

REVIEW ARTICLE

Application of Clustered Regularly Interspaced Short Palindromic Repeat—Cas12a System in Cancer Research and its Structural Basis

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Abstract: The clustered regularly interspaced short palindromic repeat-Cas12a (CRISPR-Cas12a) system is a new type of CRISPR-Cas system. As a unitary effector protein in this system, Cas12a recognizes 5'-TTTN-3' protospacer-adjacent motif and exhibits cleavage activity of double-stranded deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), supplementing the toolbox of CRISPR system. Compared to CRISPR-Cas9 system, CRISPR-Cas12a system has the advantage of high specificity, which is a promising tool for genetic manipulation in the basic cancer research and clinical cancer therapy. To date, three Cas12a proteins including *Acidaminococcus* sp. Cas12a (AsCas12a), *Francisella novicida* Cas12a (FnCas12a), and *Lachnospiraceae bacterium* Cas12a (LpCas12a) have been applied in transcriptional regulation or genome editing through CRISPR RNAs complementary to target DNA or RNA in cancer cells or immune cells. This review summarizes the latest applications of CRISPR-Cas12a system in cancer research and its structural basis.

Keywords: Clustered regularly interspaced short palindromic repeat-Cas12a, Protein structure, Cancer cells, Genome editing, Transcriptional regulation

1. Introduction

Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein (CRISPR-Cas) systems are composed of Cas effector proteins and CRISPR RNAs (crRNAs), existing in almost all archaea and bacteria^[1]. As an adaptive immune system, CRISPR defends against foreign hereditary material by two classes of nuclease effectors guided by ribonucleic acid (RNA). Class 1 effector utilizes multiprotein complexes^[2] and Class 2 effector depends solely on a unitary effector protein^[3]. The unique Cas effectors are classified into six types (I–VI) different systems, consisting of 32 distinct subtypes^[2].

The defense process of the CRISPR-Cas system contains the following three procedures: (1) Adaptation, a Cas protein complex cuts a fragment of the target deoxyribonucleic

acid (DNA) (a protospacer) and embeds it into the CRISPR array (where the sequence turns into a spacer); (2) processing, a precursor CRISPR RNA (pre-crRNA) is expressed and further processed to mature crRNAs; (3) interference, effector block - either a unitary large protein or another Cas protein complex, is led through a crRNA to identify and cut target DNA or RNA^[4].

Cas12a is recently identified as a novel Class 2 effector^[4]. Three Cas12a proteins including *Acidaminococcus* sp. Cas12a (AsCas12a), *Francisella novicida* Cas12a (FnCas12a), and *Lachnospiraceae bacterium* Cas12a (LbCas12a) are demonstrated to harbor the nuclease activity and have been applied in genome editing through crRNA complementary to target DNA^[4]. Compared to CRISPR-Cas9, another Class 2 system, CRISPR-Cas12a systems function differently as follows:

First, CRISPR arrays of CRISPR-Cas12a system are managed into mature crRNAs independent of the transacting CRISPR RNA (tracrRNA)^[5]. Zetsche *et al.* found that FnCas12a could efficiently cut the target DNA guided by only a transcribed mature crRNA *in vitro*^[4]. Second, Cas12a-crRNA complex recognizes a T-rich protospacer-adjacent motif (PAM) and cleaves target DNA^[6]. However, Cas9-small guide RNA complex recognizes a G-rich PAM and cuts target DNA^[7,8]. Toth *et al.* found that LbCas12a and AsCas12a could efficiently delete *DNMT1*, *PRNP*, *SPRN*, *PRND*, etc., in a mouse neuroblastoma cell line N2a through homologous recombination (HR) and two single-strand annealings (SSAs) repair pathways^[9]. Third, Cas12a generates a staggered DNA double-strand break (DSB) with sticky end and exhibits greater specificity than Cas9 in the genome editing adhibition^[10]. Mechanically, FnCas12a cuts target double-stranded DNA at position-14 of the non-target strand (nt-strand) and at 21 or 22 position of the target (t-strand) strand^[11]. Yang *et al.* found that AsCas12a and LbCas12a specifically induced disruption of a homozygously mutated *BRAF* (V600E) in a human melanoma cell line A375 with the mutagenesis percentages of 65.8% and 49.4%, respectively. However, *Streptococcus*

