A non-phosphorylated barcode adaptor (BC-A) is specifically ligated to the 5P-F2/P1 and 5P-R2/P1 products, but not to the P1/P1 product. (6) A third PCR is performed using P1/A-tail primers followed by a bead enrichment via a biotinylated capture oligo that is complementary to the A-tail primer. (7) The enriched amplicons are then subjected to next-generation sequencing.

In comparison to GUIDE-seq, TEG-seq detected more total off-target events under similar depth of next-generation sequencing (NGS) (Figure 2). Additionally, the read number for an individual target from TEG-seq is on average 10-fold higher than GUIDE-seq. This clearly indicates that TEG-seq is more sensitive and specific than GUIDE-seq. To further verify the off-target events detected by TEG-seq, we used the targeted amplicon-seq validation (TAV-seq) method for the quantification of off-target editing (Figure 3). The results showed that TEG-seq can detect low-frequency off-target events at 0.001% probability level as detected by TAV-seq.

As discussed later, TEG-seq also works efficiently in different cell types including primary T cells (Figure 6) and iPSCs (Figure 7) for the screening of off-target events that occur with clinically relevant gRNA targets, using Cas9–gRNA RNP format. Thus, TEG-seq is an unbiased genome-wide analysis method that effectively detects off-target events at low frequency in a wide range of cell types.

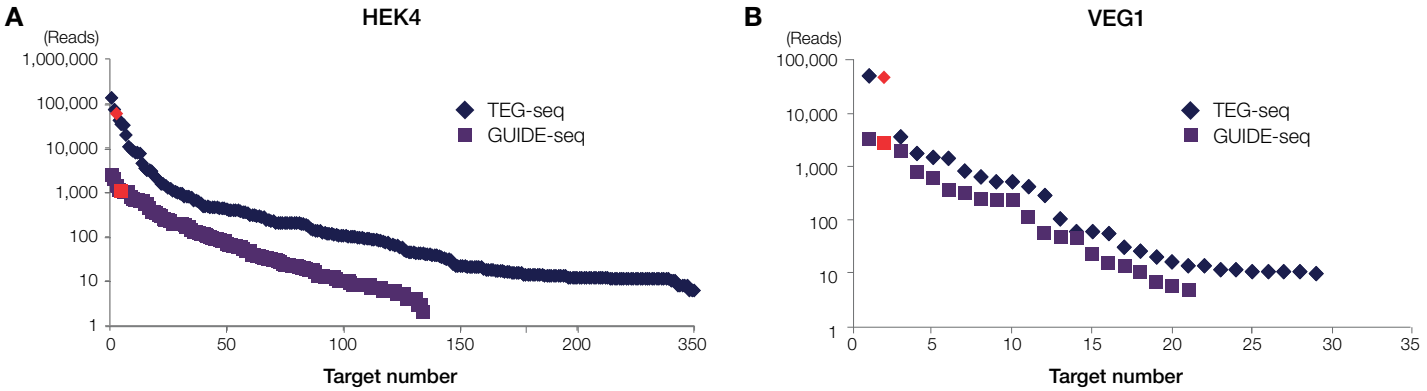

Figure 2. Comparison of TEG-seq and GUIDE-seq. Read numbers for **(A)** *HEK4* and **(B)** *VEG1* loci were plotted from individual on-target events (red) and off-target events from TEG-seq (blue) and GUIDE-seq (purple). The total off-target events detected by TEG-seq is 252 for HEK4 and 27 for VEG1, and the total off-target events detected by GUIDE-seq is 132 for HEK4 and 21 for VEG1. The read number for an individual target is also higher in TEG-seq than GUIDE-seq with a similar level of NGS sequencing depth. Cas9 and gRNA were delivered using plasmid format.

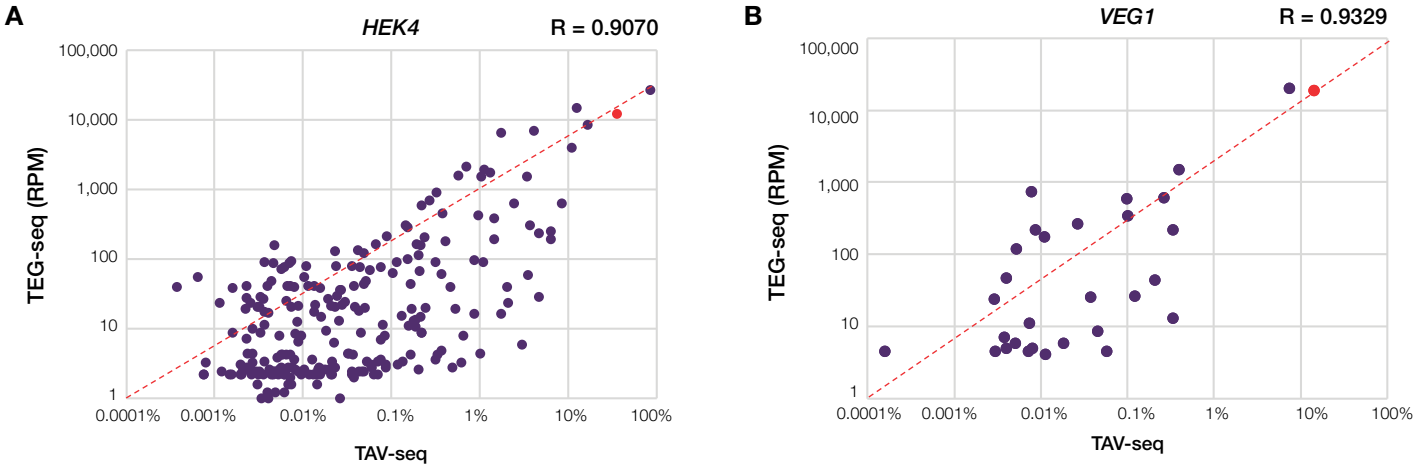

Figure 3. Comparison of off-target detection level between TEG-seq and TAV-seq. Off-target events for **(A)** *HEK4* and **(B)** *VEG1* loci were detected by TEG-seq and TAV-seq. Events detected by TEG-seq are plotted in reads per million (RPM) against the percentage of cleavage detected by TAV-seq. The correlation factor (R value) is indicated on the upper-right side of each graph. The on-target activity is indicated by red color.

Strategies to minimize off-target events through design

The CRISPR-Cas9 system is a powerful genome editing tool that only requires the presence of the Cas9 nuclease and gRNA. The Cas9–gRNA complex searches for NGG protospacer-adjacent motifs (PAMs) in the genome. When a sufficient match between the gRNA and the dsDNA target is detected, the Cas9 nuclease cleaves the DNA and produces a double-stranded break (DSB). While the CRISPR-Cas9 system typically cleaves the genome at the target site with high efficiency, cleavage at undesired sites with mismatches of one to several bases can occur. These undesired cleavage events are known as off-target effects and should be minimized to help prevent undesired side effects. Several factors can be leveraged to strategically generate a CRISPR-Cas9 system with minimal off-target events.

