Applications of CRISPR-Cas Enzymes in Cancer Therapeutics and Detection

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Summary

Cancer is a complex disease caused by combinations of cellular genetic alterations and heterogeneous microenvironments. The use of the robust and programmable CRISPR-Cas systems has greatly improved genome editing for precision cancer modeling and enabled multiplexed genetic manipulation for cancer treatment and mutation detection. In this review, we outline the current CRISPR-Cas toolkit, and discuss the promises and hurdles to translating this revolutionary technology into effective and safe clinical applications for cancer treatment and diagnosis.

Precision Genome Editing for Cancer Therapeutics

Cancer is a complicated and highly dynamic disease [1]. Tumorigenesis comprises a multistep process involving a complex interplay between cancer cells and host immune system [1, 2]. Over the past few decades, targeted therapies brought hope for the treatment of many types of cancer. But a common complication is that in a lot of patients the drugs eventually stop working, in part, owing to the complex mutational patterns in tumors and heterogeneity within the microenvironment [2]. Recent advances in **CRISPR-Cas** technologies offer great promise for precisely interrogating both cancer cells and host immune system via an ever-growing toolkit with improved specificity and off-switch.

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Resources

iv <http://www.guidescan.com/>

Expanding the CRISPR-Cas Toolkit

CRISPR-Cas systems are grouped into six types (I–VI) and two classes (Class 1: types I, III, and IV; Class 2: Types II, V, and VI) according to the numbers and sequences of cas genes associated with CRISPR arrays [3]. The type II CRISPR systems utilize a single DNA endonuclease, **Cas9**, to recognize and cleave dsDNA substrates. In contrast, the type I and type III systems encode a multi-Cas protein complex capable of **crRNA** binding as well as target sequence degradation [4]. Since the development of the RNA-programmable genome editing from the type II CRISPR-**Cas9** system in *Streptococcus pyogenes* (SpyCas9) (Figure 1A) [5], a number of CRISPR-Cas tools have been rapidly developed for the use of functional genomics in mammalian cells [6, 7], cancer target screening [8], cancer modeling [9], and therapeutics [8]. Their flexibility and potency surpass techniques such as **zinc finger nucleases (ZFNs)** and **transcription activator-like effector nucleases (TALENs)** [10]. An important property of CRISPR-Cas enzymes is the ease of changing targeting specificity by changing the sequence of the guide RNA. Unlike earlier genome editing technologies, there is no need to design a new protein for every new gene edited using the CRISPR-Cas system, making diverse applications simple [11].

A catalytically deactivated Cas9 (dCas9) has been repurposed for targeted gene repression (CRISPRi) [12, 13] and activation (CRISPRa) in a genome-wide fashion (Figure 1A) [14–16]. This and other chromatin-remodeling approaches using dCas9, such as methylation or demethylation, and acetylation, [17–21] allow epigenetic regulation of endogenous gene expression without creating DSBs and provide alternative strategies for targeting cancer epigenome and transcriptional programs [22]. In addition, gRNA engineering allows delivery of functional RNAs [23], as well as multiplexing and multimerization of proteins such as transcriptional regulators or epigenetic modifiers [24]. Other emerging and particularly exciting tools are base editors, which are generated by fusing dCas9 (or Cas9 D10A nickase) with cytidine deaminase [25, 26] or an evolved version of the tRNA adenine deaminase [27] that can mediate the conversion of G•C to A•T [25, 26] or A•T to G•C [27], respectively – without dsDNA cleavage (Figure 1A). Compared with the current CRISPR-Cas9 nuclease-mediated HDR method, the base editors can be used not only to correct cancer-associated mutations with higher efficiency and fewer undesired products [25, 27] but also to create STOP codons for efficient gene disruption without DSB formation [28, 29], and thus minimizes indels. Remarkably, an expanded PAM Cas9 variant (xCas9) with broad PAM compatibility and high DNA specificity was recently developed enabling targeting at previously untargetable sites using the CRISPR-Cas toolkit [30].

Programming CRISPR-Cas9 for enhanced accuracy

In addition to expanding the CRISPR toolbox with new molecular gadgets, identifying and minimizing off-target effects induced by CRISPR-Cas systems are important to accelerate the therapeutic applications of these nucleases [31]. CRISPR-mediated genome-editing requires multiple steps including Cas9 target recognition, binding and cleavage [6]. Over the past few years, several novel methods have been developed to study the target recognition and further characterize genome-wide specificities of CRISPR-Cas9. Using *in silico* prediction of potential off-target cleavage sites followed by experimental validation of **non-homologous end joining (NHEJ)**- induced indel mutations, these mismatched sites are

found to be mutagenized at similar frequencies as the intended ones (Figure 1B) [32–34]. Based on these findings and the molecular features of sgRNA, a number of online tools, such as the CRISPR Design Toolⁱ [32], E-CRISPⁱⁱ [35], CRISPRscanⁱⁱⁱ [36], GuideScan^v [37], and Cas-OFFinder^v [38] (Table 1), were designed to take into account the sequence similarity and other parameters for the prediction of potential off-target sites (Figure 1B).