pyogenes Cas9 (SpCas9) not only edited mutant *BRAF* (41.1%) but also cut the WT *BRAF* (11.9%), indicating the off-target effect of SpCas9 in cancer cells. Moreover, the deletion of *BRAF* remarkably decreased the protein expression of BRAF and pERK1/2, induced cell death and morphological changes of A375 cells. The data manifested the potential applicability of Cas12a in gene therapy of cancer by specifically destroying gain-of-function mutations^[12]. Finally, Cas12a also possessed the activity of RNase and processed the crRNAs in mammalian cells, which increased the efficiency of Cas12a-mediated editing^[13,14]. For example, Chow *et al.* engineered a flipArrays containing a forward crRNA targeting *Nf1* and a reversed crRNA targeting *Pten*. LbCpf1-Flip system successfully deleted *Nf1* and *Pten* in KPD (a mammalian lung cancer cell line) at the rate of 43.5% and 74.7%, respectively^[15].

Looking insight into the protein sequence and structure, these Cas12a proteins shares a series of lengths between ~1000 and ~1400 amino acids and common crRNA clip-shaped structures^[4]. The functional differences between Cas12a and Cas9 mainly resulted from distinct structures. First of all, the nuclease domains of Cas9 and Cas12a belonged to different groups of transposon-encoded TnpB proteins^[4]. Cas9 contains HNH and RuvC nuclease domains. However, Cas12a only contains a RuvC-like domain (Figure 1). Furthermore, the N-terminal part of Cas12a adapts a mixed α/β structure, but Cas9 displays an unrelated helical recognition lobe^[4] (Figure 1). Thus, CRISPR-Cas12a system provides an efficient and specific tool for basic cancer research and clinical cancer therapy.

2. Gene deletion by Cas12a in cancer cells

Disruption of one gene or multiple genes by CRISPR-Cas12a system is one of the most widely used applications in cancer cells. In 2016, Kleinstiver *et al.* first reported that both AsCas12a and LbCas12a caused insertions and deletions (indels) at the entire 19 sites spanning *EMX1*, *DNMT1*, *FANCF*, and *RUNX1* gene targets in a human

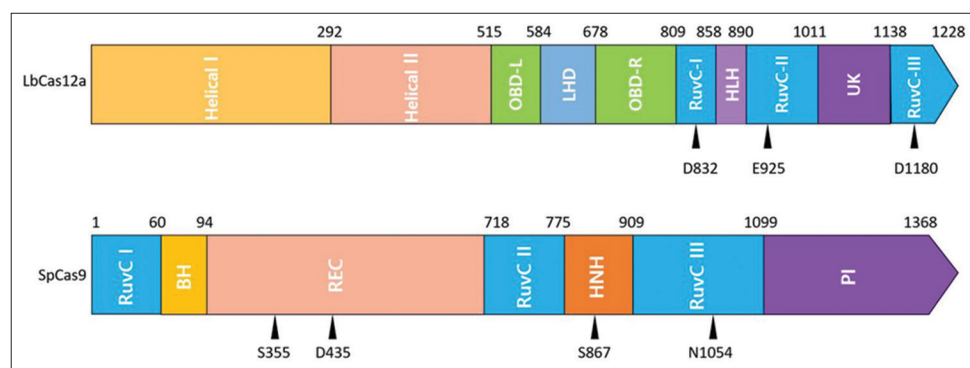


Figure 1. Schematic diagram of the polypeptide sequence and domain architectures for *Lachnospiraceae bacterium* Cas12a and *Streptococcus pyogenes* Cas9. Both the RuvC domains of LbCas12a and SpCas9 are formed by three discontinuous segments of the protein sequence. The crucial amino acids participating in target DNA recognition and cleavage are indicated by arrowheads. OBD, oligonucleotide-binding domain; LHD, helical domain; RuvC, nuclease domain; UK, domain with unknown functions; BH, bridge helix domain; REC, recognition domain; PI, protospacer-adjacent motif-interacting domain.

