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Targeting DNA With Fingers and TALENs

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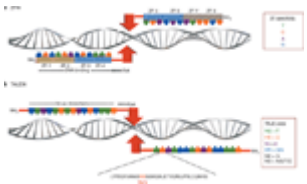
Researchers have long recognized that the random introduction of new DNA into the genome could result in unpredictable genetic effects due to bilateral influences between transgenic and endogenous sequences. Hence, precise editing or replacement of mutant genes has been a major goal of gene therapy since its inception. That goal looked possible with the publication of studies demonstrating that targeted cleavage of chromosomal sequences and enhanced homologous recombination (HR) could be achieved using chimeric molecules composed of a nuclease domain and separate, designer DNA-recognition domains.[1,2] Zinc-finger nucleases (ZFNs) are artificial endonucleases that consist of a *Fok*I cleavage domain tethered to engineered Cys_2His_2 zinc-finger, DNA-binding polypeptides.[3,4,5] ZFNs have gained considerable momentum and are widely considered the most mature nuclease technology in the gene therapy field.[6] However, after 15 years in the spotlight, a new chimeric nuclease has emerged, Transcription Activator-Like (TAL) Effector Nucleases (TALENs).[7,8,9,10] Here, we describe functional and design characteristics of ZFNs and TALENs and discuss their expanding role as tools for research and gene therapy.

Targeting with Zinc-Finger Nucleases

Each zinc finger (ZF) is about 30 amino acids, which form a ββα-fold stabilized by hydrophobic interactions and the chelation of a zinc ion, and generally binds to three base pairs.[11] Typically, arrays of 3–6 ZF modules are joined together to create a DNA-binding domain with specificity to 9–18 base pairs per ZFN monomer. Double-strand DNA cleavage requires dimerization of two *Fok*I nuclease domains. Thus, ZFNs are used in pairs with specificity to opposing DNA strands that assemble on both sides of the targeted cleavage site (panel a of **Figure 1**). This enhances the specificity of ZFN targeting not only by requiring two DNA-binding events, but also by requiring precise spacing (typically 5–6 base pairs with a 4-amino acid linker between the ZF and *Fok*I) and correct orientation between ZFNs for activity.[12,13]

Figure 1.



Comparison of zinc-finger nuclease (ZFN) and Transcription Activator-Like Effector Nuclease (TALEN) architecture. (**a**) ZFNs. Each ZFN polypeptide consists of two functional domains, a DNA-binding domain comprising a chain of finger modules (ZFs) that each typically recognize a unique 3-base pair sequence of DNA and a DNA-cleaving domain composed of the nuclease domain of the *Fok*I nuclease. *Fok*I functions as a dimer, hence when two *Fok*I nucleases bind to DNA proximal to one another they can dimerize and introduce a double-strand break. Targeted double-strand DNA cutting can be obtained by designing zinc fingers for specific sequences that flank the desired cleavage site; in the example 12 base pairs per ZFN are targeted with polypeptides containing four zinc-finger modules each (ZF-1 through ZF-4 and ZF-5 through ZF-8). (**b**) Model of a TALEN. A TAL Effector (TALE) polypeptide contains a series of typically 34-amino acid repeats, of which residues 12 and 13 [repeat variable diresidues (RVDs) shown in orange] are responsible for recognition of a specific base as shown in the box (note that there is some discussion about the precision of the RVD NK recognition of *G* and other RVDs can specify base contacts[61]). *Fok*I nuclease is fused to the C-terminal end of the protein using wild-type TALE sequence as a spacer. Several spacer lengths between the TALE binding core and *Fok*I have demonstrated activity. The number of tandem 34-amino acid repeats in the binding core defines the length of the recognition sequence, and the end of the functional DNA-binding motif. Each target sequence must be preceded by a *T* nucleotide. Two TALENs are shown to assemble on a genomic sequence in the opposite polarity to ZFNs to form a heterodimeric cleavage complex.

