The Development and Use of Zinc-Finger Nucleases

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Abstract Zinc-finger nucleases (ZFNs) were the first of the targetable nucleases to be developed and exploited for genome engineering. They have proved remarkably effective, enhancing the frequency of gene targeting several orders of magnitude. The modularity of DNA recognition by zinc fingers has made it possible to design ZFNs for a wide range of genomic targets in a remarkable assortment of organisms and cell types. Use of this platform helped define the parameters and approaches for nuclease-stimulated genome manipulation. Although much of the territory has been ceded in the last few years to the more easily designed TALENs and CRISPR/Cas nucleases, successful ZFNs are still in wide use in a number of applications, including current clinical trials.

Keywords Zinc-finger nucleases (ZFNs) • Nonhomologous end joining (NHEJ) • Homologous recombination (HR) • Gene targeting

Introduction

If you are a geneticist, you have two ways to proceed to get a mutation in your favorite gene—forward or backward. Classically (forward genetics) you would generate random mutations, identify an interesting phenotype, and then endeavor to characterize the gene that harbored the responsible mutation, which might or might not be your gene. With the advent of methods for gene isolation and DNA sequencing, it became plausible to go the other direction (reverse genetics), first identifying the gene of interest and then attacking it specifically to generate mutations and test for phenotypes. Until relatively recently, however, the tools to do this were quite limited.

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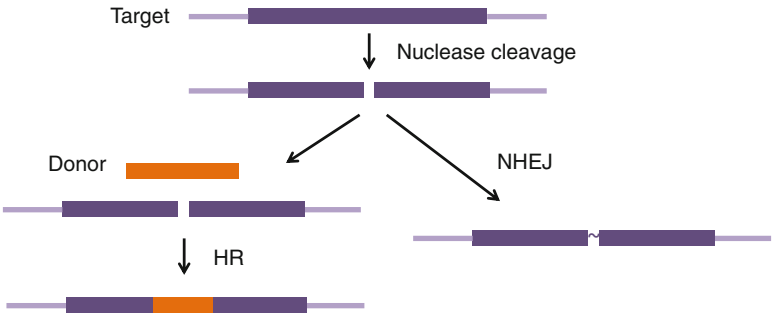

Fig. 1 Pathways of gene modification after a targetable nuclease-induced break. The target is shown as *a purple rectangle*. After nuclease cleavage, in the presence of a homologous donor DNA sequence (*orange*), the break can be repaired by homologous recombination (HR), incorporating sequences from the donor. An alternative repair pathway is nonhomologous end joining (NHEJ), which often leaves small sequence alterations at the site of the break, as indicated by the squiggle

In the 1970s and 1980s, investigators developed methods for gene targeting in yeast [1, 2] and in mice [3, 4], based on homologous recombination (HR) between an introduced DNA molecule and an endogenous target. The absolute frequency of recombination was quite low, even in yeast, but strong selection allowed recovery of the desired cells. For reasons both technical and biological, it proved difficult to extend these methods to other organisms. Beginning in the 1990s, whole genome sequences were being determined, and the desire for a facile approach to manipulate specific genes was growing. Researchers could identify sequences that they would like to alter, but had no reliable way to do so.

An important insight was the recognition that gene targeting relies on cellular DNA repair activities, and that, in normal circumstances, the intended genomic target is intact and not in need of repair. Double-strand breaks (DSBs) in chromosomal DNA constitute potentially lethal damage and must be repaired [5]. Furthermore, DSBs stimulate HR in a range of circumstances, including natural meiotic crossing over. It seemed, therefore, that the key to expanding the range and efficiency of gene targeting was to make the target more susceptible to homologous repair by breaking or otherwise damaging it. In addition to HR, DSBs are repaired in essentially all organisms by an error-prone process, called nonhomologous end joining (NHEJ). In practice, targeted DSBs lead both to local mutagenesis via NHEJ and, in the presence of an appropriate donor DNA, to targeted gene replacement (Fig. 1).

Quite a number of approaches have been taken to addressing specific genomic target sequences, and most of them are reviewed in this volume. The methods that have taken hold involve the use of nuclease proteins with separable recognition and cleavage modules. This class includes zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas RNA-guided nucleases (CRISPRs). My assignment is to review the development and applications of the pioneers among these, the ZFNs. More extensive reviews are available elsewhere [6–9].

Origins of ZFNs

ZFNs are not natural proteins, but they originated from natural components. In the early 1990s, Chandrasegaran and colleagues discovered that the Type II restriction enzyme, *Fok*I, has separable DNA-recognition and DNA-cleavage domains [10]. This observation stimulated the conjecture that novel specificities could be produced by linking the nonspecific cleavage domain to alternative DNA-binding modules. This was demonstrated first by fusion with the homeo-box from the Drosophila transcription factor, Ubx [11].