osteosarcoma cell line U2OS with editing efficiencies of 26.7% for AsCas12a and 33.8% for LbCas12a. Another study revealed that AsCas12a or LbCas12a was able to efficiently delete *DNMT1*, *PRNP*, *SPRN*, *PRND*, etc., in a mouse neuroblastoma cell line N2a through HR and two SSA repair pathways^[16]. Furthermore, Chen *et al.* developed a proximal CRISPR method positioning a catalytically dead Cas9 (dCas9) to loci adjacent to an FnCas12a target, which altered the chromatin structure and made the target recognizable to FnCas12a. They achieved the depletion of *cytochrome p450 oxidoreductase* with the mutagenesis percentages ranging from 11.5% to 18.7% in a human chronic myeloid leukemia cell line K562^[9].

Crystal structure of the gRNA and Cas12a protein was solved by the single-wavelength anomalous dispersion method using a SeMet-derived protein. Cas12a family adopted a bilobed architecture and had a triangle-shaped structure with a central positively charged channel^[7,17-19]. LbCas12a consists of three parts: Helical domain, oligonucleotide-binding domain (OBD), and RuvC domain (Figure 1). The helical domain was composed of helical I and helical II, which were packaged loosely against each other, contributing to one side of the triangle architecture^[7]. The OBD and RuvC formed another side of the triangle-shaped structure, in which three RuvC motifs (RuvC-I-III) consisted of the RuvC domain (Figure 1). In addition, the direct sequence of the Cas12a-bound crRNA adopted a short stem-loop-like structure with highly distorted fold^[7].

3. Transcriptional regulation by Cas12a in cancer cells

The gRNA for Cas12a is remarkably simple and consists of a unitary stem-loop in the direct repeat sequence. The structural- and sequence-specific features of the stem-loop were collectively recognized by AsCas12a^[20].

Lei *et al.* first found that an efficient DNA cleavage mediated by FnCas12a needed at least 18nt of guide sequence *in vitro*, which was similar to that of SpCas9 (16-17nt of spacer sequence)^[18]. In addition, alterations in the stem-loop undermined the RNA duplex and destroyed nuclease activity. However, base mutations in the loop region, except for the uracil base immediately preceding the spacer sequence, did not affect the nuclease activity^[18].

Based on the crystal structure of Cas12a, Liu *et al.* found that D908A mutation of AsCas12a (dAsCas12a) destroyed the endonuclease activity of AsCas12a, but not influenced the DNA binding activity. The authors applied dAsCas12a to fulfill the activation or repression of *DNMT1* transcription in 293T cells. Furthermore, the authors achieved significant transcriptional activation or repression of *VEFGA* in a human cervical cancer cell line HeLa using crRNA-dAsCas12a-tripartite VPR activator and crRNA-dAsCas12a-Krüppel-associated box more than 3 folds, respectively^[21].

4. Engineering crRNAs and Cas12a in cancer cells

In 2016, the complex structure of AsCas12a, crRNA, and a double-stranded DNA was resolved by Nureki group^[20]. Overall, AsCas12a exhibited an elliptical “sea conch” structure with a bilobal cavity formed by the REC and the NUC lobes^[20]. The REC1 domain (13 α -helices) and REC2 domain (10 α -helices and two β -strands) of AsCas12a developed a tiny antiparallel sheet to form a REC lobe^[20]. The RuvC, WED, PI, and NUC domains of AsCas12a formed the NUC lobe, in which the WED domain contained three separate regions (WED-I-III)^[20].

