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CRISPR-Cas9 for cancer therapy: Opportunities and challenges

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ABSTRACT

Cancer is a genetic disease stemming from cumulative genetic/epigenetic aberrations. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9-mediated genome editing technology has been extensively applied in various cell types and organisms, both in vitro and in vivo, for efficient gene disruption and gene modification. CRISPR-Cas9 has shown great promise for the treatment of cancer. However, despite its advantages and tremendous potential, numerous challenges, such as fitness of edited cells, editing efficiency, delivery methods and potential off-target effects, remain to be solved for completely clinical application. Here, we present the potential applications and recent advances of CRISPR-Cas9 in cancer therapy, and discuss the challenges that might be encountered in clinical applications.

1. Introduction

In recent years, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) -Cas9-mediated genome editing technology, as a versatile editing tool, has attracted the attention of researchers worldwide [1–4]. The CRISPR-Cas9 system consists of single guide RNA (sgRNA) and DNA endonuclease Cas9, with the former directing the latter to specific DNA sequences to cut double-stranded DNA site-specifically (Fig. 1) [5]. Currently, CRISPR-mediated genome editing has been extensively applied in various cell types and organisms to specifically edit single or multiple target genes using sgRNA for site-specific recognition [6–8]. Compared with its predecessors, such as zinc-finger nucleases (ZFNs) and transcription activator like effector nucleases (TALENs) [9,10], CRISPR-Cas9-mediated genome editing is more flexible and efficient owing to its simpler and more accurate Watson-Crick base pairing between sgRNA and target DNA (Fig. 1 **a**) [11]. Once Cas9 binds and cleaves the target DNA sequences, double-strand breaks (DSBs), located at approximately −3 nucleotides before the protospacer adjacent motif (PAM) sequence, are introduced in the genome sequence of interest and then the DNA repair machinery is initiated via the less-frequent homology-directed repair (HDR) or the predominant non-homologous end joining (NHEJ) (Fig. 1 **b**). HDR uses donor DNA template to precisely repair DSBs for gene modification with low efficiency, whereas NHEJ frequently results in genomic insertions or deletions (indels) for gene disruption with high efficiency [5,7]. Also, the CRISPR-Cas9 system can retarget new DNA sequences and introduce multiple double-strand breaks (DSBs) easily, allowing for more sophisticated gene editing via the simple alterations of the sgRNA sequence and the simultaneous expression of different sgRNAs, respectively [12]. Furthermore, activation or inhibition of the target gene transcription is achieved by the fusion of the inactive Cas9 mutant with various effector domains, termed CRISPRa and CRISPRi, respectively [13,14]. More recently, some Cas9 family members are found to have the ability to target RNA in addition to DNA [15–17]. Various RNA-targeting Cas9 (RCas9) systems have been set up and opened up novel applications such as gene expression silencing at the post-transcriptional levels [15,18], intracellular transcript imaging [19], and combatting RNA virus infection [20].

Cancer is a genetic disease stemming from cumulative genetic/epigenetic aberrations. Due to the complexity of its mechanism, current cancer therapies are limited, emphasizing the need for alternative therapeutic approaches. Technological advances, such as CRISPR-Cas9-mediated genome editing technology, allow one to precisely manipulate almost any given genomic sequence, enabling functional elucidation of genes involved in carcinogenesis and correction of cancer-causing mutations [6,11,21,22]. Thus, this technology holds potential to treat diseases including cancer [23,24]. However, despite its advantages and potential, how CRISPR-Cas9 editing tools are efficiently

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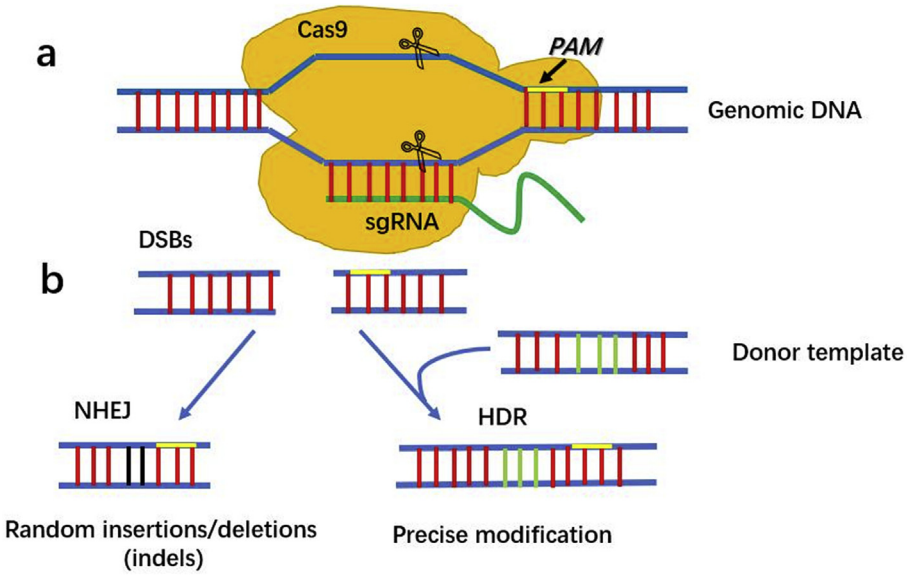