Although the *in silico* and targeted sequencing method can predict some potential off-target sites, it has limitations to reveal all the possible sites in a genome-wide manner. Thus, in addition to simply whole-genome sequencing (WGS) of CRISPR-targeted cells, several unbiased approaches for genome-wide assessment of off-target sites have been developed (Figure 1B) [31], including cell-based integrase-defective lentiviral vector (IDLV) capture [39], genome-wide unbiased identification of DSBs enabled by sequencing (GUIDE-seq) [40], and high-throughput genome-wide translocation sequencing (HTGTS) [41], biochemical label-based BLESS [42] and BLISS [43] and in vitro screening assay such as CIRCLE-Seq [44] (Table 2). Importantly, the majority of the detected off-target sites identified by these genome-wide approaches were not predicted by some of the abovementioned algorithms, indicating that a comprehensive examination of off-targeted sites is still indispensable. Also, the effects of human genetic variation on creating and destroying PAMs and on- or off-target sites also need to be taken into account to minimize risk of adverse outcomes and treatment failure from CRISPR-based therapy [45].

Following the establishment of the Cas9 off-target mutagenesis landscape, enthusiasm grew quickly for programming the CRISPR-Cas9 system to enhance its specificity (Figure 1B). One approach is to rationally engineer Cas9 to construct different variants based on the structural information [46, 47] to reduce non-specific DNA contacts and others. Two variants, high-fidelity (SpCas9-HF1) [48] and enhanced specificity (eSpCas9(1.1)) [49] Cas9, exhibit precision genome-editing in human cells with reduced off-target mutations by both genome-wide and targeted sequencing methods. Further structural and biophysical analysis of these variants identified a non-catalytic domain, REC3, that is within Cas9 and is important for target complementarity and catalytic competence [50]. Disruption of this domain leads to a Cas9 variant (HypaCas9) that has hyper-accurate performance and without compromised efficiency in human cells [50].

Disabling Cas9 and reducing its off-target effects by anti-CRISPR system

Anti-CRISPR systems are an exciting new addition to the CRISPR-Cas toolkit (Figure 1C) [51]. The anti-CRISPR proteins were first discovered in a related group of *Pseudomonas spp.* Phages [52]. Anti-CRISPR proteins 1–5 (AcrF1–5) initially identified from these phages were shown to inactivate one type of CRISPR–Cas systems without perturbing the expression of *cas* genes or the production of mature crRNA molecules, suggesting that they can directly bind to and inactivate CRISPR–Cas machinery [52]. To date, 21 families of anti-CRISPR proteins that inhibit **type I and type II CRISPR-Cas systems** have been identified

ⁱ<http://crispr.mit.edu/>

ⁱⁱ<http://www.e-crisp.org/E-CRISP/designcrispr.html>

ⁱⁱⁱ<http://www.crisprscan.org/>

^v<http://www.rgenome.net/cas-offfinder/>

[51], for which some have well described mechanistic details. For example, the anti-CRISPR protein AcrIIC1 was shown to bind the CRISPR-Cas9 HNH endonuclease domain preventing conformational motion that leads to target cleavage to inhibit Cas9 (Figure 1C) [53]. In contrast, the anti-CRISPR proteins AcrIIC3 [53] and AcrIIA4 [54–56] block different types of CRISPR-Cas9 from binding to target DNA by inducing the dimerization of Cas9 [53] and mimicking double-stranded DNA [54–56], respectively (Figure 1C).

These results demonstrate the potential utility of anti-CRISPR proteins as off-switches for CRISPR-Cas activity. This is particularly important for therapeutic applications where anti-CRISPR proteins can be harnessed to not only reduce off-target editing [56] but also to disable Cas9 activity [53–56]. Although further optimization is required to apply anti-CRISPR systems for the clinical use, they provide promise to add another security checkpoint for CRISPR therapeutics.

