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EDITORIAL

CRISPR-Cas12 and Cas13: the lesser known siblings of CRISPR-Cas9

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CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) technology has become a keystone in genetic engineering as an invaluable tool to understand precise and molecular mechanisms of disease development, drug resistance and single-cell biology (Fang and Wang 2016; Wang and Wang 2017; Wang et al. 2018). CRISPR is divided into class I and class II, with 6 divisions and finally over 30 different subtypes (Koonin et al. 2017). Of those, type II-A CRISPR-Cas9 (Csn1) was found to cut DNA with a guide RNACas12a (Cpf1) from the bacterium *Francisella novicida* was and differentiated itself from Cas9 by relying on a "T-rich" PAM sequence, requiring CRISPR RNA (crRNA), and "stagger" cutting when targeting DNA, making it ideal for multiplexed genome editing as one vector can carry additional crRNAs. Furthermore, it can cleave DNA multiple times as the recognition sequence is not affected by post-repair. Other emerging systems, Cas13a (C2c2) and Cas13b (C2c6) are specialised at RNA interference (Shmakov et al. 2017). It is important to recognise key differences of functions between Cas12 and Cas13 and hurdles to be overcome prior to therapeutic application.

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One of obstacles before clinical application is to ascertain the mechanisms of type V effectors regarding target recognition and cleaving. Cas12 and Cas9 share similar structural shapes with minor deviations except for the RuvC domain which is superimposable. A T-rich PAM sequence is required such as 5′-TTN-3′ when DNA is cleaved. A distinct difference of Cas12a property is the presence of a unique fold domain due to lack of an HNH domain, similar to the RuvC domain. The positively charged central channel of a nuclease (NUC) lobe may determine the cleaving of the target strand after catalytic residue mutations in the RuvC domain of Cas12a in the bacterium *Acidaminococcus* sp. inhibited the cleaving in target and non-target strands (Yamano et al. 2016). Although Cas12b lacks an HNH domain and possess an NUC domain that determines target strand cleavage activity similar to Cas12a, the structure of the NUC domain of Cas12b is distinctly different from a Cas12a NUC domain. Both Cas12a and Cas12b use the RuvC-like domain to cleave DNA strands after significant conformational changes related to the initial cleaving of the non-target strand. Mutations in the NUC domain in *Francisella novicida* resulted in incomplete inactivation of the target strand cleaving. This highlights the possibility that DNA cleavage activity was determined by the RuvC-like domain instead of the NUC domain that was initially proposed. The NUC domain participates in guide target binding (Swarts et al. 2017). The size of Cas12b is significantly smaller than those of commonly used

Cas9 and Cas12a; making it an ideal candidate for adeno-associated virus (AAV)-mediated experimental in vivo delivery and clinical application with less off-target effects than Cas9.

Dynamic effects of off-target genome editing should be paid special attentions to and are considered one of major obstacles for clinical application (Luo 2019; Wang and Wang 2019; Li et al. 2019; Chen 2019; Liang and Huang 2019). Off-target editing is also a critical issue in the Cas13 system, although Cas13 is already applied to the knockdown of transcripts, editing of base RNA and live-cell transcript imaging. CRISPR-Cas13 consists of four subtypes, Cas13a, Cas13b, Cas13c and Cas13d (VI-D) and differentiates itself from Cas12/Cas9 by lacking a DNAse domain which is replaced by two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains externally. Target specificity is determined by a 28–30-nt spacer and requires a guide RNA of ~ 64 nt. Uniquely to Cas13, collaboration activity post-recognition occurs and subsequently there is non-specific degradation of surrounding transcripts. Although this phenomenon occurs in bacteria, it does not exhibit itself in plant and mammalian cells resulting in its potential development as an application for RNA targeting. A potential field for application is designing it to specifically and accurately carry out diagnostics by utilizing it to cleave fluorescent reporters on target recognition (Gootenberg et al. 2017, 2018). Cas13a and Cas13b show their potentials as RNA knockdowns whereas orthologs of Cas13d could control endogenous transcript splicing with the possibility of in vivo delivery due to its small size (~ 930 aa) which is the smallest size for a class 2 CRISPR effector (Konermann et al. 2018). The application of varying analytic methodologies and editing methods is related to off-target effects. One of the key issues faced in RNA engineering in Cas13d is the difficulty in designing RNA-binding domains which can be targeted into desired cells. Additionally, some CRISPR systems can be reprogrammed to target and identify 20–30 nt RNAs. However, the major limitation is in size (~ 1200 aa) preventing them from being viable in in vivo delivery as they cannot be packed in AAV (Abudayyeh et al. 2016).