Fig. 1. The mechanisms of CRISPR/Cas9-mediated genome editing. a. The sgRNA guides the Cas9 nuclease to specific genomic sequences via standard Watson Crick base pairing. Then, Cas9 nuclease introduces double-stranded breaks (DSBs) close to the protospacer adjacent motif (PAM). **b.** DSBs can be repaired by two DNA repair pathways. One is the error-prone non-homologous end joining (NHEJ), resulting in small random insertions or deletions (indels) for gene knockout experiments. Another is the precise homology-directed repair (HDR), resulting in precise genome modification at the site of the DSBs for gene knock-in experiments using a homologous donor template. DSBs, double-stranded breaks; sgRNA, single-guide RNA; PAM, protospacer adjacent motif.

delivered to targeted cells in vivo and how to avoid or reduce unintended off-target effects remain major challenges, which are crucial for its clinical applications [25,26]. In this review, we present the potential applications and recent advances of CRISPR-Cas9 in cancer therapy. Also, we will discuss the challenges that might be encountered in clinical applications.

2. Application potentials of CRISPR-Cas9 in cancer therapy

2.1. (Epi)genome editing of cancer cells for therapeutic purposes

Given that cancer is a genetic disease stemming from cumulative genetic/epigenetic aberrations, it is rational for us to envisage that correcting the oncogenic genome/epigenome aberrations through CRISPR-Cas9 might represent a promising therapeutic strategy against cancer (Fig. 2 **a**).

In a cellular model of bladder cancer [27], for example, the expression of sgRNA and Cas9 was controlled by a cancer-specific hTERT (human telomerase reverse transcriptase) promoter and an urothelium-specific hUP II (human uroplakin II) promoter, respectively. Based on this method, Cas9 and sgRNA were co-expressed only in bladder cancer cells to activate suppressors such as *p21*, *E-cad* and *hBax*, which resulted in anti-proliferative and pro-apoptotic effects in a cancer cell-specific way. A study by Aubrey et al. showed that knockout of MCL-1 gene in human Burkitt lymphoma (BL) cells induced the apoptosis of BL cells at a higher frequency through the lentiviral CRISPR-Cas9 system, thereby leading to obvious tumor growth impairment in a human BL xenograft model via repeated induction of sgRNA [28]. Similarly, PKC (protein kinase C), as a tumor suppressor, was impaired in patient-derived colon cancer cell lines. With the CRISPR technology, the correction of PKC mutation reduced tumor growth in a xenograft model [29]. Interestingly, knock-in of a suicide gene HSV1-tk via Cas9 into the chromosomal breakpoints of the fusion genes caused cell death and decreased tumor size in human prostate and liver cancer models [30]. These results demonstrate that knockout of genes involved in the proliferation and survival of cancer cells remarkably reduces cancer cell growth and promotes apoptosis, thereby inhibiting tumor growth. Alternatively, via CRISPR-Cas9-mediated homologous recombination, knock-in of a therapeutic transgene at a desired location induce cancer cell death. Besides, this technology can also be adapted to modulate cancer epigenome, suggesting the feasibility as a promising epigenetic therapy [31].

Due to the heterogeneity of cancer, genomic aberration profiles are different not only in tumors among patients, but also in tumors during different stages or from different sites within a patient, which renders (epi)genome manipulation in cancer highly challenging. Moreover, this strategy requires fairly high editing efficiency because the unedited cells possess growth advantage over the edited ones. The former proliferates more rapidly than the latter, nullifying the therapeutic effect quickly. Also, it is of importance to select the appropriate delivery methods, especially in vivo delivery.

2.2. Combating carcinogenic virus infection

Carcinogenic virus infection is a critical factor in the occurrence of cancer, such as human papillomavirus (HPV) in cervical cancer, hepatitis B virus (HBV) and hepatitis C virus (HCV) in liver cancer, and Epstein-Barr virus (EBV) in nasopharyngeal carcinoma, Hodgkin's lymphoma and Burkitt's lymphoma. Due to the initial antiviral role as bacterial adaptive immunity, the CRISPR-Cas9 system has inherent potential for the defense and clearance of viral infection. Utilizing the viral genome-specific Cas9-sgRNA, viral oncogenes can be directly targeted and eliminated as well as the genes required for viral maintenance and replication, all of which contribute to mutations in the viral genome, suppress viral oncogene expression and ultimately induce cancer cell death (Fig. 2 **b**). As such, combating viral infection and eliminating these pathogens, can interrupt or even reverse carcinogenesis, which brings dawn to virus-related cancer patients.