The CRISPR-Cas Systems for Cancer Therapeutics

In addition to shaping the future of cancer diagnostics, CRISPR-mediated *ex vivo* and *in vivo* genome editing holds immense promise for combating cancer. Although the conventional nuclease-based gene targeting technologies, TALENs and ZFNs [11], have been explored for therapeutic gene editing previously, CRISPR-based cancer therapeutics already represent the majority (79%) of the genome editing cancer trials worldwide (CRISPR: 11 studies; TALENs: 2 studies; ZFNs: 1 study; clinicaltrials.gov^{vi}). Here, we summarize the CRISPR-Cas9-based therapeutic strategies targeting human cancers and discuss the challenges in translating the CRISPR-Cas9 system into clinical use.

Ex Vivo Gene Editing for Immunotherapy

Recent advances and success in the field of cancer immunotherapy highlight the therapeutic potential of **engineering chimeric antigen receptor (CAR) T cells**. While CAR-T cell therapy targeting CD19, a cell surface molecule found in most leukemias and lymphomas, has shown promise in the treatment of chemorefractory or relapsed B-cell malignancies with high remission rates in patients [57, 58], treating certain liquid and solid tumors still remains challenging [59]. Moreover, CARs targeting CD19 are not without any deleterious consequences, which include reversible toxicities, such as B cell aplasia, neurotoxicity, and cytokine release syndrome (CRS) [60]. To further enhance the efficacy and safety of T-cell therapeutics, gene editing technologies, including ZFNs, TALENs and CRISPR-Cas9, have been recently used to genetically modify primary human T cells.

Compared with ZFNs and TALENs, the CRISPR-Cas9 system [5] provides more rapid and efficient genetic manipulations [61, 62] with multiplexed editing capability [63] simply by viral gene delivery systems or physical transfection methods such as electroporation of Cas9 and sgRNA expression constructs [64], or **Cas9 ribonucleoproteins (RNPs)** [61, 65]. This superior advantage allows rapid T-cell manufacturing to boost T-cell efficacy by eliminating the genes such as programmed cell death protein 1 (PD-1) or cytotoxic T lymphocyte-associated protein 4 (CTLA-4) which encode T cell inhibitory receptors or signaling

^{vi}<https://clinicaltrials.gov/>

molecules (Figure 2A) [66, 67]. In 2016, China began the first clinical trial of CRISPR-Cas9 to treat metastatic non-small-cell lung cancer patients by disabling PD-1 in T cells [68]. Currently, there are several similar trials initiated in China to knock out PD-1 in T cells for the treatment of esophageal cancer, prostate cancer, bladder cancer, metastatic renal cell carcinoma, and Epstein-Barr Virus (EBV)-associated malignancies^{vi}. The University of Pennsylvania had also received permission from the US National Institutes of Health (NIH) Recombinant DNA Advisory Committee (RAC)^{vii} for the human use of CRISPR-Cas9 to treat melanoma, sarcoma, and multiple myeloma using redirected autologous T cells with CRISPR edited endogenous PD-1 and T-cell receptor (TCR) (Figure 2A).

In Vivo Delivery Technologies for Gene Editing

The therapeutic *ex vivo* gene editing has been performed mainly in immune or hematopoietic cells and can be limited to certain tissues. To broaden the application of CRISPR-based therapy, developing efficient methods for *in vivo* gene editing in somatic cells is indispensable. However, *in vivo* gene editing faces additional delivery hurdles compared to *ex vivo* therapy and may encounter more safety and regulatory concerns. These challenges also differ between viral and non-viral systems and require optimal procedures for these different approaches [69–71].

Viral delivery systems for CRISPR-Cas9 include lentivirus, adeno-virus, and adeno-associated virus (AAV) [72]. Among these, AAVs are currently the most advanced methodology for *in vivo* gene delivery (Figure 2B) [71, 73] with successful examples of treating mouse models of neurodegenerative diseases [74, 75], and the efficacy and safety of which have been tested in clinical trials and has been approved recently [76]. Although AAVs' single-stranded DNA genome can serve as a donor for **homology directed repair (HDR)** and it can transduce different cell types *in vivo* such as eye, skeletal, liver, neuron, and cardiac muscle [69] with low risk of genomic integration [77], their small packaging size requires multiple viruses to deliver all the components of CRISPR–Cas9 (Cas9, sgRNA and a donor DNA), which further decreases the **HDR** efficiency of this approach [72]. The persistent expression of CRISPR-Cas9 in edited cells after viral transduction may also increase the potential immune responses and undesirable off-target effects on genome stability. Thus, non-viral delivery of vectors or short-lived and preassembled Cas9 RNP complexes may provide an alternative approach to overcome these hurdles.