The identification of key mechanisms in Cas13 is critical prior to clinical implementation. Type VI locus systems are composed of Cas1, Cas2, Cas13a and a CRISPR array, as seen in *Leptotrichia shahii* (LshCas13a). It seems that Cas13a is the sole determinant for crRNA guidance as Cas1 and Cas2 are only involved in spacer acquisition. LshCas13a can identify target sequences complementary of the crRNA spacer about 22–28-nt long to cleave ssRNA. The crRNA spacer has a 24-nt-long stem-loop structure and a mononucleotide protospacer- flanking site is in the 3′-end with bias to A, U or C. Loop regions and other exposed regions were shown to prefer cleaving by LshCas13 and crRNA complex with the absence of tracrRNA. The efficacy of cleaving was also affected by the number of mismatches as two or more mismatches resulted in a significant reduction of RNA cleavage efficiency. The ssRNA target activation of the complex could non-specifically cleave ssRNA. The defence mechanism for RNA phases in *Leptotrichiabuccalis* Cas13a is that the binding of a crRNA target RNA duplex occurs in a positively charged NUC lobe. Within the NUC lobe the duplex undergoes conformational changes and HEPN2 and HEPN1 domains shift closer to activate the HEPN catalytic site of Cas13a which subsequently cleaves the single-stranded targets. However, the requirement of the pre-crRNA is still questionable, as Cas13a might exhibit cleavage activity without the presence of pre-crRNA even though cleavage activity was enhanced.

The most important feature of Cas12 in gene editing is its targeting of previously un-targetable sequences. For example, Cas12 targets T-rich PAM sequences without requests of G-PAMs. This enables scientists to have an increased scope for genome editing shown by its use in malaria parasites (Wang et al. 2019). Using Cas12a as a nucleic acid detection platform for viruses, the DNA endonuclease-targeted CRISPR transreporter (DETECTR) can achieve attomole DNA detection by combining isothermal amplification and Cas12a ssDNAse can be activated in papilloma virus samples (Chen et al. 2018). In comparison with Cas12a, Cas12b remains to be explored and emphasis must be put on addressing undesirable effects. Its requirement of high temperature makes it unsuitable for mammalian cells, since *Alicyclobacillus acidiphilus* Cas12b has its lower optimal temperature for nuclease activity in mammalian systems. Cas12i could play an important role in improving high-fidelity genome editing as it could improve double-nicking applications (Yan et al. 2019). The high specificity, stability in plasma, minimal size for AAV delivery and negligible off-target effects makes it an ideal candidate for therapeutic genome editing. Teng

et al. (2019) developed a new Cas12b-mediated DNA detection strategy (CDetection) to monitor DNA with sub-attomolar sensitivity, on basis on the non-canonical ssDNA transcleavage properties of the Cas12b nuclease triggered by targeted dsDNA. This is an excellent example of Cas12a-based application and development to detect dsDNA directly and to achieve single-nucleotide sensitivity, although there are still more improvements to reach clinical samples and show values of molecular diagnostics. The application of Cas13 as a diagnostic tool may have more significant implications in both agriculture and clinical settings. The development of Specific High-sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) demonstrated the potential of Cas13a as a detection platform (Gootenberg et al. 2017). Based on isothermal pre-amplification with Cas13a, attomole nucleotide detection is possible in viruses and bacteria such as synthetic Zika and dengue viruses. Subsequently, the updated version, SHERLOCKv2, addressed deficiencies in the previous model by achieving and increasing sensitivity (zeptomolar level), portable lateral flow strips and ability to use quantitative detection, composed of orthogonal and nucleic acid sequences forming a four-channel simple multiplex complex (Gootenberg et al. 2018). A modified SHERLOCK system was used for nucleic acid sampling in soybeans to investigate the glyphosate resistance gene and to highlight the flexibility and potential of the applications (Abudayyeh et al. 2019).

It is undeniable that CRISPR will play a crucial role in the field of genome editing and therapeutics. Although Cas9 was initially under the spotlight, the emergence of Cas12 and Cas13 provides exciting new avenues as a detection platform for viruses, potential in AAV in vivo delivery and RNA interference and regulation engineering for therapeutic and clinical future treatment. Furthermore, cytotoxicity induced by Cas9 in certain species could be bypassed by Cas12. However, the understanding of both systems still needs to be developed and further attention needs to be placed on discovering the mechanical and functional differences of each subtype. The use of Cas13 in in plant genes for nucleic acid detection could open a new field of study that would have significant implications for agricultural application. Further uses of CRISPR will be discovered as we expand and investigate our CRISPR-toolbox, and it is indubitable that CRISPR will play a major therapeutic and clinical role in the future.

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