Cervical cancer is caused mainly by HPV. The expression of HPV oncoproteins E6 and E7 induces normal cells to undergo malignant transformation and then maintain malignancy [32,33]. Some studies have confirmed that targeting the E6 and E7 genes and their promoters with the CRISPR-Cas9 achieved the inhibition of cervical cancer growth and reversal of the malignant phenotype [34,35]. However, due to the short E6 and E7 genes, selecting a unique target sequence can be challenging. Therefore, targeting these oncogenic viral genes with higher specificity can be a powerful strategy for cervical cancer.

The occurrence of hepatocellular carcinoma (HCC) is closely related to persistent HBV infection of hepatocytes. The dsDNA in the HBV virion is transformed into double-stranded covalently closed circular (ccc)DNA in the cell nucleus as a template to produce viral pre-genomic RNA and protein-coding mRNAs. Previous studies have demonstrated that CRISPR-Cas9-mediated HBV DNA editing and deletion can effectively inhibit virus production in both cells and mouse models [36–39]. However, these constructional models are not exactly analogous to the established persistent HBV infection in humans where HBV cccDNA

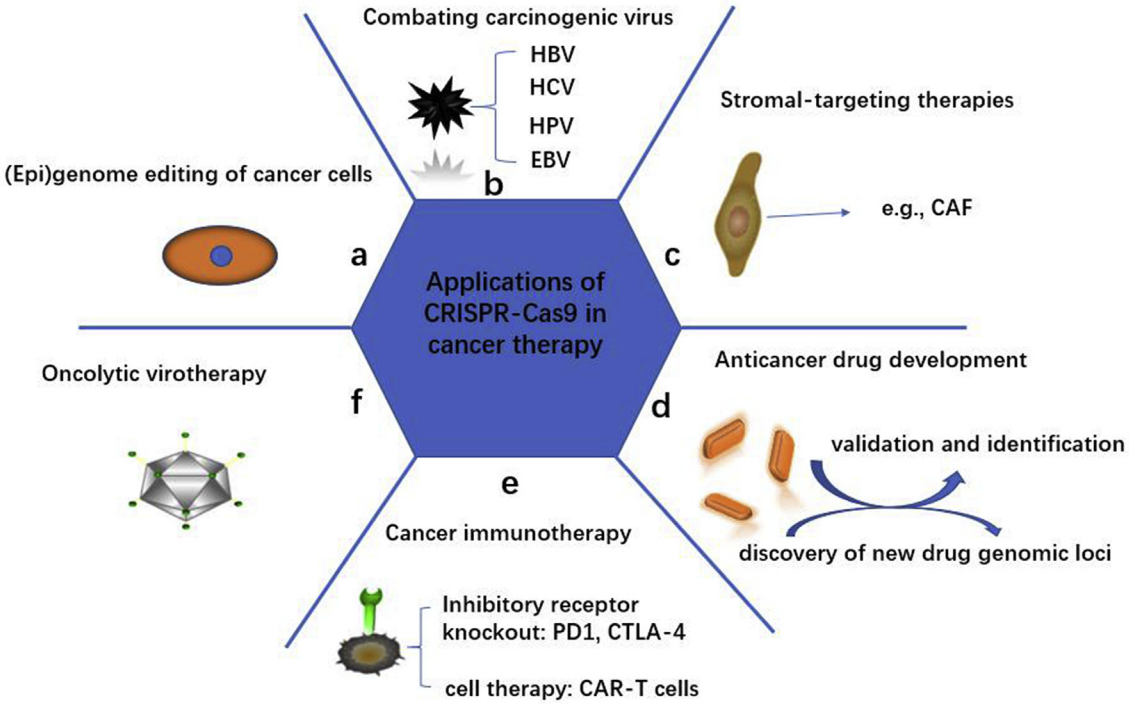

Fig. 2. Application potentials of CRISPR-Cas9 in cancer therapy. a. (Epi)genome editing of cancer cells for therapeutic purposes. Knockout of genes involved in the proliferation and survival of cancer cells remarkably reduces cancer cell growth and promotes apoptosis, thereby inhibiting tumor growth. **b. Combating carcinogenic virus infection.** Utilizing the viral genome-specific Cas9-sgRNA, viral oncogenes can be directly targeted and eliminated as well as the genes required for viral maintenance and replication, all of which contribute to mutations in the viral genome, suppress viral oncogene expression and ultimately induce cancer cell death. **c. Stromal-targeting therapies.** CRISPR-Cas9 can be used for the reprogramming of tumor stroma to achieve anticancer effects. **d. Anticancer drug development.** With CRISPR-Cas9 technology, specific drug targets are validated, resistance genes are identified and the new drug genomic loci in cancer is discovered. **e. Cancer immunotherapy.** The knockout of inhibitory receptors including PD1 and CTLA-4, using CRISPR-Cas9 technology, may be crucial to improve the efficacy of cancer immunotherapy based on T cells. Another strategy that has shown promise is cell therapy (e.g., CAR-T cell therapy). **f. Oncolytic virotherapy.** Some viruses can be genetically engineered to efficiently replicate within the host, specifically infect and kill cancer cells, and subsequently induce anticancer immune responses.

