Homing In On Increased Meganuclease Specificity

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Genome engineering and modification, in which particular chromosomal loci are precisely altered, holds great therapeutic potential, particularly in the area of cancer immunotherapy. In this context, endogenous genes must be disrupted (and additional exogenous genes must be inserted) into patient-derived primary hematopoietic cells such as T-cells or stem cells to create cells with the weaponry to attack specific cancer cell types. A variety of genome engineering systems are now available, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), CRISPR/Cas9 endonucleases, and meganucleases. Each system has unique advantages and disadvantages, and none are currently ideal for in vivo therapeutic use. Meganucleases hold great promise for gene targeting, but have not been widely used for genome engineering as they are very difficult to optimize to target specific target sequences.

As reported in *Proceedings of the National Academy of Sciences*, staff scientist Dr. Ryo Takeuchi and colleagues in the laboratory of Dr. Barry Stoddard (Basic Sciences Division) set out to develop an efficient scheme for engineering the DNA recognition specificities of meganucleases. This was a daunting task given that the DNA binding surface of meganucleases spans up to 50 amino acids that contact approximately 22 base pairs of DNA.

"Traditional methods of protein engineering and selection are generally insufficient for reprogramming such an extensive molecular interface, because the number of possible combinations of amino acids across such an extensive interface is many orders of magnitude greater than what can usually be produced and sampled in such experiments," said Dr. Stoddard. "The methods exploited in the paper allow us to increase our 'sampling frequency' of protein variants by up to 1000-fold, while also applying pressure for highly active and stable nucleases."

The authors computationally randomized key amino acid positions within meganuclease sequences and constructed libraries of DNA molecules coding for these variants. To screen these variant meganucleases, the authors used a modified *in vitro* compartmentalization (IVC) system. In this system, DNA fragments encoding both a meganuclease variant and a target site are individually expressed in droplets containing transcription/translation reagents. Thus, in each droplet, an

endonuclease is only able to cleave a target site on the same molecule as its own gene, allowing confident assignment of cleavage activity to the corresponding meganuclease. Multiple rounds of IVC were performed with increasing stringency, yielding seven final variant meganucleases.

The researchers next tested their IVC-identified variant meganucleases for cleavage in living cells using a bacterial cleavage assay. In this approach, meganucleases are expressed in bacterial cells along with a reporter plasmid containing a toxic gene and a meganuclease target site. Cleavage leads to elimination of the plasmid and cell survival. All final IVC-selected meganucleases substantially increased bacterial cell survival, indicating their in vivo effectiveness.

The authors next tested the efficacy of their meganucleases by expressing each enzyme in HEK293 cells and assessing the accumulation of small insertions and deletions (indels). They found indels at the endogenous target site for each meganuclease 2.5-34% of the time. The authors next fused each enzyme to a TAL effector domain (megaTAL), which increased meganuclease cleavage efficiency through increased DNA binding specificity. Additionally, co-expression of two MegaTALs was sufficient to generate deletion of a specified chromosomal locus.

The exquisite sequence specificity of meganucleases, particularly in the context of megaTALs, may be especially advantageous for therapeutic genome editing applications, which require exceptionally high sequence specificity to avoid deleterious off-target effects. Indeed, future work in this area will focus on the application of engineered meganucleases and megaTALs to disease-relevant systems. Said Dr. Stoddard, "the biggest areas for new questions and experiments following this work are (1) continued examination and addition of new meganuclease scaffolds to our toolkit of genome editing nucleases, and (2) demonstration of efficient genome editing (and in particular, recombinational gene modification) in primary, patient-derived human cells (as opposed to artificial cell lines)."

Citation:

Takeuchi R, Choi M, Stoddard BL. 2014. Redesign of extensive protein–DNA interfaces of meganucleases using iterative cycles of *in vitro* compartmentalization. *Proc Natl Acad Sci USA* 111(11):4061-4066.

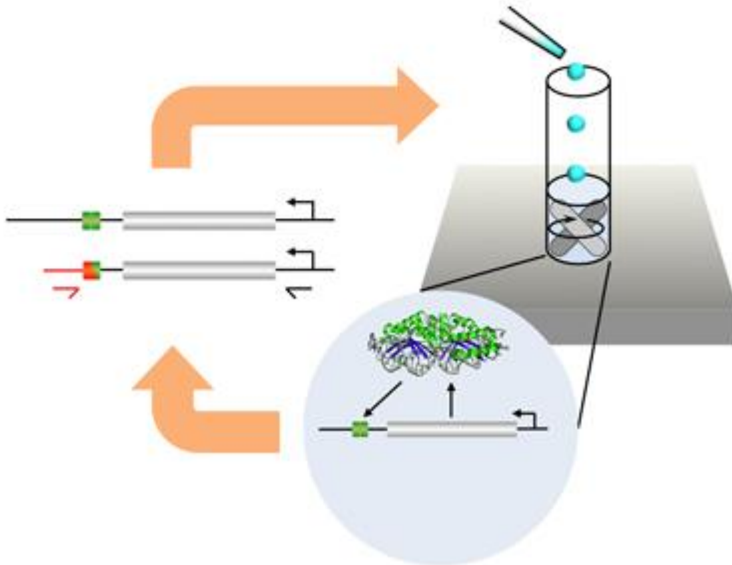

Image provided by Dr. Barry Stoddard

Schematic depiction of in vitro compartmentalization. A DNA fragment containing both a meganuclease gene (grey box) and a meganuclease target site (green boxes) is compartmentalized with in vitro transcription and translation reagents, allowing for synthesis of each meganuclease variant, within liquid droplets. Following successful cleavage, an adapter can be ligated to the cleaved target site, allowing identification of the effective variant meganuclease by PCR with an adapter-specific primer (red arrow) and a primer common to all DNA fragments (black arrow).