J Biosci Vol. 42, No. 4, December 2017, pp. 527–530
DOI: 10.1007/s12038-017-9723-4

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Series

What history tells us XLIV: The construction of the zinc finger nucleases

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Keywords. CRISPR-Cas9; homologous recombination; meganucleases; protein domains; restriction enzymes; synthetic biology; zinc finger

1. Introduction

In three previous contributions, I tried to show how complex and tortuous had been the historical process that led to the magic tool CRISPR-Cas9 (Morange 2015a, b, 2016). In particular, it was the result of an unexpected convergence between the study of bacterial and archaeal sequences later shown to be involved in the immunity against bacteriophages, and the slow development of highly specific endonucleases. In this second root of the development of the CRISPR-Cas9 magic tool, zinc finger nucleases and their designer, Srinivasan Chandrasegaran, had the major role. In three articles published between 1992 and 1996 in the *Proceedings of the National Academy of Sciences* of the United States, he conceived and developed a radically new type of endonuclease (Li *et al.* 1992; Kim and Chandrasegaran 1994; Kim *et al.* 1996). He also described the multiple potential applications of these new tools. It was a wonderful study in synthetic biology, before the name was introduced at the beginning of the 2000s. First, we must describe two other lines of research and groups of researchers whose results were necessary for the development of Chandrasegaran's project, but also gave it its full significance.

2. From splicing to homologous recombination: the work of Bernard Dujon and his colleagues

Piotr Slonimski's lab in which Dujon was trained was the first to characterize the complex splicing process occurring in yeast mitochondria, and to provide evidence that, in contrast to nuclear introns, some mitochondrial introns encode proteins involved in the splicing process (Lazowska *et al.* 1980). Among these proteins were discovered endonucleases involved in the insertion of the introns at a precise position in the mitochondrial genome (Jacquier and Dujon 1985). One of these endonucleases, I-Sce I, was expressed in *E. coli* after a modification of some of its codons, and was shown to recognize a 18 bp sequence (Colleaux *et al.* 1988; Monteilhet *et al.* 1990). The initial purpose of this work was to extend the ensemble of restriction endonucleases that had been so important for genetic engineering through the purification and characterization of meganucleases that cut DNA at a limited number of positions in the genome. They could be used to map genomes, a step considered as essential before genome sequencing. In 1992, Dujon and his collaborators showed that it was possible to introduce cleavage sites for I-Sce I in the yeast genome, and to use them to obtain large fragments of the yeast chromosomes (Thierry *et al.* 1991; Thierry and Dujon 1992). This procedure was used to map yeast chromosome XI.

But these meganucleases were not stable, and their yield was low. In addition, there was limited hope of extending their number. This is probably why Dujon decided to use these endonucleases for a different project: to characterize the mechanisms of homologous recombination. In particular, did this process involve a single-strand or a double-strand break? In 1992, Dujon and his collaborators compared the effects of two endonucleases, I-Sce I and the endonuclease HO involved in the conversion of the mating type (Plessis *et al.* 1992). They demonstrated that both endonucleases

increased the level of homologous recombination, confirming the role of a double-strand break in this process. The conclusions and the application of the previous observation were reported in an article published in 1993, where the favourable effect of a double-strand break on homologous recombination was extended to plant cells (Puchta *et al.* 1993). Previous work had demonstrated that a double-strand break was not only an early step in homologous recombination, but was also the rate-limiting step in this process: introducing breaks into DNA was the way to favour homologous recombination. In 1994, another group drew the same conclusions from experiments done on mammalian cells (Rouet *et al.* 1994). In 1995, Dujon's group confirmed the previous observation – stimulation of homologous recombination – not on a plasmid as in the 1994 experiment, but on a sequence inserted in a chromosome (Choulika *et al.* 1995).

Interest in meganucleases changed dramatically. Instead of being tools to cut genomes at precise positions for mapping before sequencing, they could now be used to increase the level of homologous recombination in organisms in which it was too low, thus making the experiments long and tedious or preventing any medical application in humans.

3. Zinc finger motifs, and the modular organization of proteins

Thanks to the new genetic engineering tools, a large number of DNA-binding proteins were isolated and characterized in prokaryotes and eukaryotes in the 1980s, and their structure was determined at the beginning of the 1990s. Many of them were perfect illustrations of a modular organization of proteins: the DNA-binding domain was distinct from the other functional domains. The independence of the different modules was rapidly exploited in the double-hybrid technique, which finds the partners of a given protein (Fields and Song 1989). Wide-ranging application of this technique later allowed the construction of interactomes, a complete description of the interactions between proteins in a cell or in an organism.

Different families of DNA-binding proteins were described, harbouring different types of DNA-binding motifs, helix-loop-helix, helix-turn-helix, zinc fingers, etc. The name 'zinc finger' came from the existence in these proteins of short sequences of amino acids that bind a zinc atom and are viewed as fingers sensing the DNA molecule.