largely exists in hepatocytes. To address this, the researchers used an HBV-encoding plasmid to produce large amounts of HBV cccDNA in mice, which is more realistic to simulate chronic HBV infection in vivo [38]. Anti-HBV CRISPR was introduced by tail-vein hydrodynamic injection and significantly reduced HBV cccDNA in the liver [38]. Also, targeting of HBV antigen-encoded regions via CRISPR-Cas9 inhibited HBV replication and played an antiviral effect [40,41]. As mentioned above, the current treatment only partially scavenges the virus. The key to eradicating HBV from infected hepatocytes is the cccDNA molecule, indicating that further studies are still needed.

In addition to HBV, HCV also promotes the development of HCC. Price et al. engineered the Francisella novicida Cas9 (FnCas9) to precisely target the viral RNA genome of Hepatitis C virus (HCV) via an RNA-guided RNA recognition, which decreased viral protein production and suppressed HCV infection [20]. Because FnCas9 can target both RNA and DNA, this versatile endonuclease may be amenable to combat multiple types of viruses simultaneously, such as both HBV and HCV.

Moreover, EBV correlated to a variety of malignancies, such as nasopharyngeal carcinoma, Hodgkin's lymphoma and Burkitt's lymphoma [42,43]. EBV mainly infects B cells and epithelial cells. Targeting the EBV genome with CRISPR-Cas9 was proven to be feasible [44–47]. For instance, in a Burkitt's lymphoma patient-derived B cell line with latent EBV infection, seven anti-EBV gRNAs were introduced simultaneously by transfection to disrupt the EBV genome [44]. As a result, cell proliferation was inhibited and apoptosis was induced. Also, the viral load was reduced. Similarly, using the lentiviral transfection method, CRISPR-Cas9 was introduced to target the EBV genes that were necessary for episome maintenance [46]. The majority of the EBV genome in EBV-positive lymphoma cells was lost, even up to 95%.

These proof-of-principle studies demonstrate the potential of CRISPR-Cas9 in the prevention and treatment of cancer with viral infection. However, the high variability of viral targets presents a challenge to these therapeutic approaches and targeting multiple pivotal loci simultaneously in the viral genome may be crucial.

2.3. Stromal-targeting therapies

Cancer cells and stromal cells (e.g., fibroblasts) influence and remodel each other during carcinogenesis, which thus forms a dynamic and symbiotic relationship that fuels cancer progression and treatment resistance. CRISPR-Cas9 can be used for the reprogramming of tumor stroma to achieve anticancer effects (Fig. 2 **c**).

In one study, comparing the fibroblasts in normal ovarian tissue with fibroblasts in advanced ovarian cancer, Yang et al. found that genes involved in glutamine synthesis were overexpressed in cancer-associated fibroblasts (CAFs) [48]. Inactivation of glutamine synthetase (GS, an enzyme required for glutamine production) effectively inhibits the growth of cancer cells, whereas inactivation of GS in cancer cells does not affect the ability of CAFs to support the growth of cancer cells [48]. Thus, we propose that CRISPR-mediated matrix GS knockout may be cost-effective in inhibiting tumor growth and achieving anticancer effects. Similarly, pancreatic cancer is extremely abundant in stroma that has been regarded as a physical barrier controlling the effective penetration of chemotherapeutic drugs into the cancer cells [49,50]. Sherman et al. revealed that a key regulator of pancreatic stellate cells is the vitamin D receptor (VDR) [51]. In a tumor model, VDR activation caused the reprogramming of reactive stroma and reduced fibrosis-

associated inflammatory markers, which contributed to increasing drug (gemcitabine) availability and inhibiting cancer progression [51]. Thus, the use of CRISPRa technology can enhance VDR activation, stroma reprogramming, and effective drug penetration, to achieve anticancer effects.

Compared with cancer cells whose (epi)genetic heterogeneity and dynamics are hard to follow, stromal cells are easier to genetically manipulate. Moreover, genetically edited stromal cells may not suffer from fitness disadvantage as therapeutically edited cancer cells.

2.4. Anticancer drug development

Identifying novel cancer therapeutic targets is complementary to cancer therapy. Currently, the development of anticancer drugs needs to address key issues such as validation of drug targets, identification of drug-resistant genes, and discovery of new drug genomic loci (Fig. 2 **d**).