Non-viral methods such as lipid and polypeptide nanoparticles are in various stages of clinical development for RNA-based therapeutics (Figure 2B) [70]. In contrast to AAVs, nanoparticle-based delivery of CRISPR–Cas components has high loading capacity for nucleic acid cargos without the risk of genomic integration and effects from persistent expression of CRISPR-Cas9 [78]. Cas9–sgRNA RNP complexes can be efficiently delivered by cationic lipids into the mouse inner ear cells *in vivo* [79] to ameliorate hearing loss [80], enabling potential future applications for treating skin cancers like melanoma. Co-delivery of Cas9 mRNA and sgRNA is also possible by synthetic zwitterionic amino lipids (ZALs) and has been successfully used for gene-editing in the liver, kidneys, and lungs of

^{vii}<https://osp.od.nih.gov/biotechnology/recombinant-dna-advisory-committee/>

engineered mice [81]. Nanoparticle-mediated delivery of Cas9–sgRNA RNP complexes has been also reported in the human osteosarcoma cell lines U2OS xenograft [82]. Moreover, a new vehicle composed of gold nanoparticles conjugated to DNA and complexed with cationic endosomal disruptive polymers was shown to deliver Cas9 RNP complexes plus donor DNA and can induce HDR to correct the DNA mutation of Duchenne muscular dystrophy in mice [83].

In addition to nanoparticles, Cas9 RNP complexes can be directly delivered across the cell membrane by co-delivery with small peptides or modifying the electrostatic charge of the protein (Figure 2B) [84, 85]. For example, efficient neuronal editing in the mouse brain mediated by delivery of engineered Cas9 RNP complexes with multiple SV40 nuclear localization sequences has recently been described [85], indicating future therapeutic use of Cas9 RNP complexes to treat neurodegenerative disease and potentially brain tumor. A thermostable Cas9 was also recently identified from the thermophilic bacterium *Geobacillus stearothermophilus* (GeoCas9), allowing an increased lifetime of Cas9 RNP complexes in human plasma [86].

Together, these therapeutic approaches using CRISPR-Cas9, including *ex vivo* and *in vivo* genome editing, will greatly facilitate the rapid genetic manipulation of immune or cancer cells. Future efforts to build CRISPR-based gene perturbation circuits into CAR-T cells for increased programmability can also lead to more efficient and safe CRISPR therapies and, consequently, to programmable and smart cellular medicines for cancer treatment.

The CRISPR-Cas Systems for Cancer Detection

While a number of CRISPR-Cas effectors have been discovered to date, only two Class 2 proteins, **Cas9** [6, 7] and Cas12a (Cpf1) [87], are widely used and their applications are mainly in genome editing. Recently, extensive research has been conducted to discover new Cas protein variants and unveil their functions to open up possibilities for novel applications such as nucleic acid detection.

Dual RNase Catalytic Activity of Cas13a

The **type VI Class 2 CRISPR-Cas effector**, Cas13a (formerly C2c2), was computationally identified based on genomic proximity to the highly-conserved *casI* gene [88]. Since its bioinformatics-based discovery, Cas13a homologs have been reconstituted biochemically from bacterial species including *Leptotrichia shahii* (LshCas13a), *Leptotrichia buccalis* (LbuCas13a), *Leptotrichia wadeii* (LwaCas13a), and *Lachnospiraceae bacterium* Cas13a (LbaCas13a). Cas13a is an RNA-guided RNA-targeting single-component enzyme [89]. Unlike most CRISPR systems that target dsDNA, Cas13a possesses two **higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains** that target single-stranded RNA (ssRNA) [89]. Cas13a also contains a functionally distinct nuclease responsible for catalyzing **crRNA** maturation to form a Cas13a:crRNA complex competent for target RNA binding [90, 91]. Binding to a complementary single-stranded RNA, also called the activator-RNA, activates the HEPN-nuclease for both target and general ssRNase activity (Figure 3A) [89, 90]. The activity of the HEPN-nuclease is inhibited by partial occlusion of the HEPN active site until binding to the activator-RNA occurs, effectively making the

activator-RNA the allosteric switch for RNase activity (Figure 3A) [92, 93]. While the mechanism appears conserved between homologs, Cas13a enzymes can be functionally separated into two distinct subtypes based on their processing activity and HEPN-nuclease nucleotide preference [91]. Later applications of Cas13a exploit pre-crRNA maturation, general RNase activity and different nucleotide cleavage preference for multiplexed RNA interference [94], target detection through reporter cleavage [90, 95] and multiplexed target detection [96], respectively (Figure 3A).