1. **Delivery format of the CRISPR-Cas9 system:** The delivery format of the Cas9–gRNA complex influences the system's clearance time and duration of nuclease expression. The use of Cas9–gRNA RNP complex containing purified Cas9 protein results in an initially high level of complex followed by rapid decay or clearance. As such, the Cas9–gRNA RNP has less time to cause undesired off-target effects. Therefore, the use of the Cas9–gRNA RNP format is recommended because it offers a high level of editing efficiency combined with faster clearance, resulting in minimal off-target effects.
2. **Optimization of gRNA design:** The use of high-scoring gRNA can help reduce the off-target events associated with the CRISPR-Cas9 system. Many genome editing design tools are available, including the Invitrogen™ TrueDesign™ Genome Editor, that enable researchers of all experience levels to easily design, select, and order reagents for accurate and successful gene editing experiments. Based on several criteria, including the probability of off-target activity, the design tool assigns a score to each gRNA. The higher the score, the less potential for off-target events. However, limitations such as the availability of PAM sites, proximity to target loci, and overall efficiency could preclude researchers from identifying gRNAs with low off-target events.
3. **High fidelity of the Cas9 enzyme:** The use of high-fidelity Cas9, an enzyme that is engineered to demonstrate improved specificity, can reduce the occurrence of off-target events. The CRISPR-Cas9 system is an extremely powerful tool that has completely transformed cell engineering as we know it. While the wild-type Cas9 nuclease can achieve high editing efficiency in a wide variety of cell types, the high editing efficiency of the wild-type Cas9 nuclease comes at an expense of increased off-target effects. The same properties that make the wild-type Cas9 nuclease so effective in cutting the genome at the desired locus inherently make it an effective tool at cutting the genome at undesired locations.

To improve the specificity of the wild-type Cas9 protein, we set out to engineer a high-fidelity Cas9 nuclease variant that would retain as much of the original on-target editing efficiency as the wild-type Cas9 nuclease, but demonstrate improved specificity at the same time. The next section outlines the steps we took to build a high-fidelity Cas9 variant that strikes the right balance between on-target editing and increased specificity.

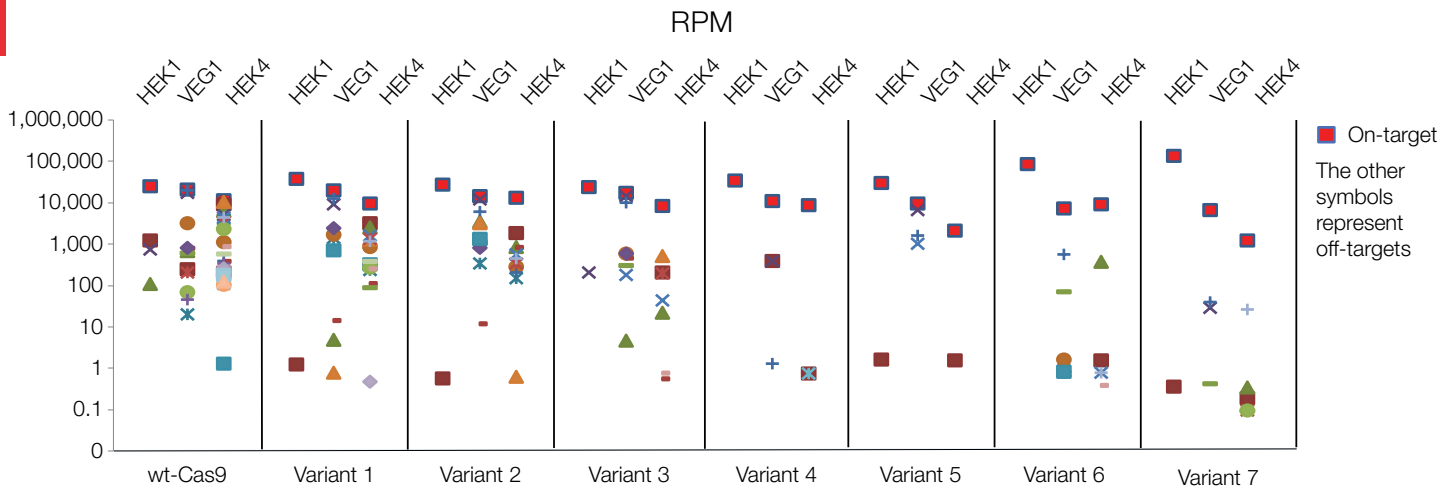

Figure 4. Genome-wide off-target screening for Cas9 variants using TEG-seq. Three commonly studied gRNAs (targeting *HEK1*, *VEGFA1*, and *HEK4*) that represent low, medium, and high potential off-target events were mixed with Cas9 protein and cotransfected in HEK293 cells. Invitrogen™ TrueCut™ Cas9 Protein v2 (wt-Cas9) was used as a control in a parallel screen with 7 high-fidelity Cas9 variants. Samples were barcoded using the Ion Xpress™ Barcode kit and sequenced using the Ion S5™ XL System. The in-house–developed Motif_Search tool was used for off-target analysis. RPM from each barcoded sample was calculated and plotted in log scale (y-axis). Red squares are on-target events, and all other markers are off-target events.

Screening of Cas9 mutants for improved specificity

Using TEG-seq, we set out to screen for a high-fidelity Cas9 variant with improved off-target profiles. Seven high-fidelity Cas9 candidates were identified and included in the screen from in-house engineered variants and published Cas9 candidates. Three commonly studied gRNAs targeting different loci (*HEK1*, *VEGFA1*, and *HEK4*) were selected and screened for off-target events with the Cas9–gRNA RNP delivery format in HEK293 cells. Shown in Figure 4 is an example of the Cas9 screening results where variant 4 outperformed other candidates. Variant 4 generated the least number of off-target events and lower read number (or number of actual cuts) at each individual off-target site compared to other Cas9 candidates in the panel.

Variant 4 was selected for further verification and compared to Sniper-Cas9 (a recently published high-fidelity Cas9 [9]) and a high-fidelity Cas9 from another supplier (Supplier I). Table 1 shows one example of the TEG-seq data on *HEK4*. Although Sniper-Cas9 and Supplier I generated less off-target events compared to TrueCut Cas9 Protein v2 (wt-Cas9), they both generated much higher off-target events compared to variant 4.* Data from Table 1 suggested that our high-fidelity Cas9 candidate, variant 4, generated 80% less off-target cleavage sites compared to TrueCut Cas9 Protein v2. Variant 4 became the new Invitrogen™ TrueCut™ HiFi Cas9 Protein.