The crRNA of AsCas12a was composed of a 24-nt guide sequence (G1-C24) and a 19-nt building (A19-U1)^[20]. The G1-C20 nucleotides of crRNA and dC1-dG20 nucleotides of t-strand consisted of a 20-bp RNA-DNA heteroduplex^[20]. In addition, the PAM duplex was formed by the dG(10)dT(1) nucleotides of t-strand and dC(10*)-dA(1*) nucleotides of nt-strand^[20]. The crRNA displayed a pseudoknot structure, rather than an ordinary stem-loop architecture deduced from the sequence of nucleotide^[22]. The conserved sites of crRNA were A(18), U(16), U(10), and U(1) among the CRISPR-Cas12a systems^[20].

The crystal information of Cas12a-crRNA-target DNA complex depicts the exact structure and interaction between each other, providing the possibility of engineering crRNAs or Cas12a to enhance the cutting efficiency or expand the application scope. Li *et al.* reported that cr3'5F crRNA possessing five 2'-fluoro ribose and pseudouridine-modified AsCas12a mRNA enhanced gene-cutting rate by 127% and 177% compared to unmodified crRNA and plasmid-encoding AsCas12a, respectively. Similarly, pseudouridine-modified LbCas12a mRNA and cr3'5F crRNA improved mutagenesis percentages by over 300% with respect to the same control. Using this system, they also enhanced the depletion efficiencies of *AAVS1*, *DNMT1*, and *FANCF* in a human hepatocellular carcinoma cell line Hep3B and a human glioblastoma cell line U87 by 300-400%^[23].

Furthermore, Kleinstiver *et al.* reported an enhanced AsCas12a variant (enAsCas12a-E174R/S542R/K548R) RNP complexes, which could recognize various PAMs such as TRTV (TATV/TGTV), TTYN (TTTN/TTCN), and TTV (ATTV/CTTV/GTTV). Compared to wild-type AsCas12a (wtAsCas12a), enAsCas12a exhibited a 2-fold mutagenesis efficiency on sites with canonical or non-canonical PAMs in human 293T cells. Compared to wtAsCas12a, enAsCas12a elevated the mutagenesis frequencies on sites with canonical PAMs in a human osteosarcoma cell line U2OS and the gene cutting efficiencies on sites with non-canonical PAMs in a human primary T cells^[24]. Taken together, the engineering of crRNA and Cas12a mRNA could elevate the genome editing efficiency of CRISPR-Cas12 system in cancer and immune cells.

5. Recognition and detection of DNA by Cas12a in cancer cells

Dong *et al.* revealed the mechanisms underlying crRNA recognition and crRNA-guided substrate binding of LbCas12a by deciphering the crystal structure of LbCas12a-crRNA complex^[7]. Specifically, crRNA adopted a stem-loop-like structure with a highly distorted fold^[7]. These data established a foundation for LbCas12a engineering to augment its specificity and efficiency for genome editing. Beyond a powerful genome editing tool in the basic study of cancer research, CRISPR-Cas12a exhibits bright prospect in clinical DNA detection, gene therapy, and immunotherapy.

Yan *et al.* presented breaks labeling *in situ* and sequencing (BLISS) to determine endogenous and exogenous DNA DSBs in cancer cells, including a human osteosarcoma cell line U2OS and a chronic myeloid leukemia cell line KBM7^[25]. BLISS could identify the DSBs induced by AsCas12a and LbCas12a, and assess the genome-scale off-target effects of them. They also observed that Cas12a displayed higher specificity than Cas9^[25]. In addition, Chen *et al.* engineered LbCas12a, AsCas12a, or FnCas12a with a crRNA and an ssDNA activator, which could facilitate non-specific ssDNA cleavage. Based on LbCas12a ssDNase activation and isothermal amplification, the authors also proposed a method termed DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR) and realized attomolar detection of DNA. DETECTR could specifically and rapidly detect human papillomavirus in cell lines (HPV types 16-SiHa and HPV types 18-HeLa) and patient samples (>90% agreement), presenting a convenient and rapid platform for cancer diagnosis^[26].