With CRISPR-Cas9 technology, specific drug targets are validated and resistance genes are identified. It was demonstrated that the exportin-1 cysteine 528 residue was the principal target of selinexor in cancer cells [52]. For example, the application of computer analysis and transcriptome sequencing techniques revealed that kinesin-5 A133P mutation may be the cause of ispinesib resistance [53]. To confirm this, Kasap et al. utilized CRISPR-Cas9 to generate the A133P mutation in HeLa cells, which resulted in increased resistance to ispinesib in mutated cancer cells [53]. It was also validated that kinesin-5 in cancer cells was a direct target of ispinesib [53]. Similarly, CRISPR-Cas9-mediated SMARCB1 gene knockdown conferred resistance to the chemotherapeutic drug doxorubicin, indicating that SMARCB1 was a drug resistance-related gene [54].

Moreover, CRISPR-Cas9 system has been widely applied to discover new drug genomic loci in cancer by targeting functional protein-encoded exons [55]. The CRISPR-Cas9 knockout library containing 64751 unique sgRNAs, was implemented in melanoma cells to seek new and more plausible candidate genes whose deletion conferred resistance to vemurafenib, a BRAF protein kinase inhibitor [56]. Also, CRISPR-Cas9 technology was applied to screen and identify therapeutic targets for genetic vulnerabilities of acute myeloid leukemia (AML) genomes [57]. KAT2A was selected as a candidate for downstream research and the inhibition of KAT2A was proposed as a therapeutic strategy in AML. These results are exhilarating.

Overall, the CRISPR-Cas9 tool offers great promise in validating drug targets, identifying drug resistance genes and finding new drug targets due to its high efficiency and relative simplicity. This will be conducive to the development of anticancer drugs.

2.5. Cancer immunotherapy

Owing to the clinical benefits it has achieved in multiple cancers, cancer immunotherapy is a rapidly expanding field nowadays. Instead of attacking tumor directly, cancer immunotherapy unleashes adaptive or innate immunity to attack cancer by various strategies (Fig. 2 **e**).

One strategy that has been successfully translated to the clinic is to unleash T cells against tumors by blocking immune checkpoints such as cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1) and programmed cell death 1 ligand 1 (PD-L1) [58–60]. Therefore, the knockdown of these genes including PD-1 gene and CTLA-4 gene, using CRISPR-Cas9 technology, may be crucial to improve the efficacy of cancer immunotherapy. CRISPR-Cas9 has recently been used in the genome editing of primary human T cells in which PD-1 expression were reduced by CRISPR-mediated indel mutations [61]. Also, Su et al. showed that precise knockout of the PD-1 gene remarkably decreased PD-1 expression via electroporation of plasmid-encoded sgRNA and Cas9 into human T cells, thus enhancing T cell immune responses to cancer cells and the ability to effectively kill cancer cells [62]. Clinical trials about CRISPR-mediated PD-1 gene knockout have been carried out in China for cancer treatment, such as castration resistant prostate cancer, muscle-invasive bladder cancer, and metastatic renal cell carcinoma (ClinicalTrials.gov Identifier: NCT02867345, NCT02863913, and NCT02867332), aiming to further evaluate the efficacy and safety of PD-1 knockout in T cells.

Another strategy that has shown promise is cell therapy (e.g., CAR-T cell therapy). It manipulates immune cells in vitro by genetic engineering, and then these cells were administrated to patients in order to combat against cancer. Previous studies have demonstrated the tremendous success in treating leukemia with anti-CD19 CAR-T cells [63,64]. Due to the excellent efficacy in hematological malignancies, some researchers are working on the development of CAR-T cells for solid tumors [65–68]. CRISPR-Cas9 system, built on the ease of design and flexibility, can disrupt multiple genomic sites simultaneously and thus yield universal CAR-T cells that are deficient in endogenous T cell receptor (TCR), HLA class I (HLA-I) and PD-1 [69]. As such, we propose that the prospective CAR-T cell therapy may evolve to be a more reliable, effective and safer therapy, which benefits from the combined gene modification involving endogenous T cell receptor, histocompatibility and inhibitory receptors. We can even speculate that gene editing may be deployed to introduce site-specific CAR itself through HDR.

2.6. Oncolytic virotherapy

Oncolytic virotherapy is an emerging therapeutic option for cancer patients [70,71]. Some viruses can be genetically engineered to efficiently replicate within the host, specifically infect and kill cancer cells, and subsequently induce anticancer immune responses (Fig. 2 **f**). Talimogene laherparepvec (T-VEC), derived from herpes simplex virus type 1 (HSV-1), is the first FDA-approved oncolytic viral therapy against advanced melanoma. It can specifically target cancer cells and promote granulocyte macrophage colony-stimulating factor (GM-CSF) production to enhance anticancer immunity [70]. In addition, T-VEC in combination with immune checkpoint inhibitors (e.g., anti-CTLA-4 and anti-PD-1) demonstrated the enhanced efficacy and safety [71,72]. Oncolytic virotherapy can also combine with conventional anti-cancer therapies such as chemotherapy, radiotherapy and targeted therapy [73–75]. In one study, for example, smac-mimetic compound therapy combined with oncolytic virotherapy (vesicular stomatitis virus) directly kill cancer cells and enhance anticancer immunity by reinvigorating exhausted $CD8^+$ T cells and promoting the accumulation and activation of $CD8^+$ T cells in the tumor [76].