* Total number of off-target sites: TrueCut Cas9 Protein v2 (wt-Cas9): 34, Sniper-Cas9: 18, Supplier I: 13, TrueCut HiFi Cas9 Protein (variant 4): 7.

Table 1. Reads per million (RPM) for off-target events detected by TEG-seq using *HEK4* gRNA in HEK293 cells.

Target	MM	Align sequence	PAM	wt-Cas9	Sniper-Cas9	Supplier I	TrueCut HiFi Cas9
On	0	GGCACTGCGGCTGGAGGTGG	GGG	25,950	112,147	57,977	41,848
Off-1	2	. . . . . GA.	GGG	23,050	26,225	6,608	691
Off-2	2	. . . . A . . . . . C . .	AGG	20,196	37,895	21,393	497
Off-3	2	. . . G. . . . . G.	AGG	18,843	8,898	1,074	7
Off-4	3	A. . T. . . . . A. .	GGG	16,942	3,890	24	0
Off-5	3	A. . . G. . . . . A.	TGG	10,310	5,654	629	0
Off-6	3	T. . . . . C. . . . . A. .	TGG	9,697	13,852	12,438	10
Off-7	3	A .G. . . . . G.	TGG	8,763	4,072	881	5
Off-8	4	. A. . . CA. . . . . A . . .	TGG	6,934	619	0	0
Off-9	3	. . . . TCA.	AGG	5,215	0	0	2
Off-10	2	. . . . . T. . . . . C.	AGG	3,113	976	0	2
Off-11	2	. . . . . G . . T	GGG	2,988	0	2,180	0
Off-12	2	. . . . . T . . . . . G	TGG	1,984	172	0	0
Off-13	2	. . . T . . . . . G . .	TGG	1,386	1,987	208	0
Off-14	2	. . . . – . . . . . g	AGG	1,272	0	0	0
Off-15	3	A . A . . . . . T	TGG	1,182	0	0	0
Off-16	3	CC . . . . . G	GGG	1,128	0	0	0
Off-17	3	T . . . . . T . . . . . A . . .	GGG	1,014	2	2	0
Off-18	3	. . T . . . CT	TGG	908	0	0	0
Off-19	3	. C . . . . . A . . . A	AGG	869	0	0	0
Off-20	3	. . . . . g . . A . . C	TGG	800	344	718	0
Off-21	3	. . . T . C . . A	GGG	744	0	0	0
Off-22	3	. . . . . A . A . . . . G	GGG	628	0	0	0
Off-23	4	A . . . . . A . . . . . GA	AGG	609	676	0	0
Off-24	3	. A . . . . . A . . . A	GGG	550	0	0	0
Off-25	3	T . G . . . . . a . . .	AGG	511	271	114	0
Off-26	2	. . . . . G . . . . . C . .	GGG	498	2,145	0	0
Off-27	4	. A . . . C.T. A	AGG	414	182	0	0
Off-28	3	. . . . . G . . . . . G . . A . .	GGG	320	0	353	0
Off-29	2	. . . . . A . . G	GGG	216	0	0	0
Off-30	4	. . . TG . . . . . CA	AGG	211	0	0	0
Off-31	2	. . A . . . . T	CAG	194	287	0	0
Off-32	3	. . . . . G . A . . . . –	TGG	135	0	0	0
Off-33	3	. C . . . . . G . . . . . G	GGG	80	0	0	0
Off-34	3	. . A . . . . G . . . . . G	GGG	42	0	0	0

To further evaluate the effectiveness of TrueCut HiFi Cas9 Protein in a more diverse set of cell types, particularly in therapeutically relevant primary T cells, we conducted additional off-target screening to compare TrueCut HiFi Cas9 Protein against TrueCut Cas9 Protein v2 (wt-Cas9) and enzyme from Supplier I. Twenty-one gRNAs were selected targeting four therapeutically relevant genes (*CD52*, *TRAC*, *TRBC*, and *PD1*) in T cells (Figure 5). Some of these gRNAs have been evaluated for CAR T cell gene therapy [10-12]. To demonstrate the difference in fidelity between the three Cas9 proteins, we intentionally included three gRNAs (TRBC-4, PD1-4, and PD1-5) that had low score from *in silico* analysis to represent gRNAs with high predicted off-target potential. In general, TrueCut HiFi Cas9 Protein generated much fewer off-target events and lower off/on ratio at individual off-target sites compared to TrueCut Cas9 Protein v2 and enzyme from Supplier I across different probability scales (Figure 5B).