Application of Cas13a for Nucleic Acid Detection and Targeting

An active Cas13a HEPN-nuclease will turn over multiple ssRNA substrates, a biochemical behavior that can be leveraged for signal amplification in target detection by coupling Cas13a activation to specific ssRNA reporter cleavage resulting in liberation of a quenched fluorophore [90]. Expanding on this, Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) platform was developed as a tool for nucleic acid detection (Figure 3B) [95]. SHERLOCK includes recombinase polymerase amplification (RPA), T7 RNA polymerase transcription from DNA to RNA and Cas13a general RNase activity. The method starts with RNA sequence amplification via **RPA or reverse transcriptase RPA (rt-RPA)** before incubating the sample with Cas13a and reporter probes, and fluorescence is then measured (Figure 3B). Paper-spotting and freeze-drying does not significantly affect the sensitivity of SHERLOCK. SHERLOCK can detect nucleic acids in patient serum or urine down to low attomolar concentrations, allowing the detection of tumor mutation in cell-free DNA (cfDNA). In mock cfDNA samples, SHERLOCK can detect two cancer mutations, EGFR L858R and BRAF V600E, under low allelic fraction with single-base mismatch sensitivity [95]. In addition to *in vitro* RNA target detection, catalytically inactive LwaCas13a (dLwaCas13a) retains its RNA-binding activity such that it can be coupled to a fluorescent probe to enable live cell RNA tracking [94]. This provides an alternative method to recognize and visualize RNA.

Apart from target detection, LwaCas13a was successfully functionalized in live cells to achieve significant knock-down in both mammalian and plant cells [94, 97]. Additionally, LshCas13a was successfully introduced into a plant cell enabling RNA interference [97]. Moreover, multiplexed gene suppression is possible given that Cas13a processes its own crRNA [90, 94]. Surprisingly, the general RNase activity that causes RNA degradation was not detected in mammalian [94] and plant cells [94, 97], possibly due to the choice of target transcripts and the role of substrate localization in cis- versus trans-activity in cells. Based on these data, Cas13a has been proposed as a tool for *in vivo* RNA manipulation.

Multiplexed Nucleic Acid Detection Using Cas12a and Cas13

After the engineering of Cas13a for detection purposes, a previously characterized Cas12a has been found to have previously unknown catalytic activity that enables it to be utilized as an alternative detection tool [98, 99]. Cas12a (Cpf1) [87] is a class V CRISPR system that does not require a trans-activating crRNA (tracrRNA) for activation and can process its own crRNA [100]. Cas12a and other type V CRISPR effectors consist of only one **RuvC endonuclease domain**, unlike Cas9 proteins, which contain both the RuvC and HNH endonuclease domains. Cas12a also differs from Cas9 in terms of **protospacer adjacent**

motif (PAM) sequence and target cleavage, in which the product generates a staggered 5' overhang. The dsDNA cleavage activity of Cas12a was recently found to be the result of its general ssDNase activity on a bound dsDNA substrate (Figure 3A) [99]. Akin to Cas13, target activated Cas12a cleaves ssDNA strands in *trans*, that is, cleaving sequences that are not complementary to its guide RNA (Figure 3A). Given Cas12a's non-specific ssDNA *trans*-cleavage activity, it has been engineered into a DNA detection tool [99]. Coupled with isothermal amplification by RPA for higher sensitivity, binding to the crRNA-complementary DNA sequence by *Lachnospiraceae* bacterium ND2006 Cas12a (LbCas12a) triggers cleavage of an unrelated ssDNA-fluorophore-quencher (ssDNA-FQ) reporter, generating a fluorescent signal. This detection system, termed DNA Endonuclease Targeted CRISPR *Trans* Reporter (DETECTR) [99], can detect DNA sequences with attomolar sensitivity. DETECTR is able to differentiate between two "high risk" types of human papillomavirus (HPV), HPV16 and HPV18, in both cell mixtures and patient samples [99].

While both SHERLOCK and DETECTR provide specific and sensitive detection, they are limited by their qualitative readout and dependence on fluorescent signals [96]. In the enhanced SHERLOCKv2 detection system [96], the readout signal was made quantitative by lowering primer concentration to avoid saturation. On the other hand, detection was made visible by transferring the system to lateral strips (Figure 3C). Thus, only cleaved reporter particles would escape accumulation on the first line and form a second line, allowing direct visualization of detection results (Figure 3C). By employing different cleavage preference of different Cas proteins, SHERLOCKv2 further enables multiplexed detection in a single reaction by combining Cas12a, Cas13a and Cas13b, each carrying out reporter substrate cleavage at a different sequence (Figure 3D). The detection signal was further enhanced by addition of the CRISPR-associated enzyme **Csm6**, which is activated by the product of Cas13 activation [96]. Same as its predecessor, SHERLOCKv2 can detect EGFR L858R mutation or the exon 19 deletion from non-small cell lung cancer (NSCLC) patients [96].