Meanwhile, repetitive structural modules, called zinc fingers, were identified in a number of eukaryotic, DNA-binding transcription factors [12, 13]. The structure, determined by Pavletich and Pabo [14], of a set of three fingers bound to their cognate site confirmed the modularity of recognition and the coordination of a single zinc atom by two histidine and two cysteine residues in each finger. The principal contacts made by each finger were to three consecutive base pairs in the DNA [15] (Fig. 2). In his second chimeric restriction enzyme, Chandrasegaran fused two different sets of zinc fingers provided by his colleague, Jeremy Berg, to the *Fok*I cleavage domain and again demonstrated redirected cutting [16]. These fusions were the first ZFNs.

Information available in 1996 suggested that a wide range of DNA sequences could be specified by zinc fingers and that there might even be a code of recognition [17, 18]. The latter prospect has not been borne out [19], but the former certainly has. By design, by selection, and by characterizing natural fingers, researchers have established an extensive catalog of individual fingers and combinations that recognize many different sequences. The establishment of a code has been foiled by the fact that fingers that perform well in one context do not routinely function well in others [20, 21].

Characterization of ZFNs

Experiments by Smith et al. [22] and Bibikova et al. [23] determined the requirements for ZFN cleavage, both in solution and in cells. The *Fok*I cleavage domain must dimerize to be a functional nuclease [22, 24]. Apparently some aspect of dimer formation in the natural restriction enzyme is lost in the zinc-finger fusion. As a consequence, the weak dimer interface alone promotes association only at very high protein concentrations [24]. To achieve efficient cleavage by ZFNs, two sets of zinc fingers are required, each linked to a cleavage domain monomer and directed to sequences in close proximity on the target DNA [22, 23] (Fig. 2). At high local concentration, dimerization is favored and cleavage occurs.

ZFNs were able to cleave a chromatin substrate in intact cells and to stimulate homologous recombination [23]. This was demonstrated using synthetic substrates injected into *Xenopus* oocytes, and it was important because the bacterial *Fok*I

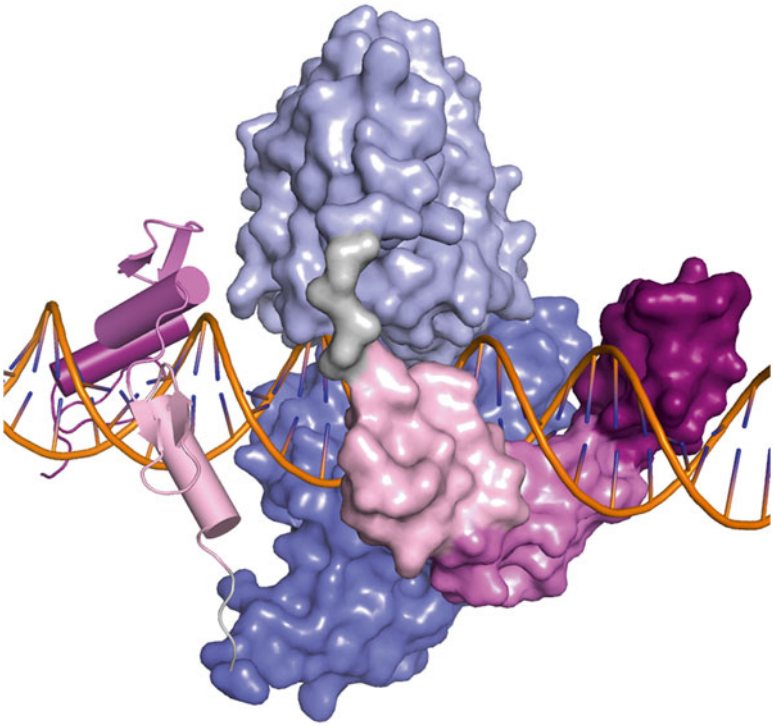

Fig. 2 Model of a pair of three-finger ZFNs bound to DNA. Each zinc finger is in a shade of *pink*, in *ribbon* representation on the *left* and space-filling representation on the *right*. The *Fok*I nuclease domains are in shades of *blue*, and the linker between these and the finger sets are shown in *gray*. The DNA axis runs horizontally through the figure, and the backbone is in *orange*. The zinc finger binding sites are 6 bp apart. This composite model was assembled using Protein Database submissions 1MEY and 2FOK [22]

nuclease would not normally see sequences in this context. How the ZFNs recognize sequences in chromatin is still not known, but there is no indication that this presents a limitation to their effectiveness. Using the oocyte system, the optimum spacer between zinc finger binding sites was shown to be 6 bp, when the linker between the binding and cleavage domains is reduced to four amino acids [23]. This linker is now in common use, and the preference for a 6-bp spacer has been validated in multiple studies [25, 26], although a spacer of 5 bp, and even 4 bp, works in some situations.

