Synthetic and Systems Biotechnology 3 (2018) 217–228

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: http://www.keaipublishing.com/synbio

Blossom of CRISPR technologies and applications in disease treatment

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ARTICLE INFO

Keywords:
CRISPR technologies
CRISPR-based tools
CRISPR-based therapy

ABSTRACT

Since 2013, the CRISPR-based bacterial antiviral defense systems have revolutionized the genome editing field. In addition to genome editing, CRISPR has been developed as a variety of tools for gene expression regulations, live cell chromatin imaging, base editing, epigenome editing, and nucleic acid detection. Moreover, in the context of further boosting the usability and feasibility of CRISPR systems, novel CRISPR systems and engineered CRISPR protein mutants have been explored and studied actively. With the flourish of CRISPR technologies, they have been applied in disease treatment recently, as in gene therapy, cell therapy, immunotherapy, and antimicrobial therapy. Here we present the developments of CRISPR technologies and describe the applications of these CRISPR-based technologies in disease treatment.

1. Introduction

At the beginning of this century, genome editing technology has been developed gradually via the exploitation of ZFNs (Zinc finger nucleases) [1] and TALENs (Transcription activator-like effector nucleases) [2]. However, the complexity of reconstruction of different enzymes corresponding to different targets, and the huge size of these proteins limited their applications in human disease treatment [3]. After the emergence of CRISPR technology derived from the bacterial adaptive immune system, the researchers' abilities to sculpt the genome and program the gene expression have been enhanced to another level [4]. The prevailing CRISPR technologies have augmented the researchers' abilities in genetic/genome engineering across a variety of life forms. Especially, the emerging CRISPR-Cas9 system has been recruited and optimized in enormous genome editing and transcription regulation applications, unprecedentedly facilitated the advancements in therapeutic applications (Fig. 1). Due to the simplicity of operation and huge potential to be engineered and applied in therapeutic areas, various CRISPR-based tools have been developed, such as gene editing, epigenome editing, base editing, transcriptional regulation, and nucleic acid detection. With the expansion of these tools, novel diagnosis approaches and disease treatments have been designed and carried out in practice.

In this review, we will first state the most recent advances in the development and applications of CRISPR technologies that could be applied in disease treatment and then summarize and enumerate the applications in disease treatment with the help of these CRISPR-based tools.

2. Development of CRISPR systems

CRISPR system was first discovered in 1987 [5]. However, the wide use of CRISPR technologies has not started until 2013. In the past 5 years, CRISPR has renovated not only the molecular biology field, but also the medicine and biotechnology fields [4]. After the technologies were first successfully applied in mammalian genetic modifications, scientists around the world participated positively in the development of this technology at their own expertise.

2.1. The discovery of CRISPR systems

The CRISPR systems are adaptive immune systems widely spread in bacteria and archaea. The discovery of CRISPR could be dated back to 1987, when Dr. Nakata's group [5] found a special repeat sequence in *Escherichia coli* (*E.coli)* which was later named as CRISPR (clustered regularly interspaced short palindromic repeat) after two decades later in 2002 [6]. At the same time, the genes located adjacent to the repeat sequences were identified as CRISPR-associated (Cas) genes. The CRISPR array and Cas proteins are the basic components necessary for the adaptive immunity process. The subsequent unraveling of the molecular details of CRISPR-Cas systems became clearer since 2007 [7–9]. In 2012, the purified Cas9 was proved to cleave target DNA *in vitro* with

Peer review under responsibility of KeAi Communications Co., Ltd.
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https://doi.org/10.1016/j.synbio.2018.10.003
Received 1 September 2018; Received in revised form 9 October 2018; Accepted 10 October 2018
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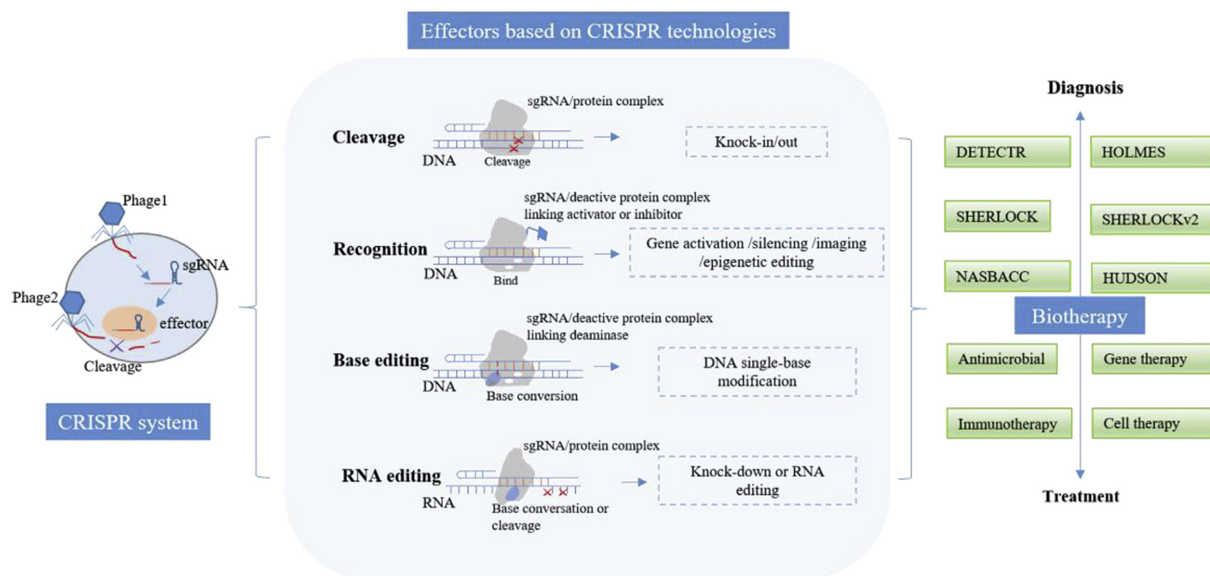

Fig. 1. Overview of the development of the CRISPR systems and their applications.

the guidance of crRNA/tracrRNA [10,11]. Later in 2013, the type II CRISPR Cas9 system showed genome editing ability in mammalian cells [12,13]. Since then, several CRISPR-Cas single effector enzymes have been characterized and engineered for use in various organisms [14,15].