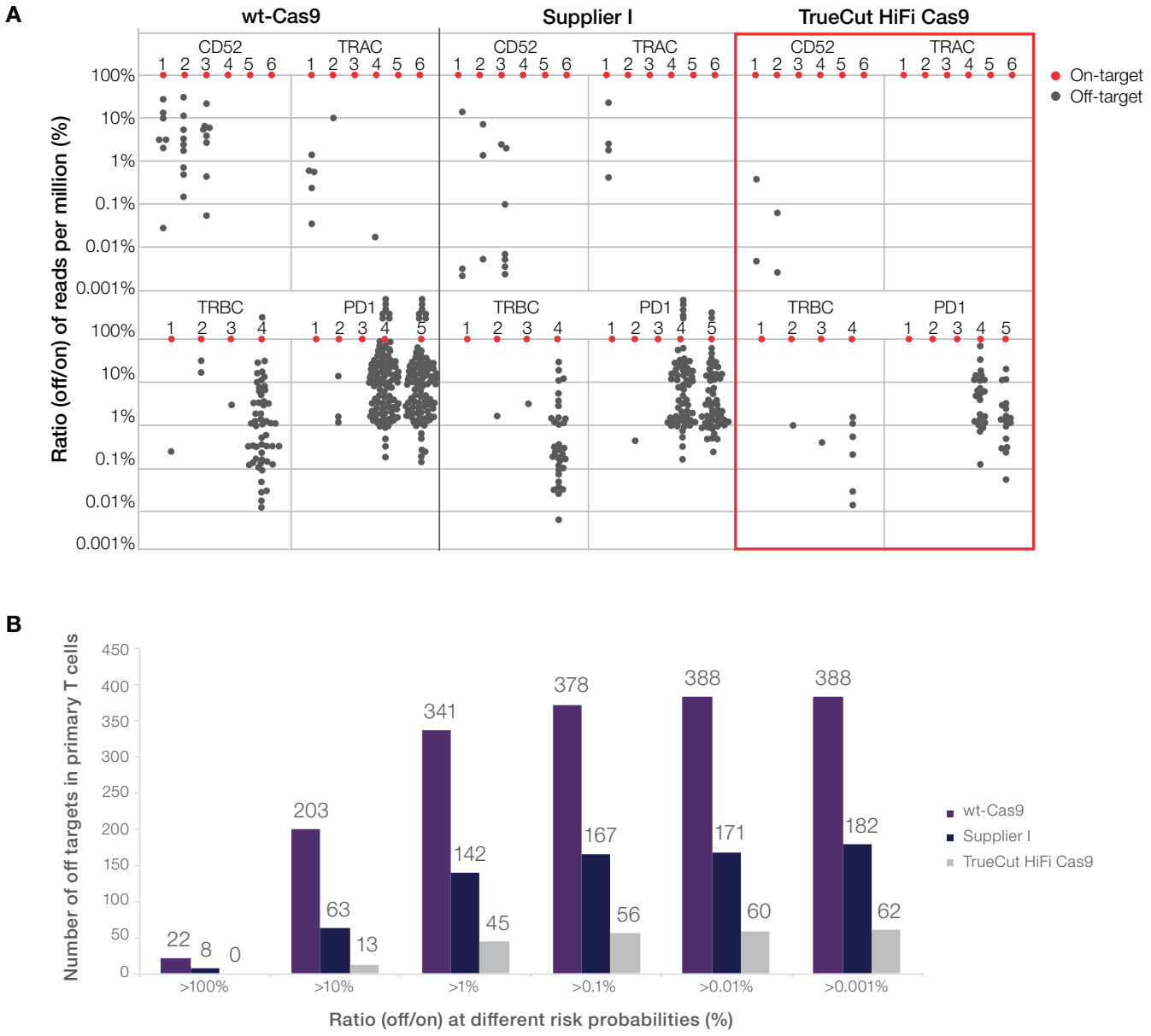

Figure 5. Genome-wide off-target events detected in T cells by TEG-seq and their off/on ratios at different percentile scales. (A) The 21 gRNAs targeting four genes *(CD52*, *TRAC*, *TRBC*, and *PD1*) were individually cotransfected with TrueCut Cas9 Protein v2 (wt-Cas9), enzyme from Supplier I, or TrueCut HiFi Cas9 Protein. The number underneath each gene name is the gRNA ID number. The off/on ratio was calculated based on RPM from individual off-target events divided by the RPM from the corresponding on-target events. Red dots are on-target events normalized to 100%. Gray dots are the off/on ratio. The gray dots above the red dots are off-target events with reads higher than on-target events. **(B)** The total number of off-target events in different percentiles based on risk probability.

Similar performance analysis was also conducted in iPSCs to demonstrate the difference in fidelity between the three Cas9 proteins. Genome-wide off-target screening was performed in iPSCs on 4 gRNAs: one gRNA targeting a commonly studied *HEK4* target, two gRNAs targeting two SNPs in the hemoglobin β subunit (*HBB*) gene that cause sickle cell disease, and one gRNA to knock out *BLC11A*, as a potential cure for sickle cell disease. As shown in Figure 6, off-target events were detected in 3 gRNAs (*HBB1*, *HBB2*, and *HEK4*) while no off-target events were detected from *BCL11A* gRNA (data not shown). Similar to its efficiency in other cell types, TrueCut HiFi Cas9 Protein also generated fewer off-target events and lower off/on ratio for individual off-target sites compared to TrueCut Cas9 Protein v2 (wt-Cas9) and protein from Supplier I.

A

HBB1			**Reads per million (RPM)**		
Target	Align sequence	PAM	wt-Cas9	Supplier I	TrueCut HiFi Cas9
On	CTTGCCCCCACAGGGGCAGTAA	CGG	141,922	258,580	284,917
Off1	TCA.....	GGG	126,583	970	132
Off2	T.....T.G.	CAG	13,100	15,871	1,229

HBB2			**Reads per million (RPM)**		
Target	Align sequence	PAM	wt-Cas9	Supplier I	TrueCut HiFi Cas9
On	CTTGCCCCCACAGGGGCAGTAA	AGG	431,212	356,556	452,904
Off1	...AA.....	TGG	246,927	3,153	319
Off2	..A..a.....C.....C.....	GGG	929	228	7
Off3	G.....A.	AGG	118	2,197	0

HEK4			**Reads per million (RPM)**		
Target	Align sequence	PAM	wt-Cas9	Supplier I	TrueCut HiFi Cas9
On	GGCACTGCGGCTGGAGGTGG	GGG	82,924	147,851	159,782
Off1	...G.....G.....	AGG	149,096	848,833	8,695
Off2	.....GA.....	GGG	151,950	390	105
Off3	A.G.....G.....	TGG	246,887	927	124
Off4	.....T.....C.....	AGG	118,633	119	65
Off5	.....A.....C...	AGG	12,949	8	0
Off6	.....G.....C...	GGG	3,005	21	0
Off7	T.....C.....A...	TGG	1,734	0	0
Off8	.....–G.....g.....	AGG	99	0	0
Off9	.....T.....G...	TGG	52	0	0

B

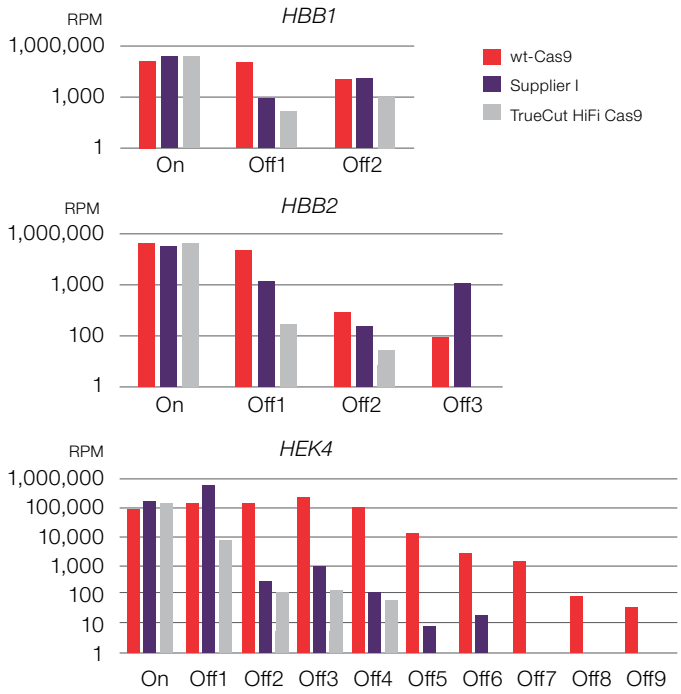

Figure 6. Off-target events detected in iPSCs with two gRNAs targeting two SNPs in *HBB* and one gRNA targeting *HEK4*. (A) TEG-seq data table containing the sequence and RPM for all on- and off-target events from each gRNA. **(B)** Bar graph representation of the RPM results for on- and off-target events from the table. TrueCut HiFi Cas9 Protein showed higher fidelity compared to TrueCut Cas9 Protein v2 and protein from Supplier I.