Full figure and legend (53K)

The resulting double-strand breaks in a DNA sequence can be repaired by either of two mechanisms, nonhomologous end joining (NHEJ) or HR.[14,15] NHEJ often results in small deletions or insertions (indels) to cause missense and/or nonsense mutations that truncate or mutate the encoded protein. Consequently, NHEJ-mediated mutagenesis is used for targeted disruptions of genetic loci (*e.g.*, gene knockout). Alternatively, HR allows for either precise modification of a target sequence or precise introduction of a specific sequence (*e.g.*, a wild-type sequence that leads to gene repair) into the targeted site. In mammals, a double-strand DNA break can stimulate HR of an exogenous DNA sequence within about 100 base pairs of the double-stranded DNA break.[16] Consequently, both targeting DNA cleavage close to a deleterious mutation and supplying either a double-stranded or single-stranded template DNA sequence can repair a damaged gene. Thus, genetic engineering has transitioned from nearly random addition of genes and expression cassettes to defined editing of a genetic material.[17,18]

ZFNs provided, for the first time, an efficient and relatively simple platform for inducing site-specific mutations or modifications of genomes, particularly for organisms for which this technology was lacking. About 20 abstracts on ZFNs were presented at the 2011 Annual Meeting of the American Society for Gene and Cell Therapy,[19] which indicates the considerable interest in using these targeting agents directly for gene therapy and indirectly for modifying human embryonic stem cells and induced pluripotent stem cells for cell therapy.[20,21,22,23] In terms of vertebrate models for gene and cell therapy, ZFNs have been used successfully for germ line knockout of genes by induction of DNA double-strand breaks and NHEJ in zebrafish,[24,25] mice,[26] rats,[27,28] rabbits,[29] and pigs[30,31] and *Caenorhabditis elegans*.[32] These achievements are particularly important, because they suggest the possibility of achieving or improving the frequencies of gene targeting and HR that are stimulated by chromosome breaks in animal models where gene targeting is difficult.

Clearly ZFNs are a very powerful resource for gene editing; however, there are some complicating issues with the design and application of ZFNs. First, some ZFNs have been associated with cytotoxicity, presumably due to cleavage at nontargeted sites.[33,34,35] Because

cleavage at off-target sites can occur when ZFN monomers form homodimers, off-target activity has been ameliorated with structure-based design of the *Fok*I dimerization interface that blocks homodimerization.[36,37,38] Second, it was quickly appreciated that it was difficult in some cases to engineer ZFNs to have the desired binding specificities.[39] ZFNs assembled using ZFs that recognize known triplets do not always have the desired sequence specificity when assembled into arrays.[40] This problem necessitated assembly of multiple ZFs that target the same base pairs followed by testing and selection of combinations for greatest ZFN specificity and efficiency. One solution is Oligomerized Pool Engineering (OPEN) in which reagents currently available can be used to create ZFNs that recognize sites about every 200 base pairs of *random* genomic sequence.[41,42] Hence, there should be multiple target sites in an average gene. An alternative approach for efficient production of ZFNs is Context-Dependent Assembly (CoDA),[43] which uses an archive of validated two-finger units derived from selection and are known to work well when positioned adjacent to each other. With available CoDA two-finger units, ZFNs can be constructed that recognize approximately one site in every 500 base pairs of *random* genomic sequence. Others have identified successful combinations of naturally derived and engineered ZF modules which is used to guide modular assembly predicted to have a targeting range of 1 in 125 base pairs of *random* genomic sequence,[44] although subsequent studies have indicated unexpected failure rates based on simple modular assembly of ZFNs.[39]

The fundamental issue boils down to targeting range; that is, the precision at which efficient cleavages can be introduced in a DNA target. For inactivation of a gene *via* the NHEJ pathway, the site of ZFN cleavage is not as important as specification of the cleavage site for HR-directed gene correction. Hence, for applications to humans, OPEN is more versatile and looks preferable to CoDA. However, OPEN is far more arduous and time-consuming. In addition, mammalian gene conversion frequency is reduced as a function of increasing distance from a DNA double-strand break (>80% reduction 100 base pairs from the double-strand break).[16] Thus, some loci of interest to gene therapists may not be modified efficiently using open source ZFN technology.