CRISPR-Cas systems are divided into Class 1 and Class 2 based on major differences between the Cas proteins involved in the binding guidance and target cleavage, and further classified into six types based on the "signature genes" [16–18]. Type I, III, and IV systems are considered as Class 1 systems based on their multi-subunit effector complexes; while the single, large, multi-domain effectors of Type II, V, and VI systems are grouped into Class 2 [16]. The classification efforts are not completed as the researchers are still actively exploring other CRISPR systems and the classification needs to be refined when more information based on comparative genomic analysis, structures, and biochemical analysis of CRISPR components is gained. The most up to date classification of CRISPR-Cas systems was summarized in Koonin et al.'s review [19]. The most widely used systems were the Type II system containing Cas9 from *Streptococcus pyogenes* and Type V system containing Cas12a (previously called Cpf1) [20] from *Acidaminococcus* sp (AsCas12a), *Lachnospiraceae bacterium* (LbCas12a) and *Francisella novicida* (FnCas12a).

2.2. The exploitation of CRISPR-based tools

CRISPR system was first regarded as a powerful genome editing tool in eukaryotic cells in 2013 [12,13]. Soon after, numerous applications arose beyond genome editing, such as targeted gene interference (CRISPRi) and activation (CRISPRa), live cell chromatin imaging, base editing, epigenetic modulation, and nucleic acid detection (Table 1).

2.2.1. Genome editing tools

The ability to introduce desired changes into genomes at precise locations possesses tremendous value not only for molecular biology, but also for medicine and disease therapy. CRISPR systems, especially the Type II and V systems, have revolutionized the genome editing field due to its preeminent features, including higher sequence specificity, artificial guided targeting, unique adaptive nature and superior editing efficiency. As genome editing tools, CRISPR-Cas9 and CRISPR-Cas12a have been used in various organisms successfully, including mammalian cells, *Saccharomyces cerevisiae*, *Escherichia coli*, *Streptomyces*, *Corynebacterium glutamicum*, *Cyanobacteria*, plants and so on [21–28]. However, the further popularization and applications are limited due to their recognition mechanisms, the fidelity of editing and the size of those proteins. The CRISPR-Cas systems recognize the target genome region through searching for protospacer-adjacent motifs (PAMs) firstly. The PAMs for SpCas9 and AsCas12a/LbCas12a are restrictive in NGG and TTTN respectively [29,30], and the fidelity of the CRISPR systems is not high enough. Thus they may induce unwanted off-target phenomenon [31]. To solve these problems, researchers created libraries of the Cas9 and Cas12a mutants to screen for variants with higher specificity and broader PAM recognition ability, which will be discussed in part 2.3. The fidelity of genome editing could also be enhanced by using shortened sgRNAs [32], the dCas9-*Fok*I fusion [33] or the inducible transient expression strategies [34–36]. The length of *SpCas9* is about 4.2 kb, which may hinder its application in gene therapy as the recombinant adeno-associated virus (rAAV) vectors can only hold about 4.7 kb generally. The "split-Cas9" which contains two parts of the Cas9 protein separately could be packed in the rAAV separately to solve this problem [37]. Moreover, the delivery efficiency of the Cas9 nucleases in different cell lines can affect genome editing efficiency. A receptor-mediated delivery strategy showed increasement in editing efficiency by up to 29-fold [38].

2.2.2. Gene expression tools

Increasing efforts have been directed to re-engineer the already well-characterized Cas9 proteins. The catalytically deactivated CRISPR associate proteins (such as dCas9) with different fusion proteins are repurposed with different functions, such as genome transcriptional regulation. The dCas9 or DNase-dead Cas12a (ddCas12a) can bind the target sequence strongly with the help of guide RNA, which can interfere with transcriptional initiation or elongation via blocking RNA polymerase binding, or transcription factor binding, resulting in down-regulation of target genes [39,40]. The repression of gene expression would be stronger when dCas9 or ddCas12a were fused with transcriptional repressors, such as KRAB (Krüppel-associated box) domain in mammalian cells [41], SRDX in plants [42] and RD1152 in yeast [43]. Interestingly, some type VI RNA-guided RNA-targeting CRISPR-Cas proteins, such as *Leptotrichia wadei* Cas13a (LwaCas13a) and CasRx from *Ruminococcus flavefacien* XPD3002, can decrease the target gene expression by binding the transcripts to interfere with the RNA transcription [44,45]. On the contrary, the dCas9 or ddCas12a fused with one or more transcriptional activators, such as VP16, VP64, p65AD, or the synthetic gene activators [46], could up-regulate target gene

Table 1
The CRISPR-based tools with different effectors.

CRISPR	Fused Effector	Example	Application	Ref.
dCas9/ddCas12a/Cas13	None	dCas9	Down-regulation	[39,44]
dCas9/ddCas12a	Transcriptional repressor	dCas9-KRAB	Down-regulation	[41]
dCas9/ddCas12a	Transcriptional activator	dCas9-VP64	Up-regulation	[188]
dCas9	Fluorescent proteins	dCas9-EGFP	Chromatin imaging	[52]
nCas9	Cytidine deaminase domain	APOBEC–XTEN–nCas9–UGI	C·G to T·A conversion	[67]
nCas9/ddCas12a	Adenosine deaminase domain	nCas9-ABE 7.0	A·T to G·C conversion	[68]
dPspCas13b	Adenosine deaminase domain	dPspCas13a-ADAR2	A to I conversion	[89]
dCas9	DNA methyltransferase domain	dCas9-DNMT3A	DNA methylation	[101]
dCas9	Ten-eleven translocation domain	dCas9-TEF1	DNA demethylation	[101]
dCas9/ddCas12a	Histone acetyltransferase domain	dCas9-p300	Histone acetylation	[107]
dCas9	Histone deacetylase domain	dCas9-HDAC3^{R265p}	Histone deacetylation	[108]
nCas9	DNA polymerase I domain	nCas9-PolI3M	Mutation in a specified window	[126]

expression via recruiting RNA polymerase binding [41,43]. Notably, in order to relieve the delivery pressure of CRISPR-based activator, Dr. Xie's group downsized the Cas9 variants to generate "mini-Cas9" activator by deleting conserved functional domains. The size of "mini-Cas9" can be shortened by 0.7kb–1.2kb compared with the WT Cas9, the up-regulation abilities of the "mini-Cas9" in mammalian cells are efficient along with an optimized gRNA expression cassette [45].