6. Virus-mediated delivery of CRISPR-Cas12a system in primary human cells

Adenovirus (Ad), adeno-associated virus (AAV), lentivirus, and retrovirus vectors have been applied as tools of CRISPR delivery. Because AAV and Ad vectors possess the advantage of episomal nature and could insert DNA into non-transformed, dividing, or quiescent cells, they have been preferably used in the delivery process^[27]. As mentioned above, the high genome editing efficiency and low off-target effects render Cas12a to be a promising tool for primary cells editing over Cas9. Tsukamoto *et al.* constructed an Ad vector with an AsCas12a expression cassette (Ad-AsCas12a) for the 1st time. The authors applied Ad-AsCas12a system to target *AAVSI* locus in H1299 cells (a human non-small cell lung carcinoma cell line) and observed 16% indels of *AAVSI* locus. Furthermore, Ad-AsCas12a system generated 12% indels of *AAVSI* locus in primary human hepatocytes and 60% cell viability^[28]. These results indicated that the delivery of CRISPR/Cas12a system mediated by Ad vector would be a promising tool for genome editing of primary human cells.

7. CRISPR-Cas12a system in immunotherapy

The engineering of immune cells mediated by CRISPR-Cas12a system facilitates basic immunology research and immunotherapy. As mentioned above, Kleinstiver *et al.* designed enAsCas12a variant RNP complexes, which recognized expanded PAMs, enhanced genome editing activity and reduced off-target effects compared to wtAsCas12a in human cells. Specifically, the mutagenesis frequencies on targets with non-canonical PAMs mediated by enAsCas12a were significantly higher than those of wtAsCas12a in human primary T cells. Overall, enAsCas12a presents an upgraded version of Cas12a with wider application for genome editing in cancer cells and immunocytes^[24]. In another study, Dai *et al.* developed a system that AAV delivery of crRNA array combined with LbCas12a mRNA electroporation could disrupt *TRAC* and *PDCD1* sites in human primary T cells with the editing efficiencies of 78.9% and 83.6%, respectively. Furthermore, this system was able to establish a stable CAR-T cell with homology-directed repair knock-in and immune-checkpoint knockout (KIKO CAR-T cell) at high rate in single step. Specifically, 90.4% CD3⁺ CAR22⁺-sorted T cells successfully expressed CD22BBz CAR and were knocked out of *PDCD1* simultaneously. The CD22BBz CAR-T cells were highly potent in cancer cell killing and cytokine production compared with vector-transduced T cells. Collectively, the modularity of the AAV-Cas12a KIKO system provides a simple and precise approach of T-cell engineering in the cancer immunotherapy^[29].

8. Prospective

CRISPR-Cas12a system expands the toolbox of CRISPR system and has been widely applied in yeast, rice, *Arabidopsis*, zebrafish, *Xenopus*, mice and human cells for transcriptional repression or genome editing^[4,30,31]. Based on the structure of Cas12a, scientists have screened mutant strains to allow the system to perform genome editing with an extended PAM pattern and in different species^[22]. The CRISPR-Cas12a system has the advantages of high efficiency and specificity, which is widely applied in the gene deletion, transcriptional regulation, and DNA detection of cancer cells^[32,33]. More importantly, it might be a promising tool for gene therapy and immunotherapy of cancer. Further, directions of Cas12a would be the exploration of novel nuclease effectors with smaller size and higher specificity from other strains and the appropriate delivery system into the tissue or body.

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Conflicts of interest

The authors declare no potential conflicts of interest.

Author contributions

Z.Y. and W.Z. conceived the ideas of the paper, Z.Y. and K.Z. wrote the paper. T.X., S.B., Z.S., L.W., Z.Z., and C.L. revised the paper.

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