The latter motif was of a particular interest. It was described early (Miller *et al.* 1985) and corresponded to a huge family of DNA-binding proteins. NMR and X-ray diffraction studies on crystals revealed the structures of some members of this family, and provided a precise description of the interactions with their DNA targets (Pavletich and Pabo 1991). What was remarkable was that the modular organization extended within the DNA-binding domain: these domains contained multiple zinc fingers, each successively and independently recognizing three nucleotides on the DNA molecule.

The door was open to the production of DNA-binding proteins with new specificities, by selecting and assembling different zinc fingers. The objective was to shed light on the interactions between DNA-binding proteins and DNA, but also eventually to alter these interactions.

4. Srinivasan Chandrasegaran: A wonderfully successful project in synthetic biology

Chandrasegaran acquired a good training in the physical-chemical study of DNA-binding proteins (Chandrasegaran and Carroll 2016). His initial project was not original: to develop new, highly specific, restriction enzymes. But he very rapidly realized that the approaches followed until then had been dead ends. The efforts that had been made to modify well-described restriction enzymes to convert them to enzymes with a high specificity had been disappointing, and the hope of finding meganucleases comparable to I-Sce I was limited: since the role of most restriction endonucleases was to degrade foreign DNA, there was no selective pressure that might have favoured the formation of such enzymes.

So, Chandrasegaran initiated a radically new project aimed at building an artificial restriction enzyme by combining a nuclease domain with a DNA-binding domain obeying simple rules of DNA recognition, with a view to modifying it so as to adapt it to different targets.

In 1992, he selected a nuclease domain, that of Fok I, a restriction enzyme from *Flavobacterium okeanokoites* (Li *et al.* 1992). The reason was that, in this type of restriction enzyme, the sequence that was recognized and the sequence that was cut were different, and located a few nucleotides apart. It suggested that the nuclease and the DNA-binding domains were at least partially separated, and that the nuclease domain would be able to function with other DNA-binding domains. Some of these hypotheses were confirmed by experiments reported in the article.

Two years later, Chandrasegaran described the construction of a chimeric protein containing the nuclease domain and the *Drosophila* homeodomain of ultrabithorax, and demonstrated that this chimeric protein was able to cut the DNA sequences to which the ultrabithorax protein binds (Kim and Chandrasegaran 1994). This chimeric protein proved the value of the concept that guided the project. But the organization of the ultrabithorax domain is not modular.

In 1996, Chandrasegaran and his collaborators combined the Fok I nuclease domain with a DNA-binding domain containing three zinc fingers (Kim *et al.* 1996). The result was as positive as 2 years before. But with zinc fingers, the

road was now open to adapt the system to any DNA sequence.

5. The end of the story

The road was not as straight as had been imagined. The organization of zinc fingers was not completely modular, and a huge amount of work was required to improve the system. Chandrasegaran needed nearly 10 years to convince himself that the system was now ready to be used (Durai *et al.* 2005).

Meanwhile, Dana Carroll's group, in collaboration with Chandrasegaran, had demonstrated that homologous recombination was increased in *Xenopus* oocytes by the introduction of a zinc-finger endonuclease (Bibikova *et al.* 2001). The impact of this experiment was great: it was rapidly reproduced by other laboratories and in different organisms, and in 2005 the first medical applications of the new technology were conceived (Urnov *et al.* 2005).

But zinc finger nucleases were rapidly pushed out of the limelight. In 2009, the rules governing DNA recognition by the transcription activator-like effector proteins isolated from plant pathogenic bacteria were shown to be simpler than those of zinc finger proteins (Boch *et al.* 2009; Moscou and Bogdanove 2009), and the first TALENs were rapidly shown to be efficient (Christian *et al.* 2010; Miller *et al.* 2011).

In 2012, CRISPR-Cas9 offered another, much simpler way to design specific endonucleases.

6. Conclusions

The previous paragraphs described lesser known episodes of the history of editing tools that have not found the place that they deserve (see, for instance, Lander 2016). It is difficult to foresee the exact future of zinc finger nucleases, but it will probably be less brilliant than that of CRISPR-Cas9. However, their development was far from being useless. Zinc finger nucleases opened the way to genome editing. It was thanks to the fame that they acquired that the groups that characterized the mechanisms of action of CRISPR-Cas9 were immediately able to acknowledge the potential of this new tool (Jinek *et al.* 2012; Gasiunas *et al.* 2012).

Whatever the future of zinc finger nucleases, the work of Chandrasegaran will remain remarkable. Alone he took three decisive steps that were audacious but successful. He decided that it was time to abandon the search for naturally occurring endonucleases and instead to turn towards their construction. And for this he decided to use the modular organization of proteins and to choose among known DNA-binding domains that which was best adapted to his work.

As we have seen, the study of Chandrasegaran's work does not provide recipes for success. But it does give clues to the way scientific knowledge progresses. It was a combination of deep originality and smart exploitation of recent observations. The leap that was his decision to build an artificial restriction enzyme was combined with rapid use of recent results on the modular organization of proteins and the way they interact with DNA. Chandrasegaran rapidly perceived a niche favourable to the development of his work. His strength lay in the link that he was able to establish between an ambitious project and the opportunities created by the proliferation of molecular descriptions.

Acknowledgements

I am indebted to David Marsh for his critical reading of the manuscript.

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