2.2.3. Live cell chromatin imaging tools

Eukaryotic genomic DNA is organized three-dimensionally in cells as chromatin, which dynamically interacts with proteins and other chemicals. The information and behavior of such structures are still unclear in various cellular processes [47–49]. Taking advantages of the live cell imaging tools, researchers can reveal the dynamic processes and explore the interactions of the mechanical factors and the signals [49,50]. The fluorescence in situ hybridization (FISH) was a major strategy for live cell imagining before 2013 [51]. However, FISH had some drawbacks as it cannot be used to visualize dynamic processes in living cells [51,52]. The CRISPR-based live cell imaging tools emerged recently with the dCas9 fused with different fluorescent proteins [20,53,54]. Generally, with the guidance of the optimized sgRNA, dCas9 fused with EGFP bound to the repetitive elements of the genome. After the EGFP complex gathered to these loci, the fluorescence of the EGFP would display and show robust green [52]. However, the non-repetitive genomic sequences seem to be difficult to be imaged by CRISPR-based imaging tools as the fluorescence signal of a few dCas9-sgRNA complexes at the target region is not sufficient for detection [55]. Researchers can add more counts of sgRNAs of the nearby loci to alleviate this problem, but this method cannot be repeated biologically easily as delivery of lots of sgRNAs is challenging and may increase the off-target rate [55]. To solve this problem, researchers engineered the sgRNA scaffolds with up to 16 MS2 binding motifs which can bind to the bacteriophage MS2 coat protein (MCP). The MCPs were tagged with fluorescent proteins and thus the motifs would be labeled with fluorescence. This approach can achieve imaging of repetitive and non-repetitive genomic regions [55]. It was difficult to image the different interchromosomal or intrachromosomal loci. Co-expression of dCas9 orthologs fused with different fluorescent proteins may be a good idea [56]. However, imaging multiple genomic loci simultaneously is limited by the need of spectrally distinct fluorophores. Combining CRISPR-based imaging with FISH, tracking multiple genomic elements simultaneously was successful [57]. Further, the multiple dynamic genomic elements imaging was achieved by the "track first and identify later" method. They first labeled and tracked chromosomal loci in live cells with the CRISPR-based imaging, then barcoded those loci by DNA sequential FISH in fixed cells [58].

2.2.4. Base editing tools

As many hereditary diseases are caused by single nucleotide mutation, the ability to perform precise genome base editing holds tremendous value in therapeutic areas. When dCas9/nCas9 or ddCas12a is fused with natural or engineered deaminase enzymes, they become base editors [40,59–62]. The base editors can directly convert C·G to T·A or A·T to G·C without introducing double stranded DNA breaks [63–68]. The first example was reported in 2016 showing the C·G to T·A conversion achieved by dCas9/nCas9 fused with the cytidine deaminase enzyme named rat APOBEC1 or fused with APOBEC1 and Uracil DNA glycosylase inhibitor (UGI). The cytidine deaminase catalyzes cytosine (C) deamination and generates uracil (U) that has the base-pairing properties of thymine (T). The Cas9 nickase (nCas9) replaced the dCas9 to generate the BE3 (APOBEC–XTEN–nCas9–UGI) with improved activity and accuracy [67]. Nearly at the same time, the Target-AID system also achieved specific point mutation, with dCas9/nCas9 fused with the activation-induced cytidine deaminase PmCDA1 from sea lamprey and the UGI [69]. Over the past few years, these base editors have been upgraded [70,71]. For example, the HF-BE3 was generated from protein engineering [72], the BE4 and BE4-Gam were created via altering the linkers of BE3 and increasing the copies of UGI [73,74], the eA3A-BE3 was obtained by replacing the rAPOBEC1 with engineered human APOBEC3A (eA3A) domain of BE3 [75,76] and so on. Later, the editing window could be narrowed by engineering the BE3 [63] and be broadened by BE-PLUS which combines the BE and the SunTag systems [77]. The genome-targeting scopes of the BEs have also been broadened by replacing the dCas9/nCas9 with other CRISPR-associated proteins or the variants [63,78]. To deal with low efficiency caused by methylated regions or CpG contexts, researchers developed the human APOBEC3A-based BEs [79]. In 2017, A·T to G·C conversion was implemented by the adenine base editors (ABEs). The ABE7.10 employing optimized bacterial adenosine deaminase tethered to nCas9 catalyzes adenosine (A) hydrolytic deamination and generates inosine (I) which is read as guanine (G) by polymerases [68]. Over the past few years, researchers applied the base-editors to lots of species and organisms, such as yeast [69], plants [40,80–83], bacteria [62,84], rats [85], zygotes [86], embryos [87,88] and so on. Remarkably, an RNA targeting base editor named REPAIR (RNA Editing for Programmable A to I Replacement) can achieve A- > I editing of RNA. This base editor incorporates the catalytically inactive Cas13b from *Prevotella* sp. *P5-125* (dPspCas13b) fused with the adenosine deaminase mutant acting on RNA (ADAR2) deaminase domain ($ADAR2_{DD}$) (E488Q/T375G) [89], and its applications have been demonstrated in yeast [90].

2.2.5. Epigenome editing tools

The molecular mechanisms of heritable gene expression changes that cannot be attributed to changes in DNA sequence information are classified into epigenetics, while all post-translational chromatin modifications in the genome are defined as epigenome. The epigenomic features includes DNA methylation, histone modifications, chromatin variations and non-coding RNAs, which reveals unprecedented insights into gene regulation and genome organization [91,92]. Still, their functional roles are not fully elucidated [93]. Therefore, specific

epigenome mapping tools are expected to assist researchers to uncover the mysterious functions of chromatin modifications [94]. With careful design and optimizations, these tools can precisely modulate gene expressions [95], thus they could be potentially used for epigenetic caused disease treatment, such as glioblastoma [96] and Fragile X syndrome (FXS) [97]. DNA methylation and demethylation are the most widely studied phenomena of epigenome. To explore the potential of CRISPR tools in epigenome area, the dCas9 fused with the catalytic domain of distinct DNA methyltransferases (DNMTs) were designed and locus-specific DNA methylation was achieved [98,99]. Meanwhile, the dCas9 fused with the catalytic domain of ten-eleven translocation (TET) family enzymes achieved locus-specific DNA demethylation in mammalian cells [100–102]. Other than DNA methylation/demethylation, histone modification is another major epigenomic features. The dCas9 fused with the KRAB domain or other protein domains could increase the tri-methylation level of histone H3 at lysine 9 (H3K9me3) specifically, finally result in target gene down-regulation [103,104]. Similarly, the dCas9 fused with the domain of histone demethylases, such as the catalytic domain of LSD1, resulted in a substantial local loss of the active enhancer markers H3K4me2 and H3K27ac, and finally improved the target gene expression [105]. Similar to the histone methylation and demethylation processes, locus-specific histone acetylation and deacetylation can be achieved by dCas9 or ddCas12a fused with the domains of acetyltransferases and deacetylases respectively [106–108].