Genome Modification with Tal Effector Nucleases

Fifteen years after the introduction of ZFNs, an alternative approach for introducing chromosomal breaks at selected sites was developed. TALENs are novel fusion proteins that, like ZFNs, consist of assembled DNA-binding motifs coupled to *Fok*I nuclease.[7,8,9,10,45,46,47] The DNA-binding motifs come from proteins secreted by plant pathogens in the bacterial genus *Xanthomonas*. The proteins activate genes within infected plant cells to improve the environment for the invading pathogen.[8] The proteins, TAL effectors, have nuclear localization signals and an acidic transcription-activation domain. The DNA-binding motifs of TAL effectors consist of a tandem repeat of typically 34 amino acids. Each repeat appears to bind to a single base pair based on a simple cipher[7,9] shown in panel b of the **Figure 1**. The cipher can be used to predict the specificity of a TAL effector polypeptide. Residues 12 and 13 of the 34-amino acid repeats, referred to as repeat variable diresidues (RVDs), define binding to a specific base.[7,9] This code was deciphered by Boch *et al.*[7] where it was demonstrated that artificial TAL effectors targeted to novel sequences could activate transcription, thereby opening the door to a variety of TAL effector-based genome engineering applications. Since then, sequence-specific DNA-binding proteins with predicted binding specificities have been generated economically in a matter of days, using molecular biology methods practiced by most laboratories.[48,49,50,51,52,53,54,55] The activities of custom-designed TALENs in human cells have efficiencies of NHEJ-induced mutagenesis ranging up to 45% of transfected cells.[47,56] In

addition, TALENs have been used to create NHEJ modifications in *C. elegans*,[32] zebrafish,[51,52] and rats.[57] As with ZFNs, TALEN-mediated double-strand breaks also stimulated HR in human cells at levels that are similar to the levels achieved with ZFNs.[22,46]

TALEN-binding sites are expected to occur about once every 35 base pairs based on criteria identified by examination of naturally occurring TALEs.[48] If this turns out to be the case experimentally, it will provide greater flexibility in the selection of target sites than open source ZFN platforms and thereby make TALENs very attractive for research in gene and cell therapy. Furthermore, the apparent lack of context dependence and 1:1 correspondence of repeat variable diresidues with defined single base pairs, in contrast to ZF modules for which there are a multiplicity of ZFs for a given triplet of base pairs, with complex contextual interaction, makes modular assembly of TAL effector proteins more straightforward. Remarkably, the success rate for generating active TALEN pairs using simple design parameters (*i.e.*, spacer length and adhering to characteristics of natural TALEs) has been as high as with ZFNs using open source technology.[22,51,56,57,58] Several groups have developed reagents and protocols for simple, rapid modular assembly that make TALENs broadly available to all investigators.[48,49,50,51,53,54,55,58]

Future Directions

Extraordinary progress in gene-editing targeting technologies and the recent emergence of TALENs as an alternative, open source gene-targeting platform, supports the prediction that gene-editing will continue to gain momentum for generation of models and therapeutics. The gene therapy community has now 10 years of accumulated experience using ZFNs for precise modification of human genomes and those of model organisms.[13] At this time, three promising ZFN-based therapies have entered clinical trials for treatment of diabetic neuropathy, AIDS, and glioblastoma. Despite considerable excitement revolving around TALENs, it is too early to predict that they will replace or rival ZFNs for gene therapy. Additional studies are required to: (i) develop efficient means of delivery (TALENs are typically 1,200+ amino acids in length), (ii) define immunogenicity of TALENs, and perhaps most important, (iii) characterize the specificity of TALENs. Each TALEN monomer typically specifies 15+ base pairs for a combined 30+-base pair-target sequence, which is predicted to be unique in the human genome. However, unbiased studies of ZFN specificities have revealed shortcomings of off-target predictions using computer algorithms that search for nearest matches.[59,60] As with ZFNs, early studies reveal that TALENs can bind degenerate sequences and have demonstrated activity at related off-target sites.[56,57] Hence, unbiased experimental assessment of off-target sites will be necessary for all ZFNs and TALENs intended for gene therapy. Whether or not TALENs withstand the vigorous standards of clinical use remains to be seen. However, considering their ease in manufacture and reliability in function, we expect increasing adoption of TALENs by the research community. This bodes well for the development of new genetic models and effective therapies for our most prevalent congenital diseases.

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