The high-fidelity Cas9 variant that we identified retains sufficient on-target editing efficiency for standard use in genome editing experiments while significantly reducing off-target events commonly observed when using the wild-type protein. The use of the TrueCut HiFi Cas9 Protein is especially beneficial when it is necessary to use a suboptimal gRNA option due to the limited availability of PAM sites near the cut site. TrueCut HiFi Cas9 Protein is also beneficial in applications where off-target events can result in undesired phenotypes or loss of functional gene activity.

Conclusion

The CRISPR-Cas9 system is a widely adopted genome editing tool with broad applications from basic research to therapeutics. While it can achieve high editing efficiencies, off-target events must be minimized to prevent undesired phenotypes or loss of functional gene activity, which is especially detrimental for therapeutic applications. As a result, accurate detection of off-target events is essential, and appropriate design choices must be made to minimize off-target events. Here we demonstrated the effectiveness of TEG-seq as an *in cellulo* analysis method, with 10-fold more sensitivity and specificity compared to GUIDE-seq. We later leveraged TEG-seq for the identification of a high-fidelity Cas9 (TrueCut HiFi Cas9 Protein) that exhibited superior off-target profiles compared to TrueCut Cas9 Protein v2 and another supplier's high-fidelity Cas9 enzyme in a wide range of cell types, including primary T cells and iPSCs.

Genome editing products and services

For more information on TrueCut HiFi Cas9 Protein, go to **thermofisher.com/cas9**

For gRNA design and ordering, go to **thermofisher.com/trueguide**

For TEG-seq services, go to **thermofisher.com/engineeringservices**

References

1. Crosetto N, Mitra A, Silva MJ et al. (2013) Nucleotide-resolution DNA double-strand break mapping by next-generation sequencing. *Nat Methods* 10:361–368.
2. Frock RL, Hu J, Meyers RM et al. (2015) Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol* 33:179–187.
3. Tsai SQ, Nguyen NT, Malagon-Lopez J et al. (2017) CIRCLE-seq: a highly sensitive *in vitro* screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nat Methods* 14:607–614.
4. Kim D, Bae S, Park J et al. (2015) Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods* 12:237–243.
5. Wang X, Wang Y, Wu X et al. (2015) Unbiased detection of off-target cleavage by CRISPR-Cas9 and TALENs using integrase-defective lentiviral vectors. *Nat Biotechnol* 33:175–179.
6. Tsai SQ, Zheng Z, Nguyen NT et al. (2015) GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol* 33:187–197.
7. Tang P-T, Ding B, Peng L et al. (2018) TEG-seq: an Ion Torrent–adapted NGS workflow for *in cellulo* mapping of CRISPR specificity. *Biotechniques* 65:259–267.
8. Anderson KR, Haeussler M, Watanabe C (2018) CRISPR off-target analysis in genetically engineered rats and mice. *Nat Methods* 15:512–514.
9. Lee JK, Jeong E, Lee J (2018) Directed evolution of CRISPR-Cas9 to increase its specificity. *Nat Commun* 9(1):3048.
10. Stadtmauer EA, Fraietta JA, Davis MM et al. (2020) CRISPR-engineered T cells in patients with refractory cancer. *Science* 367(6481):eaba7365.
11. Graham C, Jozwik A, Pepper A et al. (2018) Allogeneic CAR-T cells: more than ease of access? *Cells* 7(10):155.
12. Schumann K, Lin S, Boyer E et al. (2015) Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. *PNAS* 112(33):10437–10442.

Find out more at **thermofisher.com/crispr**

ThermoFisher SCIENTIFIC

Advancing CAR T cell therapy with CTS TrueCut Cas9 Protein

Advancing cell therapy products to reach the clinic starts with the selection of the right reagents. Not only do the reagents need to be of sufficient quality to comply with rigorous regulatory standards, but they also need to be manufactured with scalable processes to meet future clinical demands. Under the Gibco™ Cell Therapy Systems™ (CTS™) brand, Thermo Fisher Scientific offers a broad array of high-quality products specifically designed for use in cell therapy research applications. From media, reagents, growth factors, and enzymes to selection beads and devices, all Gibco™ CTS™ products are manufactured in compliance with the 21 CFR Part 820 quality system regulation and/or are certified to ISO 13485 and ISO 9001. The adherence to stringent quality standards allows for a seamless transition from bench to clinic.

Chimeric antigen receptor (CAR) T cell therapy, first approved by the U.S. Food and Drug Administration (FDA) in 2017, is a rapidly growing field in cancer therapy and involves the isolation and activation of T cells from a patient's blood for *ex vivo* genetic modification. The engineered T cells are then infused back into the patient to enable T cell–mediated cytotoxicity as treatment. CRISPR-Cas9 is one of the most commonly leveraged nonviral editing tools for engineering T cells for therapeutic applications. Thermo Fisher now offers the Gibco™ CTS™ TrueCut™ Cas9 Protein for use as an ancillary material in cell and gene therapy applications.

We take a deeper look at the CTS TrueCut Cas9 Protein, from its detailed quality specifications to its performance in primary T cells. We assessed the performance of the CTS TrueCut Cas9 Protein (CTS Cas9) against our flagship, research-grade Invitrogen™ TrueCut™ Cas9 Protein v2 (RUO Cas9) to confirm that the manufacturing scale-up of the enzyme to GMP standards had no significant impact on the product performance.

CTS TrueCut Cas9 Protein—quality control and specifications

CTS TrueCut Cas9 Protein is a GMP-grade recombinant Cas9 protein manufactured in compliance with standards for *Ancillary Materials for Cell, Gene, and Tissue-Based Products,* including USP <1043>, Ph.Eur. 5.2.12, and ISO 20399-1, -2, -3, following the principles of 21 CFR Part 820 in an FDA-registered manufacturing site. In addition to having extensive traceability documentation, the product is also subjected to aseptic manufacturing, extensive safety testing, and sterile filling to eliminate potential contaminants that may affect the safety of cell-based therapies. CTS Cas9 is provided in large pack sizes (2.5 mg and 5.0 mg) at a high concentration (10 mg/mL) in a transfection-ready format for electroporation; its specifications are shown in Table 1.

Table 1. Representative quality specifications for the CTS TrueCut Cas9 Protein.