Designing ZFNs

As noted above, the modularity of zinc finger recognition does not translate into a simple code. Many groups have derived new fingers and finger combinations, both by design and by selection. Simply making new combinations of well-characterized

fingers sometimes works, but not reliably [21, 27–29]. A number of researchers have established selection schemes [30–33], have identified fingers that work well together [34, 35], and have endeavored to understand what governs successful recognition [36]. Many active and specific ZFN pairs have been derived (e.g., see [8, 36]), but still no simple method for producing new ones has emerged.

The Klug group demonstrated a number of years ago that constructing DNA-binding arrays with units of two fingers enhanced their specificity [9, 37]. Six-finger arrays constructed with extended linkers between finger pairs are more sensitive to mismatches to the DNA, perhaps because one mismatch destabilizes the entire two-finger unit [37]. The most extensive library of zinc fingers and finger pairs is maintained by Sangamo Biosciences, Inc., and ZFNs based on this collection are marketed by Sigma-Aldrich (http://www.sigmaaldrich.com/life-science/zinc-finger-nuclease-technology.html). The price of these reagents has decreased substantially in recent years, but they are still rather expensive. The advantages are that Sigma does all the design and testing and ultimately provides validated ZFNs.

The first ZFNs for a genomic target displayed significant toxicity when expressed at high levels [38], due to promiscuous binding and cleavage [39], and this issue has continued to trouble many new designs. Increasing the number of fingers in each monomer is one approach to ameliorating this effect, but that is not always sufficient [40, 41]. The toxicity is frequently a property of one ZFN within a pair, and it appears to be due to inadequate specificity, leading to homodimerization and cleavage at unintended, off-target sites [39]. A major step forward was the introduction of substitutions in the dimer interface of the cleavage domain that prevent formation of homodimers, while allowing the necessary heterodimerization [42, 43]. The second generation of these obligate heterodimer modifications [44] also corrects a deficit in cleavage efficiency seen with the first generation, and these are now in common use with ZFNs and TALENs [45].

In many situations with experimental organisms, cleavage and mutagenesis (by NHEJ) at off-target sites is a tolerable nuisance, since the effects can be minimized by backcrossing, by complementation, or by use of independently derived alleles (e.g. [46],). In applications to humans, however, the issue is more concerning. Methods to detect off-target mutagenesis have been developed, based on determination of in vitro binding and cleavage specificity [9, 47–49]. Ultimately, procedures are needed that detect where secondary mutations are actually made in cells and organisms. [50] Whole genome sequencing would have to be very deep, since rare mutations can be selected from a cell population when introduced into patients, and such mutations can have severe effects [51].

The Utility of ZFNs in Gene Targeting

The first experiments in an intact organism showed that ZFNs directed to a genomic sequence in *Drosophila melanogaster* could stimulate local mutagenesis by NHEJ [38] and sequence replacement by HR. [52] This was followed by experiments with human cells in culture, using synthetic [53] or natural [41] targets, and by studies in

several model organisms. By now, ZFNs have been used successfully in more than 25 different species, from yeast to butterflies to humans [7, 8].

Each new organism, cell type or end use requires careful consideration of how the nucleases and, in cases where HR is desired, the donor DNA, will be delivered. This concern applies to the other targeted nucleases as well, and the lessons learned from ZFN studies have contributed to the accelerated progress with TALENs and CRISPRs.

Because the genomic changes induced by ZFNs are permanent, only transient expression is required. In cultured mammalian cells, investigators have delivered ZFNs by plasmid transfection [41, 53], viral vectors [54–56], mRNA transfection, and even direct protein addition to the culture medium [57]. The latter approach seems to work because of the high intrinsic positive charge on the ZFNs, and it is applicable to a range of cell types, albeit with variable efficiencies. Long, double-stranded donor DNAs can be introduced on plasmid or viral vectors, while short, single-stranded donors are usually simply added to the medium [54, 55, 58, 59].

For situations in which manipulation of whole organisms is the goal, genome alterations in the germ line must be achieved. The cells in which the germ line is most accessible are typically in the very early embryo. Injection of ZFN mRNAs at this stage has proved successful in a wide range of organisms, including insects [60–63], fish [64–68], frogs [69], sea urchins [70, 71], mice [72–74], rats [75–77], and rabbits [78]. In the cases where HR products were reported [60, 71, 73–75], the donor DNA was simply included in the injection mix. In pigs and cows, genome modified animals were produced by in vitro mutagenesis of cultured somatic cells followed by nuclear transfer to enucleated eggs [79, 80].