2.2.6. Nucleic acid detection tools

Rapid and precise sensing of trace amount of nucleic acids is of vital significance for clinical diagnosis and human health. The CRISPR-Cas9 system was firstly used for nucleic acid detection to discriminate different Zika viruses. The CRISPR system was combined with the RNA amplification technique known as NASBA (nucleic acid sequence-based amplification) to generate a method named NASBACC [109]. In the same year, the newly discovered CRISPR system named CRISPR-C2c2 was adapted for nucleic acid detection as well. The *Leptotrichia buccalis* C2c2 (LbuC2c2) is an RNA-guided non-specific RNase active protein. LbuC2c2 catalyzed efficient target RNA cleavage only when such substrates could base pair with a complementary sequence in the crRNA. 0.01 nm target RNA can be detected distinctively by simply adding the quenched fluorescent RNA reporter and the LbuC2c2 with specific crRNA [110]. Later, a method named SHERLOCK (Specific High-Sensitivity Enzymatic Reporter unLOCKing) which can discriminate nucleic acid of attomolar concentration was reported [111]. In 2017, a research group developed HOLMES (one-HOur Low-cost Multipurpose highly Efficient System) platform for nucleic acid detection with the help of Cas12a (Patent CN 201710573752). In 2018, the HOLMESv2 which could be performed in one pot was used for nucleic acid detection with the help of Cas12b and Loop-Mediated Isothermal Amplification (LAMP) [112,113]. In April 2018, the *Science* press reported three nucleic acid detection tools with the incorporation of CRISPR tools, including DNA endonuclease-targeted CRISPR trans reporter (DETECTR) [114], SHERLOCKv2 [115] and heating unextracted diagnostic samples to obliterate nucleases (HUDSON) [116]. DETECTR was based on the Cas12a that has target-activated, non-specific ssDNase cleavage property. It identified the HPV64 and HPV16 successfully with high sensitivity and specificity [114]. SHERLOCKv2 consisting of Cas13, Cas12a, and Csm6, can achieve multiplex nucleic acid detection with tremendously improved sensitivity. The quantitative measurement of input is as low as 2 attomolar [115]. The HUDSON system was designed based on SHERLOCK. The viral particles would be lysed first and the high level of ribonucleases found in bodily fluids would be inactivated with the help of heat and chemical reduction before performing the SHERLOCK steps. The advantage of HUDSON is that it is time-saving and does not require the nucleic acid extraction step [116].

2.2.7. Other tools

The CRISPR system can be redesigned to do a great deal of unimaginable work. Basically, Cas9 and Cas12a can be regarded as RNA guided DNA endonucleases. With the help of ligases or hosts, they have been used to clone large fragments [117–120]. Together with large pooled single-guide RNA (sgRNA) libraries, CRISPR-Cas proteins or fusion proteins coupled with the effectors can serve as screening tools [121], such as screening cancer-related genomic ribonucleotides [122], identifying fetal hemoglobin regulator [123] and so on. The CRISPR-Cas system can also serve as a nucleic acid mutation toolkit [124]. Taking advantages of the CRISPR system, researchers can create recording tools [125] and evolutional tools with nCas9 (D10A) combined with the fidelity-reduced *Escherichia coli* DNA polymerase I [126].

2.3. The novel CRISPR systems

CRISPR has been regarded as an indispensable toolkit for genetic manipulations. However, the frequently used CRISPR nucleases still have some limitations, such as the restriction of PAMs and the low fidelity. The feasible solutions to these problems include mining alternative CRISPR systems or engineering the current available CRISPR-associated nucleases to create a number of variants with specific biochemical properties such as altered PAM specificity or reduced off-target cleavage efficiency.

2.3.1. Mining new CRISPR systems

Apart from the first well characterized CRISPR-Cas9 system, a number of new CRISPR systems were reported from 2015 to 2017 [19] (Fig. 2), presenting the widespread diversity of such systems. This diversity provides researchers with many variations of Cas proteins that may be explored for different applications. In 2015, Shmakov et al. discovered three distinct Class 2 CRISPR/Cas systems that were different from the Type II Cas9 [127], naming as Type V-A, V-B, and V-C with the representative CRISPR-associated nucleases of Cas12a, C2c1, and C2c3, respectively [128,129]. In 2016, two novel CRISPR-CasX and CRISPR-CasY systems were reported, which were among the most

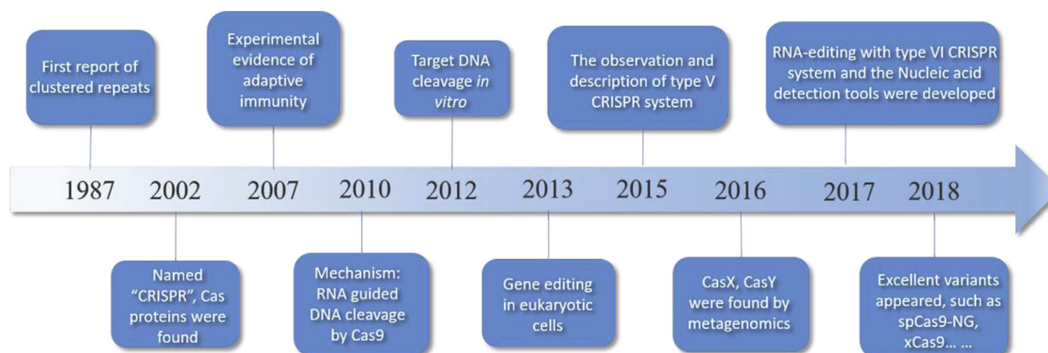

Fig. 2. A brief time line of key discoveries of the CRISPR systems.

compact systems yet identified [130]. The CRISPR-associated proteins in these two systems are renamed as Cas12e and Cas12d. Another Type VI CRISPR enzymes are RNA guided RNA nucleases. There are three unique Type VI CRISPR proteins: Cas13a (C2c2), Cas13b, and Cas13c, each of which has two HEPN RNase domains [131]. With the continuously published new data sets of bacterial or archaeal genomes, we believe more and more future exploration of these diverse CRISPR systems would be envisioned.