Assay	Specification
Purity by RP-HPLC	≥95.0%
Purity by SDS-PAGE	≥95.0%
Aggregates	≤5.0%
Concentration	10 mg/mL
Identity by HPLC-DAD	Conforms
Identity by SDS-PAGE	Conforms
pH	7.0–7.8
Activity (*in vitro*)	≥90% cleavage of a DNA reference
Residual DNase	Less than the limit of quantification (<LOQ)
Residual RNase	<LOQ
Residual host-cell protein	<LOQ
Residual host-cell DNA	<LOQ
Endotoxin	<10.0 EU/mg
Sterility	No growth
Mycoplasmas	Negative

Stability of the CTS Cas9 and RNP complex

The CTS Cas9 and the ribonucleoprotein (RNP) complex (i.e., the complex of Cas9 protein with guide RNA) were assessed for their stability under various conditions. First, the CTS TrueCut Cas9 Protein was serially diluted over a range of concentrations, and then subjected to five freeze-thaw cycles. Cleavage activity was then measured using an *in vitro* cleavage assay. No significant change to the cleavage activity was observed after five freeze-thaw cycles, compared to the control samples without any freeze-thaw cycles, as seen in Figure 1. Additionally, the stability of the RNP complex was assessed at various time points (0, 10 min, 1 hr, 4 hr, and 16 hr) to simulate normal use conditions. The RNP complex was serially diluted, and cleavage activity was measured using an *in vitro* cleavage assay. No significant impact to cleavage activity was observed at different time points, as seen in Figure 2.

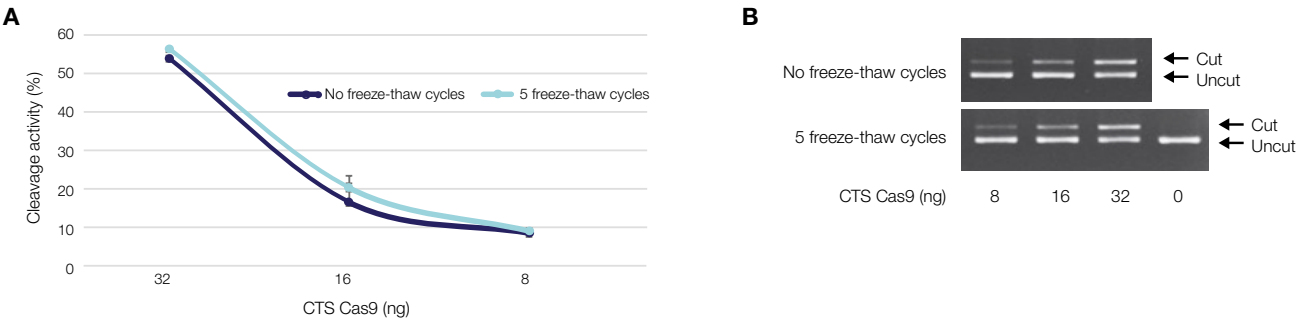

Figure 1. CTS Cas9 remained stable after five freeze-thaw cycles, as measured using an *in vitro* cleavage assay. (A) CTS Cas9 was serially diluted over a range of concentrations, and the cleavage activity was measured. The various amounts (8 ng, 16 ng, 32 ng, and 0 ng) of CTS Cas9 were incubated with excesses of gRNA (40 ng) targeting the *HPRT* gene, and a plasmid (300 ng) containing an *HPRT* sequence, for 10 minutes at 37°C. **(B)** The cleavage reactions containing uncut and cut plasmids were resolved on an agarose gel and quantitated using the Invitrogen™ iBright™ 1500 Imaging System. Reactions were performed in triplicate.

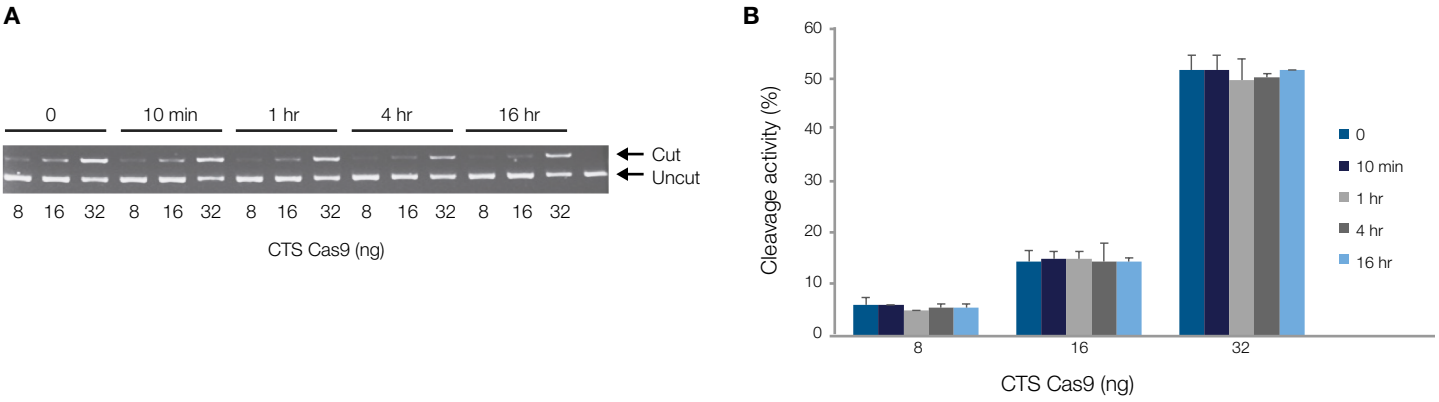

Figure 2. CTS Cas9–RNP complex maintained cleavage activity at room temperature over various time intervals. CTS Cas9 was serially diluted over a range of concentrations, and the cleavage activity was measured. The various amounts (8 ng, 16 ng, and 32 ng) of CTS Cas9 were mixed with an excess of gRNA (40 ng) targeting the *HPRT* gene, and incubated for different times from 10 minutes to 16 hours at room temperature. The samples were then incubated with an excess of plasmid (300 ng) containing an *HPRT* sequence, for 10 minutes at 37°C. **(A)** The cleavage reactions containing uncut and cut plasmids were resolved on an agarose gel and quantitated using an iBright Imaging System. **(B)** The cleavage activity plotted as a bar graph. Reactions were performed in triplicate.

Product consistency—CTS TrueCut Cas9 (CTS Cas9) vs. TrueCut Cas9 v2 (RUO Cas9)

As you transition from bench to clinic, it is important that your CTS TrueCut Cas9 (CTS Cas9) nuclease give you the same, consistent results as the TrueCut Cas9 v2 nuclease (RUO Cas9). To better assess the difference in activity between the CTS Cas9 and RUO Cas9, we leveraged an *in vitro* cleavage assay. The assay provided a direct side-by-side comparison of the proteins' cleavage activity without any cellular context. Both the CTS Cas9 and RUO Cas9 proteins were serially diluted for testing the cleavage activity. The *in vitro* assay results in Figure 3 showed that CTS Cas9 has cleavage activity comparable to that of the RUO Cas9 at all tested dosages.