Plants present particular challenges to delivery of genome engineering reagents. In some favorable cases, whole plants can be regenerated from cells or callus cultures, and the manipulations can be done in those contexts. This has worked, for example, in tobacco [81] and maize [82]. ZFN delivery in other plants has been accomplished with T-DNA transfer from Agrobacterium [83–86]. Viral vectors are also being developed [87], but no current approach is applicable to all plants.

The case of genome editing in plants nicely illustrates the fact that the biology of each system will dictate the best experimental approach and the range of outcomes. Experience with two popular experimental organisms emphasizes this potential limitation. The first applications of ZFNs to the nematode, *Caenorhabditis elegans*, achieved very good frequencies of somatic mutagenesis, but nothing in the germ line [88]. The nucleases were delivered in this study by forming extrachromosomal arrays of the transgenes, which were likely subjected to potent RNA interference in the germ line. When researchers instead used mRNA injection directly into the developing gonad, ZFN mutagenesis was observed, albeit at rather low frequency [89]. With the more efficient TALENs [89, 90], and particularly with CRISPRs [90–98], injection of DNA, mRNA and protein have all proven effective.

The other case is the zebrafish. It was among the early success stories for ZFN mutagenesis [66, 67, 99], but HR products were not readily obtained. With more efficient cleavage by TALENs, HR with both DNA oligonucleotides (oligos) and long, double-stranded donors was achieved [100, 101]. Interestingly, many of the

oligo HR products appear to be only half-homologous, half-end joined [100]. This presumably reflects the strong preference in zebrafish embryos for DSB repair by NHEJ.

ZFN Contributions

Research with ZFNs and homing endonucleases (also called meganucleases) [102] has provided critical information on how to optimize the results of targeted genome cleavage. As noted above, this includes guidance on nuclease delivery in a variety of organisms and cell types. The balance between repair by NHEJ and by HR has been addressed [60, 103], including the idea of introducing single-strand breaks (nicks), rather than DSBs, to favor HR. [104–107] Design of the donor DNA was investigated [108], and the efficacy of synthetic, single-stranded oligo donors was demonstrated [59, 109]. Homology requirements and the extent of target sequence incorporation at the target (conversion tracts) have been defined [108, 110]. A method for making insertions with only limited terminal homology was demonstrated [111]. Approaches to making a variety of more complex genomic alterations have been made, including precise deletions and inversions [59, 112, 113], gene correction by cDNA insertion [56, 114], and chromosomal translocations [115, 116]. In addition, methods for detecting and quantitating nuclease-induced mutagenesis in the absence of selection have been developed [42, 117, 118].

For many research applications, the ease of design makes TALENs and CRISPR/Cas nucleases very attractive. The CRISPRs have the added advantage that a single, constant protein is involved, and specificity is determined by guide RNAs that can be easily multiplexed—for genome-wide libraries in cell populations [119, 120], or to attack multiple genes in a single cell [121]. TALENs appear to have inherently high specificity that can be enhanced by obligate heterodimer modifications, as noted above [45, 122]. The specificity of CRISPR/Cas nucleases has been questioned [123–127], but some effective solutions have been developed. These include shortening the guide sequence to exacerbate the influence of mismatches [128], using a variant Cas9 that cuts only one strand in conjunction with a pair of guide RNAs that direct nicks to closely spaced sites [126, 129, 130], and fusing fully inactivated Cas9 to the *Fok*I cleavage domain along with two guide RNAs to promote dimerization [131, 132].

Before ceding the playing field entirely to TALENs and CRISPRs, we should note that when a single target is being attacked repeatedly, it doesn't matter what platform is being used. For applications to human therapy, to livestock and to crop plants, the development of the cleavage reagent represents a small part of the cost and effort devoted to the project. Considerations like specificity and ease of delivery then become paramount. In this regard, the smaller size of the ZFNs will offer an advantage in some circumstances. Finally, some existing ZFNs are among the most effective and specific of the nucleases currently in use [49, 114, 133–135]. ZFNs targeted to the human CCR5 gene [49, 136] have been in clinical trials for several

years [9, 137] and are proving safe and, to the extent allowed in a Phase I analysis, effective. Additional ZFN pairs have been targeted to other human disease genes [7, 8], and ones that have proved useful in specific applications like these are likely to continue to be exploited in the foreseeable future.

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