2.3.2. Engineering of WT CRISPR/Cas proteins

In addition to the discovery of new CRISPR systems, engineering WT enzymes could generate mutants with a variety of functions, further adding to the diversity of these enzymes. As for Cas9, one motivation of Cas9 enzyme engineering was the acquirement of diversiform PAMs. In 2014, the PI domains of different Cas9 were swapped to generate two chimeras: one was Sp-St3Cas9 (SpCas9 with the PI domain of St3Cas9) targeting the 5′-GGGCT-3′ PAM , whereas traditional SpCas9 targeted the 5'-NGG-3'; the other one was St3-SpCas9 (St3Cas9 with the PI domain of SpCas9), targeting 5′-GGGCG-3′ PAM, whereas traditional St3Cas9 targeted 5′-NGGNG-3′ [29]. This work not only enlarged the scope of PAM sites, but also improved the PAM specificity. In addition, the PAM-interacting motif of SpCas9 was mutagenized to create variants with different PAM recognition abilities (Table 2). Cas9 proteins from other species were also upgraded for targeting efficiency improvement or operational feasibility enhancement. For example, SaCas9 from *Staphylococcus aureus* and FnCas9 from *Francisella novicida*, were mutagenized with reasonable structure analysis of the gRNA-Cas9 complex. Variants that can recognize more relaxed PAMs: 5′-NNGRRT-3′, 5′-NNNRRT-3′ (KRH, KKH of SaCas9) and 5′-YG-3′ (a variant of FnCas9) were obtained [132,133]. Since 2015, several research groups worked on the reconstruction of SpCas9 protein, aiming not only to increase the diversity of PAM sequences, but also to improve the specificity and reduce the off-target effects. SpCas9-HF1 [134], eSpCas9 [135], HypaCas9 [136], evoCas9 [137], xCas9 3.7 [78] and Sniper-Cas9 [138] were generated in succession (Table 2). SpCas9-HF and eSpCas9 were designed according to the structure analysis of the substrate-enzyme-RNA complex. They found that 1) Disruption of SpCas9-mediated DNA contacts can alter the energetics of the complex; 2) Positive-charged groove would stabilize the non-target strand of the target DNA when it is positioned among the HNH, RuvC III, and PAM-interacting domains. Thus mutants with reduced off-target effects were obtained. HypaCas9, hyper-accurate Cas9, exhibited high genome targeting specificity without compromising on-target activity [136]. The evoCas9 [137] was obtained through random mutations in the REC3 domain. When screening the library of variants of SpCas9, researchers found that the specificity was improved and no off-target effect was observed. xCas9 3.7 was obtained with the help of the evolutionary methods named phage-assisted continuous evolution (PACE), and this variant not only improved the fidelity but also broadened the scope of PAM recognition [139]. In 2015, another variant of SpCas9, iCas9 with higher editing efficiency was applied in the multiple-gene disruptions in *Saccharomyces cerevisiae* [21]. Another variant, SniperCas9 with high specificity and activity was obtained by Sniper screen [138]. As for Cas12a remolding, attentions have been focused on the PAM recognition diversity improvement and the ddCas12a-related tools construction. From 2017 to 2018, several groups devoted themselves to engineer Cas12a with altered PAM specificities. They obtained the RR and RVR variants of As/Lb/FnCas12a by structure analysis of the complex [140–142].

3. CRISPR-mediated diseases therapy

As we stated above, CRISPR-based technologies have empowered researchers with an unprecedented toolbox that enables breakthrough discoveries and innovative methodologies. These methodologies could expand their therapeutic applications, such as the pathogen detection [143], disease modeling [144,145], and most importantly disease therapy (Table 3) [146,147]. In this section, we would discuss the CRISPR-based technologies that have been applied in *in vivo* gene therapy, cell therapy, immune therapy and bacteriophage therapy (Fig. 3).

3.1. In vivo gene therapy

Gene therapy is defined as the delivery of nucleic acid into the patients for disease treatment [148,149]. Traditional gene therapy strategies include interfering with target gene expression, replacing the culprit genes, correcting genetic mutations and so on. With the development of CRISPR technologies, the recent advances in gene therapy intrigued our great interests [150]. In 2014, the *Fah* mutation which causes human hereditary disease tyrosinemia was corrected in adult mice via CRISPR-Cas9. Three sgRNAs targeting the *Fah* (*Fah1*, *Fah2* and *Fah3*) were cloned into the adeno-associated virus (AAV) vector pX330, and an initial genetic correction rate of ~1/250 cells was observed [151]. In the same year, the CRISPR-Cas9 was successfully introduced into mice liver through adenovirus packaging and mutated the endogenous proprotein convertase subtilisin/kexin type 9 (PCSK9) gene with high efficiency. These mutations of *Pcsk9* reduced the low-density lipoprotein cholesterol level and protected against cardiovascular disease [152]. Interestingly and persuasively, three independent reports from *Science* showed that the CRISPR technologies were used to treat the Duchenne muscular dystrophy (DMD) by *in vivo* gene therapy [153–155]. The three groups delivered the CRISPR-Cas9 (SpCas9 or SaCas9) into the *mdx* mouse model of DMD through AAV vectors, and removed the mutated exon 23 from the dystrophin gene and restored dystrophin protein expression in cardiac and skeletal muscles. Improved muscle function of the DMD mice was observed. Contemporaneously, another group used the CRISPR-Cas9 to restore muscle function of the DMD mice by *in vivo* gene therapy successfully as well via electroporation-mediated delivery strategy [156]. Unsurprisingly, CRISPR-Cas12a can also correct DMD mutations in *mdx* mice [157]. Even more encouraging, reported on 30th August 2018 in *Science*, a muscular dystrophy was fixed in dog by CRISPR-based therapy [158]. These studies shed light on the future applications of CRISPR-based gene editing approaches for DMD clinical therapy. In ophthalmology, CRISPR-based *in vivo* gene therapy again successfully lowered the intraocular pressure (IOP) of mice, which is a major risk factor of primary open-angle glaucoma (POAG) [159]. The retinal degeneration in mice due to CRISPR-Cas9 mediated disruption of neural retina-specific leucine zipper protein gene (*Nrl* gene) could also be prevented [160]. In human embryos, researchers corrected the heterozygous *MYBPC3* mutation in human preimplantation embryos with precise CRISPR-Cas9 system and no off-target mutation was observed [147]. In 2018, the Cas9-GFP sgRNA-lipid complex was injected into the mouse model of human genetic deafness by canalostomy. The result showed that the injected mice had higher hair cell survival rates and lower auditory brainstem response thresholds than that of the control group [28]. Recently, two research groups applied the base editors to gene therapy. One group injected Ad vectors (adenoviral vectors) containing spCas9-BE3 and gRNAs targeting the *Pcsk9* or *Hpd* gene separately in utero [161]. Another group intravenously injected AAV-base editor systems to correct the mutation of phenylalanine hydroxylase ($Phae^{nu2}$) successfully [162].

