論文の内容の要旨

論文題目

Crystal structure of the minimal Cas9 and generation of mutants with enhanced catalytic activity

（最小 Cas9 の結晶構造解析と活性向上変異体の創出）

氏名 山田 真理

The RNA-guided DNA endonuclease Cas9 derived from the microbial type II CRISPR-Cas system associates with dual guide RNAs, crRNA (CRISPR RNA) and tracrRNA (*trans*-activating crRNA), or a synthetic sgRNA (single-guide RNA), and generates a DNA double-strand break at DNA target sites. The CRISPR-Cas9 system has been harnessed for a variety of new technologies, exemplified by genome editing. Cas9-mediated DNA targeting requires a PAM (protospacer adjacent motif), a short nucleotide sequence adjacent to the target site. Cas9 proteins and their cognate guide RNAs from different microbial species are remarkably divergent in sequences and lengths. Crystal structures of Cas9 orthologs, such as *Streptococcus pyogenes* Cas9 (SpCas9, 1,368 aa) (Nishimasu *et al., Cell* 2014; Anders *et al., Nature* 2014), *Staphylococcus aureus* Cas9 (SaCas9, 1,053 aa) (Nishimasu *et al., Cell* 2015) and *Francisella novicida* Cas9 (FnCas9, 1,629 aa) (Hirano *et al., Cell* 2016), provided mechanistic insights into the RNA-guided DNA cleavage mechanism of Cas9, and facilitated engineering of CRISPR-Cas9 to create new tools, such as Cas9 variants with enhanced fidelity (Slaymaker *et al., Science* 2015; Kleinstiver *et al., Nature* 2016) or altered PAM specificities (Kleinstiver *et al., Nature* 2015; Kleinstiver *et al., Nat Biotech* 2016), and a potent transcriptional activator (Konermann *et al., Nature* 2015). However, the action mechanisms of divergent Cas9 orthologs have not been fully understood.

Here, the author reports the crystal structures of *Campylobacter jejuni* Cas9 (CjCas9, 984 aa), one of the smallest Cas9 orthologs, in complex with sgRNA and its target DNA. A structural comparison of CjCas9 with the other Cas9 orthologs provided insights into a minimal functional Cas9 scaffold. Furthermore, the CjCas9 structure revealed an unexpected diversity in orthologous CRISPR-Cas9 systems, such as the structural diversity in tracrRNA scaffolds and the mechanistic diversity in PAM recognition. Whereas other characterized tracrRNA scaffolds

contain several stem loops, the CjCas9 tracrRNA scaffold contains a triple-helix architecture, which is distinct from other known RNA triple helices, such as the telomerase RNA subunit TER, the SAM-II riboswitch and long noncoding RNA MALAT1. Thus, these structural findings highlight the structural diversity of the tracrRNA scaffold, and expand the natural repertoire of RNA triple helices. In addition, the previously characterized Cas9 orthologs "read" nucleotide sequences in the non-target DNA strand within the PAM-containing duplex to achieve PAM recognition (for instance, SpCas9 recognizes the 5′-NGG-3′ in the non-target DNA strand). In stark contrast, these structural and functional analyses revealed that CjCas9 recognizes the promiscuous 5′-NNNVRYM-3′ PAM by reading nucleotide sequences not only in the non-target strand, but also in the target strand. The crystal structure of CjCas9 revealed an unexpected diversity of recognition mechanism of guide RNA and PAM in CRISPR-Cas 9 system.

CjCas9 has been thought to be unavailable for genome editing due to weak DNA cleavage activity in eukaryotic cells (Ran *et al., Nature* 2015). However, very recently, it was reported that genome editing using CjCas9 was successful by examining the sequence of the guide RNA and PAM (Kim *et al., Nature Commun* 2017). Since small CjCas9 has advantages such as high efficiency of introduction into viral vectors, it is expected to be applied to genome editing. However, CjCas9 has weak DNA cleavage activity in eukaryotic cells because cleavage efficiency was lower than that of SpCas9. From this background, the author conducted a search for activity enhancing mutants and succeeded in creating mutants with twice as much activity intensity as WT CjCas9. Overall, these findings improved our mechanistic understanding of the CRISPR-Cas9 systems, and provided a framework for further Cas9 engineering for its applications.