Early cancer detection ensures prompt treatment and is associated with decreased mortality. While there are several techniques available for cancer detection, they all have shortcomings in terms of specificity, sensitivity, cost and speed. Examples of conventional detection methods includes mammography and other imaging procedures. In the case of breast cancer, while mammography is the only proven detection method that reduces mortality [101], its sensitivity varies across different age and ethnic groups [101], and its accessibility is limited due to its cost. Biomarkers are alternative components for cancer detection. Prostate cancer relies on prostate-specific antigen (PSA) screening for detection. However, its high false-positive rate render it ineffective [102]. Other protein biomarkers include surface proteins from cancer cells (e.g. CD44 in breast cancer) [103] and epithelial-mesenchymal transition (EMT) markers from circulating tumor cells (CTC) [104]. Nucleic acid markers include circulating tumor-derived DNA (ctDNA) [105, 106] or microRNAs found in blood [107], mutant gene and disrupted epigenetic modification [108]. While examples of common biomarker detection tools such as ELISA, mass spectrometry and electrophoresis-based methods are highly sensitive, they rely on expensive devices and therefore their accessibility is limited [109]. Moreover, detecting target protein present in small amounts can be

challenging since proteins cannot be amplified as nucleic acid samples [109], while the sensitivity of nucleic acid detection depends on primer design and target amplification.

With high specificity and sensitivity, speed, low-cost and multiplex capabilities, the use of Cas proteins holds great potential in RNA and DNA quantification as well as multiplexed mutant detection. Many cancer types have been reported to evolve through the accumulation of genetic mutations or alterations. For example, colorectal cancer progression is characterized by WNT pathway and EGFR signaling activation, followed by TGF β signaling inactivation and loss of p53 function, eventually leading to carcinoma formation and metastasis. [110]. Changes in gene expression, such as *MYC* and *ERBB2* overexpression, also contribute to colorectal cancer progression [111]. Through multiplexed detection, CRISPR-based detection has the potential to distinguish between different stages of cancer formation, which provides insightful information for appropriate treatment. However, the varied gene expression between different tumor subgroups [112] resulted from tumor heterogeneity can pose a challenge for this kind of nucleic detection tools in terms of effective probe design. Further combination of single-cell technology and CRISPR-based detection may overcome this hurdle and provide a fast and scalable cancer genotyping platform with high resolution.

To develop effective screening tools for early cancer detection, high sensitivity and specificity with low false positive rates must be achieved. Recent technological advances in next-generation sequencing of cell-free DNA [106] or its combination with assessment of the levels of circulating proteins [113] hold promise for addressing the issues of sensitivity and specificity. These combined assays for both genetic alterations and protein biomarkers can even localize the organ of origin of detected cancers through algorithms developed from supervised machine learning [113]. In the case of CRISPR-Cas detectors, like SHERLOCKv2, false positive rates can be reduced by adding the CRISPR-associated enzyme *Csm6* during signal amplification [96]. Nevertheless, more tests are needed to precisely measure the specificity and sensitivity of CRISPR-Cas nucleic acid detection systems. If successful, they can provide alternative approaches for biomarker readout and enhance the capabilities of nucleic acid detection for cancer diagnosis.

Concluding Remarks

The expansion and improvements in CRISPR-Cas technologies enable more efficient and safer applications of CRISPR-Cas in both cancer therapeutics and detection. CRISPR-enabled *ex vivo* gene editing in T cells has entered clinical trials for different types of cancer and will soon be tested to determine whether such treatments are safe and well tolerated in patients. Beyond *ex vivo* use, recent advances in the fast evolving CRISPR-Cas technologies alleviate challenging problems of gene editing *in vivo*, including (1) efficient delivery of multiple CRISPR-Cas components using nanoparticles and RNP complexes; (2) reduced off-target effects by new variants of Cas9 and anti-CRISPR proteins; and (3) direct conversion of point mutations without DSBs by base editors to avoid low editing efficiency mediated by HDR. For detection applications, engineered Cas13 and Cas12a enzymes offer new strategies for rapid detection of tumor DNA or cancer-related viruses with high specificity and sensitivity and can be further optimized for detecting multiple target DNAs