3.2. Cell therapy

The easily accessible and programmable features of CRISPR-Cas system also spur a revolution in cell therapy. CRISPR technology was first applied in cell therapy in 2013 [163], correcting the cystic fibrosis transmembrane conductor receptor (CFTR) locus in the cultured intestinal stem cells of cystic fibrosis (CF) patients. Then it was expanded to correct clonal cells and organoids for CFTR function restoration.

Table 2
The variants of traditional Cas proteins.

Enzymes	Variants	Results	Methods	Mutated domains	ref
Sp-St3Cas9(*Streptococcus thermophiles*)	Sp-St3Cas9 (SpCas9 with the PI domain of St3Cas9)	Sp-St3Cas9: 5′-GGGCT-3′ PAM (SpCas9: NGG)	Swapped PI domains to generate two chimeras	PI	[29]
St3-SpCas9(*Streptococcus pyogenes*)	St3-SpCas9 (St3Cas9 with the PI domain of SpCas9)	St3-SpCas9: 5′-GGGCG-3′ PAM (St3Cas9: NGGNG)			
iCas9 from SpCas9	D147Y, P411T	Improved gene disruption ability	Accidentally	REC1, REC3	[21]
Variants of SpCas9	D1135E D1135V/R1335Q/T1337R(VQR) D1135E/R1335Q/T1337R(EQR) D1135V/G1218R/R1335E/T1337R(VERE) G1218R/N1286Q/I1331F/D1332K/R1333Q/R1335Q/T1337R(QQR1)	D1135E: reduced SpCas9 off-target effects VQR: 5′-NGAN-3′ PAM EQR: 5′-NGNG-3′ PAM VERE: highest activity on 5′-NGCG-3′ PAM and minimal activity on 5′-NGG-3′ PAM QQR1: 5′-TGGT-3′ or 5′-TAAG-3′ PAMs, highly specific for 5′-NAAG-3′ but a slower cleavage rate	Random mutations in the residues of PAM-interacting motif (1097–1368) for VQR/EQR/VRER, and based on the structures.	PI	[189–191]
Variants of SaCas9(*Staphylococcus aureus* Cas9)	E782K/N968K/R1015H(KKH) E782K/K929R/R1015H(KRH)	KKH KRH: 5′-NNNRRT-3′ from 5′-NNGRRT-3′ PAM	Random mutations in a region encompassing PI domain	Phosphate lock loop, TOPO, CTD	[132]
Variant of FnCas9(*Francisella novicida U112*)	E1369R/E1449H/R1556A(RHA)	RHA: 5'-YG-3′ from 5′-NGG-3′ PAM	Structure analysis of the FnCas9 and DNA/sgRNA complex	WED, PI	[133]
SpCas9-HF1	N497A/R661A/Q695A/Q926A	Enhanced specificity	Structure analysis	REC3, RuvC III	[134]
eSpCas9	K855A eSpCas9(1.0): K810A/K1003A/R1060A eSpCas9(1.1): K848A/K1003A/R1060A	Enhanced specificity and reduced off-target rate eSpCas9(1.1): enhanced specificity	Structure analysis of the complex of non-target strand groove with DNA	HNH, RuvC III	[135]
HypaCas9	N692A/M694A/Q695A/H698A	Enhanced specificity	Based on the SpCas9-HF1 and eSpCas9(1.1)	REC3	[136]
evoCas9	M495V/Y515N/K526E/R661Q(VNEQ)	Enhanced specificity, 70-fold higher fidelity of the WT	Random mutations in the REC3 domain	REC3	[137]
xCas9	xCas9 3.7: A262T/R324L/S409I/M694I/E1219V	Increased PAM diversity, and improved DNA specificity	The phage-assisted continuous evolution method	REC2, REC3, PI	[78]
Sniper-Cas9	F539S/M763I/K890N	High specificity without killing on-target activity	Random mutagenesis	REC3, RuvC II, HNH	[138]
SpCas9-NG	VRVRFRR: D1135V/L1111R/R1335V/A1322R/E1219F/G1218R/T1337R	Recognition of relaxed NG PAMs	Structure analysis	PI	[192]
AsCas12a(*Acidaminococcus* sp. *BV3L6*)	AsCas12a.RR: S542R/K607RAsCas12a.RVR: S542R/K548V/N552R	RR: TYCV; RVR; TATV PAMs, with enhanced activities *in vitro* and in human cells	Structure analysis of the complex, selected residuals in proximity to the PAM duplex	RR: WED-II, PI RVR: WED-II	[141]
LbCas12a(*Lachnospiraceae bacterium ND2006*)	LbCas12a.RR: G532R/K595R AsCas12a.RVR: G532R/K538V/Y542R	RR: TYCV + CCCC and RVR: TATV PAMs, with enhanced activities *in vitro* and in human cells	Sequence alignment with AsCas12a and structure analysis	RR: WED-II, PI RVR: WED-II	[141]
FnCas12a(*Francisella tularensis novicida*)	FnCas12a.RR: N623R/K687RFnCas12a.RVR: N623R/K629R/N633R	The highest activity of FnCas12a-RR, ~5% at one CCCC PAM site and two TYCY PAM sites	Sequence alignment with AsCas12a and LbCas12a	RR: WED-II, PI RVR: WED-II	[142]
Variant of AsCas12a	K949A	Enhanced specificity	Similar to strategies previously employed with SpCas9	BH	[141]

Table 3
Examples of disease treatment via CRISPR-based technologies.