Given that CRISPR-Cas9 has been widely applied in viral genome editing, it holds great potential in oncolytic virotherapy to enhance the efficacy and safety. For example, replacement of thymidine kinase (TK) gene with Ret finger protein (RFP) gene via CRISPR-Cas9 in vaccine virus resulted in more than 90% of RFP positive plaques [77]. Actually, the CRISPR-Cas9 system also efficiently produced virus with the deletion of N1L and A46R [78].

3. Challenges and perspectives

CRISPR-Cas9 technology has dramatically facilitated precise genome-targeted manipulation and has been extensively applied to cancer treatment in a wide variety of ways, opening new avenues for cancer therapy. However, numerous challenges concerning the efficacy and safety, remain to be addressed for completely clinical application, such as fitness of edited cells, editing efficiency, delivery methods and potential off-target effects (Fig. 3). Therefore, it is critical to identify the pivotal factors that influence the therapeutic outcome of CRISPR-mediated genome editing in cancer.

3.1. Fitness of edited cells

The first major challenge is the fitness of edited cells (Fig. 3 **a**). Compared to unedited cells, edited cells commonly possess fitness defect with weaker ability to proliferate and differentiate, resulting in

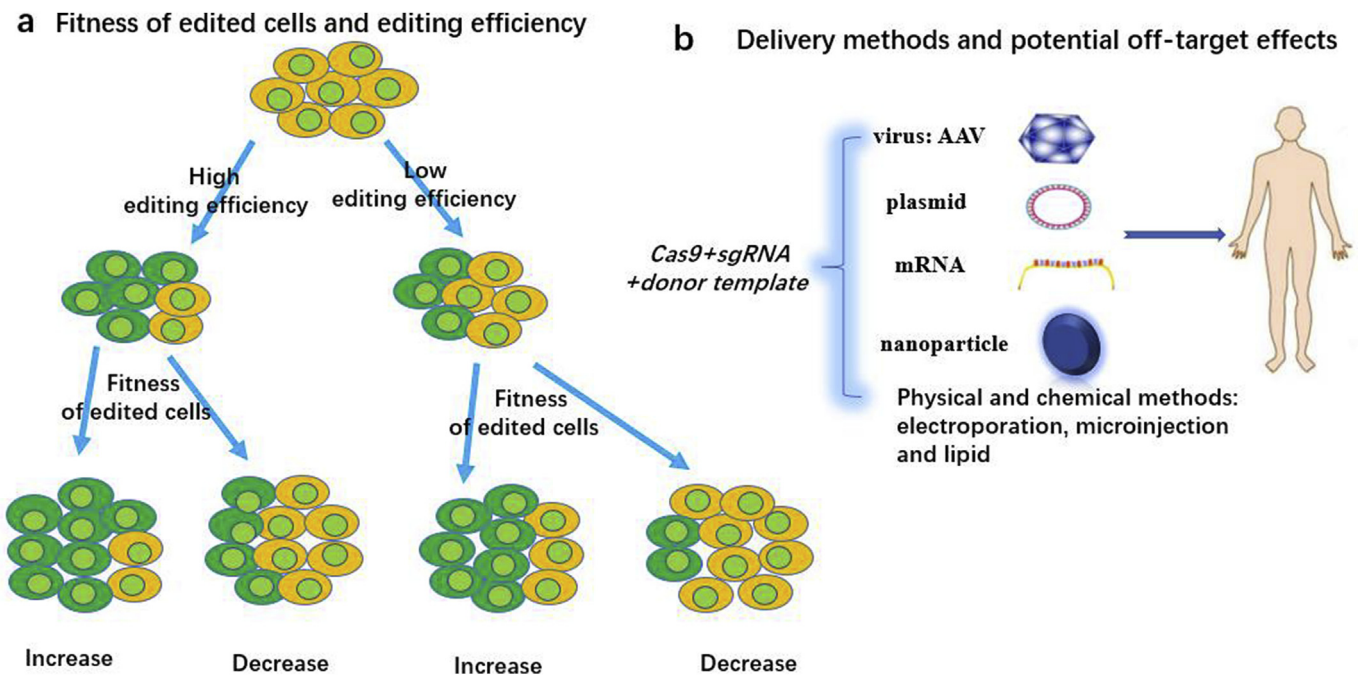

Fig. 3. The challenges of applying CRISPR/Cas9 technology. **a. Fitness of edited cells and editing efficiency.** When the editing efficiency is high, the number of cell populations carrying the desired genomic modifications will increase. However, if the editing efficiency is low, the number of edited cells will be less. Compared to unedited cells, edited cells commonly possess fitness disadvantage, resulting in lower therapeutic benefits. Conversely, if the edited cells have increased flexibility over unedited cells, rendering a selective advantage to the edited cells, the number of cells that need to be edited initially will be reduced to combat cancer. **b. Delivery methods and potential off-target effects.** Various delivery methods, such as virus, plasmid, mRNA, and nanoparticles, can be used for Cas9/sgRNA delivery. In addition, physical and chemical methods including electroporation, microinjections and lipid-mediated transfection, are available. But for in vivo delivery, it will benefit, particularly from non-viral vectors such as nanocarriers that are effective. Of note, the potential off-target effects should not be ignored.