simultaneously. This multiplexed ability is particularly important for cancer, in which the genome of tumor cells has a complex mutational landscape. Despite positive outcomes, these CRISPR-based detection methods still need to be tested in a larger cohort of patients, examined in terms of reagent stability, and compared side-by-side with current available methods for cancer diagnosis to ensure their robustness. Still, it remains to be determined (1) how robust targeted delivery of CRISPR-Cas systems to different organs will be achieved in an *in vivo* context and whether there may be side effects to normal tissues; (2) how efficient these CRISPR-Cas technologies can be used in a multiplexed manner to target and detect complex genetic alterations in different types of cancer, with minimal false-positive signals and off-target effects; and (3) how to mitigate the effects of human genetic variation and immune responses to ensure robust outcomes and minimize treatment failure of CRISPR therapy (see Outstanding Questions). Going forward, there is a tremendous need to identify bona fide nucleic acid biomarkers for diagnosis and understand what genes, especially those which are previously undruggable, are required for cancer dependency. With these information, we believe CRISPR-Cas enzymes will undoubtedly transform cancer therapeutics and detection to produce real clinical benefit.

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Glossary

CRISPR-Cas

a naturally occurring defense system found in bacteria and archaea. The clustered regularly interspaced palindromic repeat (CRISPR) system consists of RNA guide(s) for target recognition and a Cas enzyme for target cleavage.

Zinc finger nucleases (ZFNs)

artificial engineered restriction endonuclease consists of the FokI restriction enzyme and a zinc-finger binding domain that recognize DNA triplets.

Transcription activator-like effector nucleases (TALEN)

artificial engineered restriction endonuclease consists of the FokI restriction enzyme and a TAL effector DNA-binding domain that targets single nucleotide.

Cas9

a subclass of Cas proteins that are single-protein effectors with two nuclease domains targeting DNA.

Type VI Class 2 CRISPR-Cas effector

a subclass of Cas proteins that are single-protein effectors with two nuclease domains targeting RNA.

Higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains

a highly conserved family of domains that contains motifs with RNase activity.

crRNA

a RNA strand sequence containing a spacer and repeat sequence that is responsible for target recognition.

Recombinase polymerase amplification (RPA) and Reverse-transcriptase RPA (rt-RPA)

a single-tube, isothermal and low-temperature alternative to PCR. rt-RPA RNA amplification remains single-tubed by adding reverse transcriptase to the reaction.

RuvC endonuclease domain

a nuclease domain that catalyzes single-strand DNA cleavage.

Protospacer adjacent motif (PAM) sequence

short genomic sequence adjacent to the target gene crRNA recognizes. The nucleotide sequence of PAM varies across different Cas proteins.

Csm6 effector

a CRISPR type-III effector nuclease that binds to cyclic oligoadenylate or linear tetraadenylate nucleotides with 2',3'-cyclic phosphate group to activate nonspecific RNA degradation.

Engineering chimeric antigen receptor (CAR) T cells

engineered T-cells fused with synthetic antibody, which enables them to recognize cancer-related antigens.

Cas9 ribonucleoproteins (RNPs)

pre-assembled ribonucleoprotein with purified Cas9 protein and guide RNA.

Homology directed repair (HDR)

a precise DNA repair mechanism that requires homologous template at the site of double-stranded break.

Non-homologous end joining pathway (NHEJ)

a DNA repair mechanism in which the ends of the double-stranded break is directly ligated.

Type I and type II CRISPR–Cas systems

subtypes of Cas proteins that differ one another in terms of crRNA components, target recognition and PAM sequence and location.

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Outstanding Questions

How robust can targeted delivery of CRISPR-Cas systems to different organs be achieved in an *in vivo* context and will there be side effects to normal tissues?

How efficient can these CRISPR-Cas technologies be used in a multiplexed manner to target and detect complex genetic alterations in different types of cancer, with minimal false-positive signals and off-target effects?

How can we mitigate the effects of human genetic variation and immune responses to ensure robust outcomes and minimize treatment failure of CRISPR therapy?

Highlights

- New variants of Cas9 and anti-CRISPR proteins exhibit reduced off-target effects, further enabling precision genome editing for cancer therapeutics.
- Non-viral delivery of CRISPR-Cas9 using nanoparticles and Cas9 ribonucleoprotein (RNP) complexes enables efficient editing *in vivo* without undesirable effects of persistent CRISPR-Cas9 expression in edited cells.
- The *trans*-cleavage activity of Cas12a and Cas13 has been utilized as detection tools in which single-stranded reporters are cleaved upon target recognition. The systems have been used to detect cancer-causing mutations and cancer-related viruses with high specificity and sensitivity.