Disease	Species	Possible pathogenesis	Therapeutic Method	Results	Ref.
Hereditary tyrosinemia	mouse	Mutations of the fumarylacetoacetate hydrolase gene *Fah*	Delivery of the CRISPR-Cas9 system and the ssDNA donor into the mouse to correct the *Fah* mutants	Initial expression of the wild-type FAH protein in ~1/250 liver cells and rescued the body weight loss phenotype.	[151]
Cardiovascular disease	mouse	Mutations in the subtilisin/kexin type 9 gene *Pcsk9*	Delivery of adenovirus expressing Cas9/sgRNA targeting the *Pcsk9* gene in mouse liver	The mutagenesis correction rate of *Pcsk9* in the liver was > 50%	[152]
Duchenne muscular dystrophy (DMD)	mouse	Mutations in the dystrophin gene	Delivery of the SpCas9 or SaCas9/sgRNA targeting the exon 23 into the *mdx* mouse model	Excision of intervening DNA and restored the *Dmd* reading frame	[153–155]
DMD	mouse	Mutations in the dystrophin gene	Electroporation-mediated transfection of the Cas9/gRNA constructs targeting the exon 23 into the skeletal muscles of the *mdx* mice	Excision of the mutant exon 23 of *mdx* the *mdx* mice model	[156]
DMD	mouse	Mutations in the dystrophin gene	Injection of the LbCas12a mRNA, the sgRNA and the ssODN donor targeting the exon 23 into the *mdx* zygotes	The *DMD* mutations were corrected in the *mdx* mice model	[157]
DMD	canine	Mutations in the dystrophin gene	Delivery of the AAV vectors carrying SpCas9/sgRNA targeting the exon 51 into the cranial tibialis muscles	Dystrophin was restored to levels ranging from 3 to 90% and the muscle histology was improved	[193]
Primary open-angle glaucoma (POAG)	mouse	Mutations in the myocilin gene	Intraocular injection of the virus containing of SpCas9/sgRNA targeting the MYOC mutation (Y437H)	Lower IOP and further glaucomatous damage prevention	[159]
Retinal degeneration	mouse	Mutation in the *Nrl* gene	Delivery of the AAVvector cayyring CRISPR-Cas9 to postmitotic photoreceptors	The treatment substantially miproved rod survival and preserved cone function	[160]
Hypertrophic cardiomyopathy (HCM)	human embryos	Mutation in the *MYBPC3* gene	Microinjection of the recombinant Cas9 protein, sgRNA and ssODN DNA into the cytoplasm of pronuclear stage zygotes 18 h after fertilization	The heterozygous *MYBPC3* mutation was corrected without evidence of off-target mutations	[147]
Cardiovascular disease	mouse	Mutations in the subtilisin/kexin type 9 gene *Pcsk9*	Injection of the Ad vector (adenoviral vector) containing BE3 and a gRNA targeting *Pcsk9* codon W159 (Ad-BE3-Pcsk9)	The base-editing rate was 10%–15% and the indel rate was about 2%, leading to reduce the plasma *PCSK9* and cholesterol levels.	[161]
Hereditary tyrosinemia	mouse	Mutation in the fumarylacetoacetate hydrolase gene *Fah*	Injection of the Ad vector containing BE3 and a gRNA targeting the upstream *HPD* enzyme Q352 (Ad-BE3-Hpd)	The lethal phenotype of hereditary tyrosinemia type 1 was rescued.	[161]
Phenylketonuria	mouse	Point mutation in the *Pah* gene on exon 7 (c.835 T > C)	Injection of a dual AAV system and split nSaKKH-BE3 targeting Pah^{enu2} gene into the adult mice	The mutant of Pah^{enu2} gene was corrected, the phenylalanine hydroxylase enzyme activity was restored, and the light fur phenotype was reversed in Pah^{enu2} mice.	[162]
Human genetic deafness	mouse	A dominant-negative missense mutation in the *TMC1*	Injection of the Cas9–guide RNA–lipid complexes targeting the $Tmc1^{Bth}$ allele into the cochlea of neonatal $Tmc1^{Bth/+}$ mice	Higher hair cell survival rate and lower auditory brainstem response thresholds were observed	[28]
Cystic Fibrosis	organoids	Mutation of cystic fibrosis transmembrane conductor receptor (CFTR) locus	The mutant F508 del allele was corrected using the CRISPR/Cas9 mediated homologous recombination	Functionality of the corrected allele in the organoid system was demonstrated	[163]
β-thalassemia	mouse	Either point mutations or deletions in the β-globin (*HBB*) gene	Generation of the iPSC-derived hematopoietic stem cells (HSCs) from the somatic cells of patients, correction of the mutations of *HBB* gene by CRISPR/Cas9 system	No observations of tumor formation in the mice model after the iPSCs transplantation	[166]
Microbial infection	mouse	Infection of bacteria with antibiotic resistance genes	Delivery of the RNA-guided nuclease Cas9 targeting antibiotic resistance genes by the bacteriophage	The bacteria in a mouse skin colonization model was killed successfully	[169]
Tumor	mouse	Mutilple pathegenetic mechanisms	Delivery of the CRISPR-based engineered CAR-T cells (such as PD-1 disrupted CAR-T cells) into the mice model	The efficiency of the engineered CAR-T cells is higher	[172,174,179]

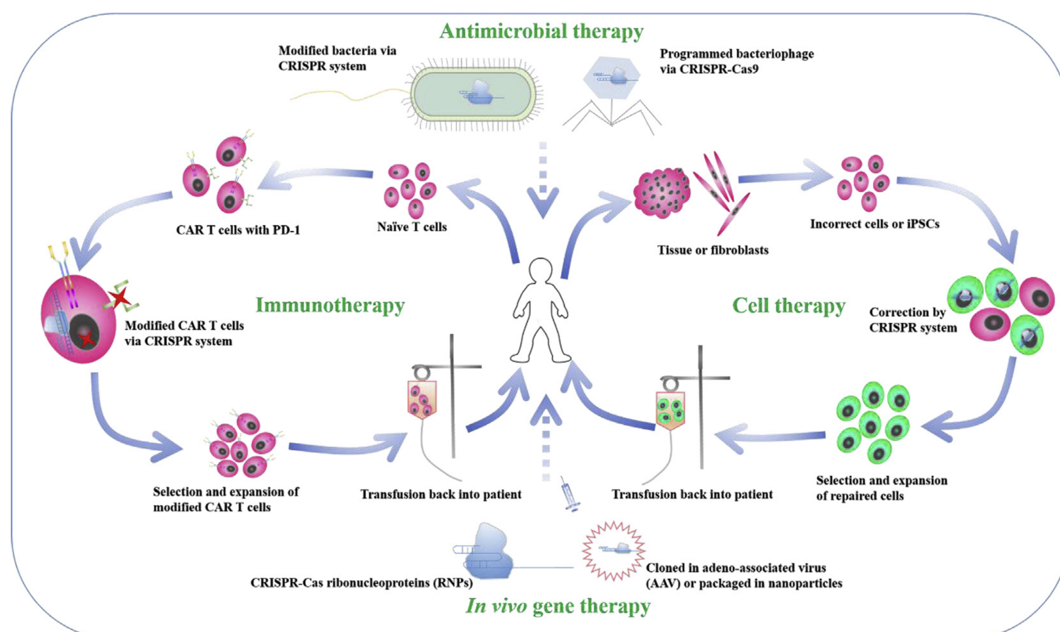

Fig. 3. Overview of the disease treatment via CRISPR-based technologies. The CRISPR systems can be used for *in vivo* gene therapy though the Cas ribonucleoproteins (RNPs) or delivered by AAV. Naïve T cells come from the patients and CAR T cells are activated by antibody-coated beads. The reprogramed CAR-T cells generated by knocking out the PD-1 or other receptors, were transfused back to the patients. The incorrect cells are isolated from the patients and iPSCs are generated by reprograming the fibroblasts, and corrected with the help of CRISPR-based technologies. The repaired cells were used for therapy.