unsatisfied therapeutic benefit. Conversely, if the edited cells have increased vitality over unedited cells, the number of cells that need to be edited initially will be reduced to combat against cancer, thus lowering the demand for high editing efficiency. However, particularly in cancer, cancer cells possess the advantage of growth, including rapid proliferation and longer survival. This, for CRISPR-Cas9, will require high editing efficiency. Therefore, this therapeutic threshold will be extremely high in that it demands modification of large numbers of cells directly. This problem can be partially solved by in vitro CRISPR-mediated genome editing. When the edited cells expand enough, they can be reinfused into the patients. However, spontaneous mutation of p53 could occur in edited cells and Cas9 can induce p53-mediated DNA damage response [79,80]. Therefore, it is pivotal to monitor the function of p53 to ensure that patient's cells have functional p53 before and after engineering.

3.2. Editing efficiency

The second major challenge is the genome editing efficiency (Fig. 3 **a**). The therapeutic efficacy of most genome editing will be enhanced as the editing rates increase. For CRISPR-Cas9, the activity of the DSB repair pathways plays a major role in controlling its editing rates. Due to the different repair mechanisms mediated by NHEJ and HDR, editing efficiency differences obviously exist not only in different cell types, but also in different cellular states. NHEJ is active throughout the whole cell cycle, while HDR preferentially operates during the S/G2 phase. Thus, NHEJ is generally more flexible and efficient for generating indels to knockout carcinogenic genes than HDR. The efficiency of HDR, as precise gene modification, is relatively sluggish depending on both homology arm length and DNA template type. HDR templates are typically single-stranded oligonucleotides or plasmids containing alleles and delivery of HDR templates has been accomplished with viral or non-viral vectors [81]. Theoretically, nanocarriers can also be exploited to deliver HDR templates analogous to Cas9 plasmids/proteins and sgRNA delivery. Based on this, we suppose that a nanocarrier could be designed to simultaneously deliver both HDR template and Cas9-sgRNA to targeted genes to improve HDR efficiency, ultimately increasing overall editing efficiency. Strategies to increase the efficiency of HDR or NHEJ repair with minimal off-target effects [25] will enhance the efficacy of cancer treatments.

3.3. Delivery methods, especially in vivo delivery

How CRISPR-Cas9 editing tool is safely and efficiently delivered to targeted cells in vivo is another huge challenge for rapid and broad implementation in clinic (Fig. 3 **b**). Ideally, successful gene modification is closely related to effective cell targeting, rapid clearance after the CRISPR system works, and minimal cytotoxicity. However, it is difficult to simultaneously achieve all of the above criteria with current delivery methods. As for CRISPR–Cas9 technology, Cas9 nuclease protein and sgRNA are required to be delivered into the nucleus of one cell. To further precise genome editing, an additional donor DNA template may also be needed.

Delivery of DNA (in the form of a plasmid or virus) or mRNA can result in the expression of Cas9 protein in the targeted cells and ultimately induce Cas9-mediated gene editing [82]. Theoretically, sufficient clinical benefits can be achieved by delivering the appropriate number of programmable nucleases in a transient manner that reduces the risk of potential off-target genome editing and immunogenicity-induced immune responses. To date, the most widely applied vectors in various studies are viral vectors such as adeno-associated virus (AAV) for the efficient in vivo delivery of CRISPR-Cas9 [83–85]. AAV has the following advantages: low immunogenicity, low risk of carcinogenesis and serotype-associated target cell specificity [86–88]. Studies have shown that each AAV serotype has its own preferential delivery efficiency for a particular cell type, which enables the researcher to select