Next, we wanted to confirm that the protein activity of CTS Cas9 observed *in vitro* is preserved when transferred into a cellular environment. Primary T cells were selected, as they offer the appropriate cellular context needed for CAR T cell therapy development. Seven gRNAs targeting four CAR T cell–related therapeutic genes—*TRAC, TRBC, PD1*, and *CD52*—were used in this experiment. The primary T cells were isolated and activated, then edited with CTS Cas9 and RUO Cas9 using the Invitrogen™ Neon™ Transfection System (10 µL kit). Cell viability and cleavage activity were measured and assessed at the genetic level using next-generation sequencing.

Figure 4A shows that there was high (>90%) cell viability post-transfection for both CTS Cas9 and RUO Cas9 proteins across all targets. This result showed that the CTS Cas9 had low toxicity to cells and was comparable to RUO Cas9. Additionally, no significant difference in cleavage efficiency was observed between the CTS Cas9 and RUO Cas9 (Figure 4B) across all target loci. The experimental results confirmed that CTS Cas9 maintains high editing efficiency and low cell toxicity comparable to RUO Cas9 across all target loci in primary T cells. The results from the cellular assays are consistent with the activity measured using *in vitro* assays.

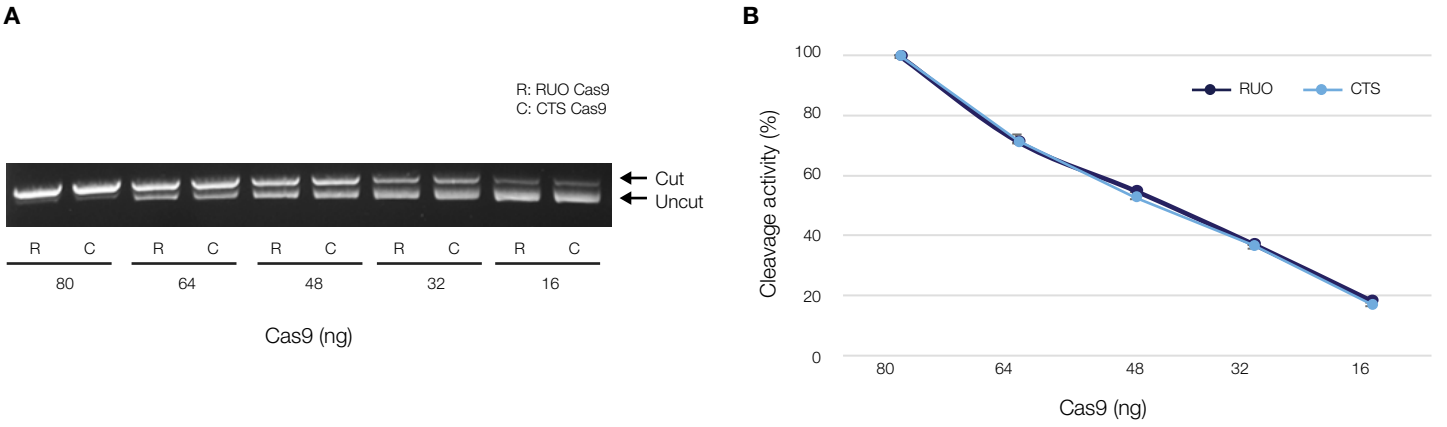

Figure 3. Comparable *in vitro* cleavage activity between CTS Cas9 and RUO Cas9. RUO Cas9 and CTS Cas9 were serially diluted (80 ng to 16 ng), and the cleavage activity was measured. Different amounts of RUO Cas9 (R) and CTS Cas9 (C) were mixed with excesses of gRNA (40 ng) targeting the *HPRT* gene, and a template plasmid (300 ng) containing an *HPRT* sequence, for 10 minutes at 37°C. **(A)** The cleavage reactions containing uncut and cut plasmids were resolved on an agarose gel and quantitated using an iBright Imaging System. **(B)** The cleavage activity plotted as a graph. Reactions were performed in triplicate.

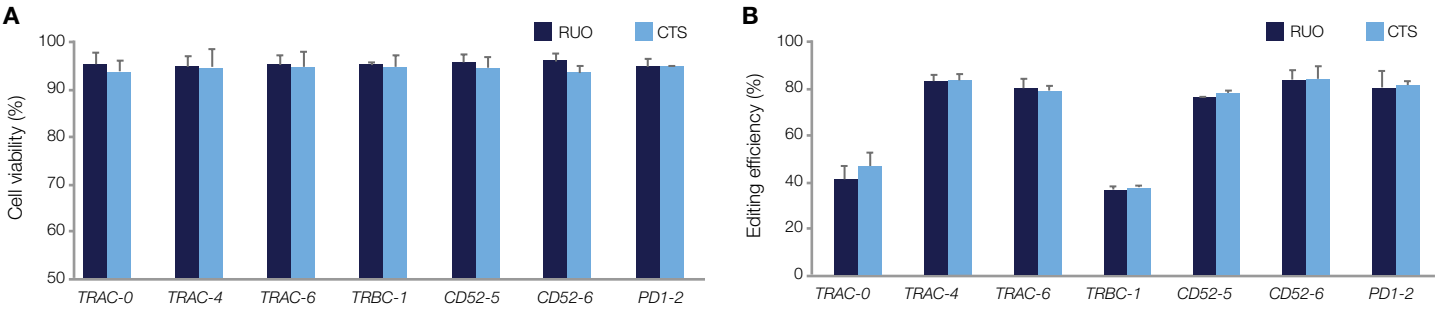

Figure 4. Comparable cell viability and editing efficiency between CTS Cas9 and RUO Cas9 in T cells. CTS Cas9 and RUO Cas9 (7.5 pmol) were each mixed with Invitrogen™ TrueGuide™ Synthetic sgRNA (7.5 pmol) to form two RNP complexes. Each RNP complex was used to transfect 500,000 T cells using the Neon Transfection System (10 µL kit). Cells were harvested after 72 hours of culture. **(A)** Cell viability was measured and analyzed by flow cytometry. **(B)** Summary of NGS-based analysis of editing efficiency as measured by targeted amplicon-seq validation (TAV) using an Ion Torrent™ NGS system for all target loci. All reactions were performed in triplicate.

Performance of CTS Cas9 at 10x process scale-up

To assess the performance of CTS Cas9 at a larger electroporation scale that is more representative of an autologous T cell development process, we used the larger-scale Neon Transfection System (100 µL kit).

Similar to the smaller-scale electroporation results in Figure 2, the cell viability from both Cas9 proteins remains high (over 90%) at the larger scale (Figure 5A). The results suggested that a 10x scale-up in the electroporation process did not negatively affect the performance of the CTS Cas9. The performance of the CTS Cas9 protein was comparable to that of RUO Cas9 at both electroporation scales.