(A) CRISPR-Cas toolkit **(B) CRISPR-Cas with enhanced accuracy** **(C) Anti-CRISPR Cas systems** **Figure 1. Precision Genome Editing for Therapeutics.**

(A) The current CRISPR-Cas toolkit. Left: Gene editors with endonuclease activity, such as Cas9 and Cas12a (Cpf1). Middle: Epigenetic editors for targeted gene repression (CRISPRi) or activation (CRISPRa) using a catalytically deactivated Cas9 (dCas9) fused to different effector domains. They allow epigenetic regulation of endogenous gene expression without creating DSBs. Right: Base editors, which are generated by fusing dCas9 (or Cas9 D10A nickase) with cytidine deaminase or tRNA adenine deaminase (dark blue) to directly convert G•C to A•T or A•T to G•C (red) without DNA cleavage in genome, respectively. (B) Enhancing CRISPR-Cas accuracy by identifying genome-wide off-target sites and programming new Cas9 variants. (C) Anti-CRISPR systems for switching off Cas9 and reducing off-target effects. Left: Anti-CRISPR protein AcrIIC1 (gray) binds to Cas9 (blue), allowing DNA binding while preventing cleavage of the target strand by blocking its active site. Right: Anti-CRISPR protein AcrIIC3 (green) binds to Cas9 (blue), inducing its dimerization and preventing target dsDNA binding.

Figure 2. Applications of CRISPR-Cas in Cancer Therapeutics.

(A) *Ex vivo* therapeutic strategies using CRISPR-based CAR-T cell therapy. Patient-derived T cells are isolated and genetic engineered with CRISPR-Cas9 to knockout endogenous PD-1 (or TCR), and then redirected into the same patient. (B) *In vivo* delivery of CRISPR-Cas for therapeutics. CRISPR-Cas9 components are delivered into the patient via either viral (Adeno-associated virus, AAV) or nonviral methods including nanoparticles and Cas9 ribonucleoprotein (RNP) complexes.

Figure 3. Catalytic Activities and Applications of Cas13a and Cas12a in Cancer Detection. (A) Dual RNase activity of Cas13a includes crRNA processing and specific and general *trans*-ssRNA cleavage. Cas12a has recently been identified to also possess promiscuous ssDNA cleavage activity, which can be activated by both single-stranded or double-stranded DNA activator. (B) The SHERLOCK system detects DNA or RNA target and releases signal through promiscuous reporter cleavage. (C) Visualizing detection readout on lateral strip. When reporter strands are cleaved, accumulation at the first line is reduced, and antibody-gold conjugates bind to protein A on the second line. (D) Multiplexed detection in one single reaction is made possible by utilizing different Cas proteins' different cleavage preference designing different reporter recognized by a single Cas protein.

Table 1.

Web-based tools for sgRNA design and off-target prediction.

Name	Description	Reference
CRISPR Design Tool	Genome-wide sgRNA identification and off-target analysis; search by entering gene sequence	[32]
E-CRISP	Genome-wide sgRNA identification and off-target analysis; search by entering gene sequence or gene name	[35]
CRISPRscan	Genome-wide sgRNA identification and off-target analysis; search by entering gene sequence	[36]
GuideScan	Genome-wide sgRNA identification for SpCas9 or for Cpf1 and off-target analysis; search by entering gene sequence, gene name or genomic coordinates	[37]
CasOFFinder	Genome-wide sgRNA identification and off-target analysis; search by entering gene sequence	[38]

Table 2.

Cell-based tools for off-target identification.

Name	Description	Reference
Integrase-defective lentiviral vector (IDLV) capture	Off-target identification by recognizing linear and double-stranded IDLV in double-stranded breaks inserted through NHEJ	[39]
Guide-seq	Cell-based off-target identification by recognizing blunt, end-protected double-stranded oligodeoxynucleotide integrated into double-stranded breaks	[40]
High-throughput genome-wide translocation sequencing (HTGTS)	Cell-based off-target identification by recognizing translocation junctions	[41]
Direct in situ Breaks Labeling, Enrichment on Streptavidin and Next-Generation Sequencing (BLESS)	Cell-based off-target identification by recognizing biochemically labeled double-stranded breaks captured on streptavidin beads	[42]
Breaks Labeling in situ and Sequencing (BLISS)	Cell-based off-target identification by recognizing biochemically labeled double-stranded breaks in fixed cells	[43]
CIRCLE-seq	In vitro off-target identification by recognizing linearized circular DNA containing a Cas9 cleavage site	[44]