Other than treating CF patients, CRISPR-Cas system has also been applied in the engineered human induced pluripotent stem cells (iPSCs) to correct disease-causing mutations, such as the β-thalassemia [164–166]. The general workflow (Fig. 3) includes reprograming of the iPSCs from patients' fibroblasts, engineering the iPSCs to correct the defect genes by CRISPR-based systems [167,168], screening for the corrected cells, verifying the function restoration of the modified iPSCs, and finally transfusing the repaired cells back to the patients.

3.3. Antimicrobial therapy

Antibiotic resistance has been a non-negligible risk to the world's health and global economy. Revitalizing phage therapy to treat pathogen bacterial infection has drawn the researchers' attentions. In 2014, a bacteriophage system containing CRISPR-Cas9 was reported to deal with the antibiotic resistance. The modified bacteriophage targeted the antibiotic resistance genes of *Staphylococcus aureus*, and then destroyed the genes and immunized avirulent *Staphylococci* to prevent the spread of plasmid-borne resistance genes [169]. Other than killing bacteria by bacteriophage directly, they designed the phages harboring the CRISPR-Cas system to invade in the bacteria by lysogenization, which targets the specific DNAs of the bacteria such as the antibiotic resistance gene. If the bacteria contain the resistance gene, the gene will be cleaved by the CRISPR-Cas system, and the antibiotic sensitivity of the bacteria would be higher. This allows programming of lytic phages to kill only specific antibiotic-resistant bacteria [170].

3.4. Immunotherapy

Being a powerful tool of genome editing, CRISPR technology holds great promise as the means for immunotherapy, especially chimeric antigen receptor (CAR) T cell-based adoptive immunotherapy [171]. T cells have the potential to target and destroy cancer cells. However, the programmed death-1 (PD-1) receptor on activated T cells can bind with the ligand PD-L1 expressed on dentritic cells (DCs) or some tumor cells, thus, decreasing the activity of T cells. To solve this problem, CRISPR-Cas9 system has been employed to reprogram the primary human T cells by destroying the function of PD-1 [172]. In order to reprogram T cells, the endogenous T cell receptor (TCR) locus was replaced with a new TCR in order to recognize the cancer antigen easily via CRISPR-Cas9 [173]. The engineered CAR-T cells improved the anti-tumor efficacy by ablating the genes of PD-1 via CRISPR-Cas9 system [174]. Due to the drawbacks of PD-1, CAR-T cells may recognize the alloantigens of the recipient, leading to graft-versus-host disease (GVHD), and may cause immunogenicity or alloreactivity because of HLA class I (HLA-I) on the surface of allogeneic T cells [175]. To eliminate these defects, multiplex genes were edited in CAR-T cells via CRISPR-Cas9, such as endogenous TCR locus (TRAC or TRBC) and β-2 microglobulin (B2M) [176,177]. The therapeutic efficacy of CAR-T cells with the lymphocyte activation gene-3 (*LAG-3*) deleted is higher than that of the untreated cells [178]. In another case, the CAR was integrated into the TRC locus precisely under the control of endogenous regulatory elements, which can reduce tonic signaling, avert accelerated T-cell differentiation and exhaustion, and also increase the therapeutic potency of engineered T cells [179]. In clinic, the immunotherapy with the modified T cells by CRISPR-Cas9 is on the way [180,181].

4. Conclusion remarks and future prospects

Within the last few years, stunning progress in the development of various CRISPR-based technologies has witnessed the revolutionary change in molecular biology field, particularly in mammalian model systems and human cells. Apart from the frequently used Cas9 system, alternative CRISPR-associated nuclease variants are continuing being discovered and characterized, providing potential improvements in precision, efficacy and delivery. Meanwhile, further optimization of currently well-characterized CRISPR systems also offers infinite possibilities in various applications. Still, there are some technical barriers that need to be marched over. As a generally applicable approach, CRISPR-based technologies hold great potential in therapeutic applications. In June 2016, the US approved the first CRISPR clinical trial [181]. Later in October in the same year, the engineered CAR-T cells were injected into the first patient at the West China Hospital in Chengdu [182]. In 2017, successful correction of the human embryos

for a pathogenic gene mutation was achieved [147], and more CRISPR-related clinic trials are being considered [180].

However, as the CRISPR revolution continues, social and ethical concerns over their use are also rising. Especially therapeutic applications of these powerful tools deserve greater considerations. The US Food and Drug administration (FDA) paused the CRISPR gene therapy trial in May, 2018 without giving any details [183]. Without doubts, CRISPR-based therapy is still in its infant stage, with several limitations to be addressed. One of the major concerns is the off-target effect. Different Cas nucleases have been engineered to improve the targeting precision [78,134–138]. Another major bottle neck for CRISPR-based gene therapy is the delivery efficiency. Other than technical barriers, social and ethical concerns also draw the public attentions. One report showed that Cas9 may trigger the adaptive immune responses in humans, but currently no feasible solution has been found [184]. Moreover, another report pointed out that Cas9 editing induced a p53-mediated DNA damage response, which is a phenotype common to cancer cells [185], and inadvertent changes to the human germ line has also been reported [186,187]. The causes of these problems are not clear yet, therefore, CRISPR-based therapy still has a long way to go. All in all, as the exploitation of CRISPR continues, we believe more and more spectacular improvements and applications could be envisioned.

Conflicts of interest

The authors have declared no conflict of interest.

Acknowledgements

We gratefully acknowledge financial support from the Fundamental Research Funds for the central Universities to YZ. L and National Natural Science Foundation of China (Grant No. 81502966).

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