HDR-based knock-in efficiency of CTS Cas9

Given the importance of precise genome editing for cell and gene therapy applications, we also evaluated the efficiency of homology-directed repair (HDR)-based knock-in (KI) of the CTS Cas9 in primary T cells. Four CAR T cell–related genes—*TRAC, TRBC, PD1*, and *CD52*—were selected, and one gRNA was used per gene. A single-stranded oligodeoxynucleotide (ssODN) was used as the donor DNA to evaluate the percentage of HDR. The electroporation was performed with the Gibco™ CTS™ Xenon™ Genome Editing Buffer, a buffer optimized for improved performance for HDR knock-in–based applications.

CTS Cas9 was comparable to the RUO Cas9 in total editing efficiency as well as percentage of indels and HDR, across all targets (Figure 5B). The total editing efficiency for both CTS Cas9 and RUO Cas9 was 60–90% and the HDR percentage was 40–70%. These results suggest that the CTS Cas9 offers high knock-in performance and results comparable to the RUO Cas9.

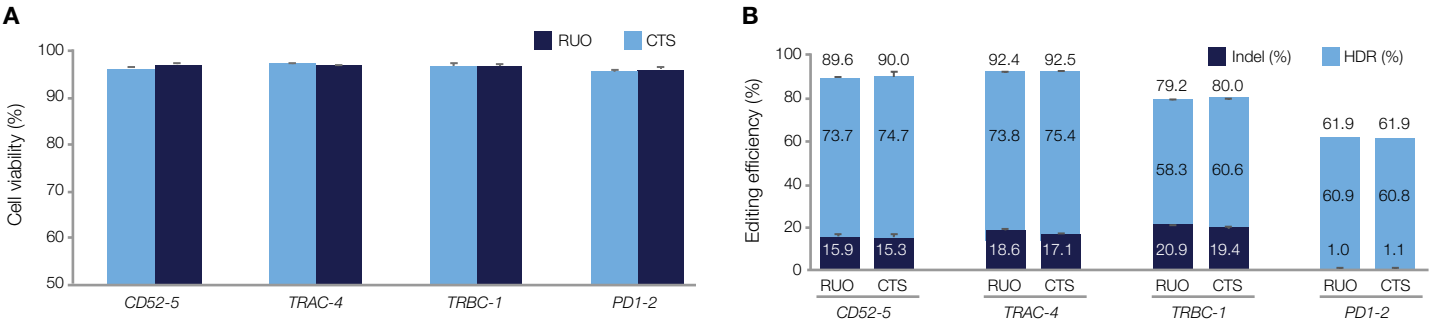

Figure 5. CTS Cas9 achieved high HDR-based knock-in efficiency at 10x electroporation scale. CTS Cas9 and RUO Cas9 (12.5 µg or 75 pmol) was mixed with TrueGuide Synthetic sgRNA (75 pmol) and ssODN donor (150 pmol) to form RNP-ssODN. Each RNP was transfected into 5,000,000 T cells using the larger-scale Neon Transfection System (100 µL kit). Cells were harvested after 72 hours of culture. **(A)** Cell viability was measured and analyzed by flow cytometry. **(B)** Summary of editing efficiency calculated as percentage of donor integration through HDR and indel as measured by targeted amplicon-seq validation (TAV) using an Ion Torrent NGS system. All reactions were performed in triplicate.

Consistency of CTS Cas9 performance across multiple material lots

To further evaluate the reproducibility of the manufacturing processes, three lots of CTS Cas9 were produced over the course of several months at full production scale. For each lot of CTS Cas9, a dilution series was made, the cleavage activity for each dose was measured, and the editing efficiency at *TRAC-4* and *CD52-6* in primary T cells was assessed. No significant lot-to-lot variation was detected at the target loci in T cells (Figure 6).

Conclusions

The application of CRISPR-Cas9 for the development of cell and gene therapy products holds great promise. In this work, we highlighted the extensive quality specifications and the consistent lot-to-lot performance of the CTS TrueCut Cas9 Protein. We also demonstrated performance of the CTS TrueCut Cas9 Protein comparable to that of our flagship, research-grade TrueCut Cas9 Protein v2, at different electroporation scales in primary T cells. This confirmed that the scale-up of manufacturing processes to GMP standards did not negatively affect the performance of the CTS Cas9. We also showed that a high level of HDR was achieved when using the Cas9 proteins with the optimized CTS Xenon Genome Editing Buffer. With the launch of the CTS TrueCut Cas9 Protein, you can now accelerate your therapeutics more confidently, knowing that Thermo Fisher can supply a high-quality product at the scale you need for your cell and gene therapy development.

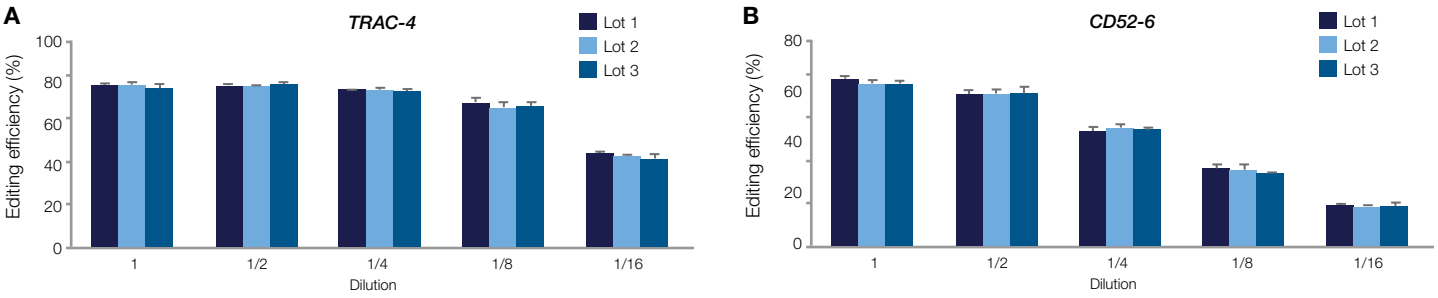

Figure 6. Consistent performance across multiple lots of CTS Cas9 in T cells. CTS Cas9 proteins from three manufacturing lots were serially diluted. Different amounts of CTS Cas9 (7.5 pmol for undiluted) were mixed with 7.5 pmol of **(A)** TRAC-4 or **(B)** CD52-6 TrueGuide Synthetic sgRNA and transfected into T cells using the Neon Transfection System (10 µL kit). The cells were lysed and amplified using a pair of primers flanking the cleavage sites, 72 hours after transfection. The amplicons were barcoded and sequenced using an Ion Torrent NGS system and analyzed using an editing efficiency analysis tool developed in-house. Reactions were performed in triplicate.

Learn more at **thermofisher.com/ctscas9**

gibco