the optimal serotype to enhance targeted delivery to the organ of interest [89]. Although AAV-mediated Cas9 delivery performs well in a laboratory setting, some concerns still exist with the use of AAV in the clinical setting. For instance, expression of important genes may be inadvertently disrupted when the transgene is integrated into the target genome. The Cas9 gene is 4–7 kB in size, and the amount of DNA that can be encapsulated in the virus is limited [90], since the maximum capacity of AAV is around 4.7 kb. Therefore, it is difficult to construct a virus that can encode such a large gene, its promoter and gRNA. Also, the long-term presence of viral-mediated delivery system (months or even years) may increase the accumulation of off-target cleavage. Better temporal control of CRISPR activity can be realized by delivery of purified Cas9–sgRNA ribonucleoproteins (RNP), as their rapid degradation (within a few hours) ensures a shorter active window with a higher editing efficiency compared to DNA- or RNA-based delivery methods [91]. Electroporation was widely utilized for RNP delivery [61], which has successfully introduced CRISPR-Cas9 RNP into cells in vitro. Other common methods for nucleotide transfection have been demonstrated for Cas9 RNP delivery such as microinjections and lipid-mediated transfection. The preformed RNP complex was microinjected into animal embryos to produce genome-edited animals with a success [92,93]. Since the Cas9-sgRNA complex itself is anionic, the cationic lipofection reagents can be used to deliver Cas9-sgRNA complex, which results in up to 80% genetic modification in cultured cells with higher specificity and about 20% in mouse inner ear hair cells [94].

The most suitable candidate vectors may be the non-viral in vivo delivery of nucleases in the direct mRNA or protein forms that abrogate the transcriptional process. Compared with plasmid DNA or viral vectors, non-viral delivery including nanoparticle-mediated delivery may have some potential advantages, enabling the more accurate control of the duration of dosing, the decreased long-term expression of nuclease, the reduced risk of off-target effects, and the minimization of potential side effects such as tumorigenesis [81]. All of these can support the rapid, safe and efficient genome editing in vivo. However, nanoparticle-mediated protein delivery is even more challenging than nucleic acid delivery owing to the tough process from packaging into small particles, to maintain biological activity, and then to prevent its degradation before entering the nucleus. Therefore, any non-viral delivery material should be well tolerated, biocompatible and non-immunogenic, and have the ability to deliver effective Cas9-sgRNA to the nucleus for genome editing.

3.4. Off-target effects

In addition, potential off-target effects deserve our attention and should not be ignored (Fig. 3 **b**). Previous studies have shown that the CRISPR-Cas9 system can frequently generate indels at undesired genomic loci [25,95]. Continued genetic modification will increase the risk of off-target cleavage and reduce the specificity of editing, possibly resulting in unwanted mutations and potential toxicity. To apply CRISPR-Cas9 in vivo safely, endonuclease-induced off-target events should be minimized because indel formation at unintended loci may affect cell viability or instead promote tumorigenesis. Intensive research has been conducted to limit off-target events and improve the specificity [25]. Possible methods for reducing the risk of Cas9-mediated off-target include the use of paired Cas9 nickases, truncated gRNAs with shorter protospacer complementary regions, and high-fidelity Cas9 endonucleases. Modification of the Cas9 protein to alter PAM preferences or enhance target DNA recognition can also be used to decrease off-target effects and thus enhance the on-target specificity [8,96–98]. Moreover, a synthetic switch was built to self-regulate Cas9 expression in both the transcription and the translation steps [99]. The synthetic switch could simultaneously inhibit transcription and translation, thus rapidly attenuating the Cas9 expression. The restricted Cas9 expression minimized the off-target effects while increasing high efficiency and on-target indel mutation. Also, the synthetic switch can be integrated into viral vectors for self-regulating Cas9 expression, which provides a new “hit and run” strategy for in vivo genome editing. Of note, various factors, such as the number, type and stage of editing cells, and the expression pattern of the genome editing system [81], may affect the specificity. Some explorations have been conducted to assess off-target effects and thereby enhance the genome editing specificity [8,100,101]. It is worth expecting that the future CRISPR-Cas9 technology will not only reduce the possibility of off-target, but also maintain the on-target efficiency and specificity.

4. Concluding remarks

Despite promising advances in the clinical application of CRISPR-Cas9 [102], it is still a long way to completely translate CRISPR-Cas9-mediated genome editing technology in human patients for targeting cancer-related genes. Notably, the ever-changing mutational profiles resulting from cancer evolution and heterogeneity render it hard to follow and rectify the “culprit” mutations, not only with CRISPR-Cas9 systems, but also with any (epi)genome editing tools currently available. Thus, it is imperative that further research should improve editing efficiency, confer more fitness to edited cells, increase the target specificity and decrease potential off-target effects. Moreover, the potential host immune response induced by CRISPR-Cas9 components remains to be resolved [103].

In summary, CRISPR-Cas9 technology, as a powerful editing tool, has tremendous therapeutic potential for increasing our anticancer approach, albeit with some challenges. We are optimistic that gene editing therapy will herald a new era in the field of cancer. Also, future cancer research should enable engineers, physicists, and chemists to work with clinicians and biologists for obtaining the state-of-the-art understanding of the various properties of cancer, including genetic and biochemical properties. We believe that the continuous advances and innovations in CRISPR-Cas9 technology will increase safety and effectivity of therapeutic strategies and bring the promise to patients with cancer in the future.

Conflicts of interest

The authors disclose no potential conflicts of interest.

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