REVIEW

Progress and prospects: Zinc-finger nucleases as gene therapy agents

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Zinc-finger nucleases (ZFNs) are powerful tools for experimental gene manipulation. A number of recent papers have shown how this technology can be applied effectively to models of human gene therapy. Significant target genes and useful methods of ZFN delivery have been reported. Important strides have been made in minimizing toxic side effects observed with some ZFNs, which bodes well for their ultimate safety. New tools are available for the design and testing of ZFNs for new target genes. Applications of ZFNs to stem cells have been described, and genuine gene therapy trials appear to be on the immediate horizon.

In brief

Progress

- Zinc-finger nucleases are very powerful gene modification tools.
- New ZFNs have been derived for new genomic targets.
- Additional methods of delivering ZFNs to human cells have been explored.
- Several types of editing have been achieved, including large insertions.
- Modifications of the cleavage domain reduce toxicity.
- New methods allow screening of new ZFNs before use in human cells.

Prospects

- Gene therapy procedures based on ZFN-induced modifications of stem cells seem imminent.
- It will be important to improve methods to detect and minimize off-target effects that could be deleterious.
- Methods for making and testing new ZFNs will continue to improve, making the technology more widely accessible.

Zinc-finger nucleases are powerful gene modification tools

The ultimate maneuver in gene therapy is to replace a defective gene with a normal allele at its natural chromosomal location. In principle, this has several advantages over providing a complete therapeutic gene, for example, with a viral vector. Because it is usually the case that only a small portion of the gene must be altered, correcting a gene at its normal location obviates the need to include full coding sequences and all required regulatory sequences in the therapeutic DNA. Expression of the corrected gene is very unlikely to be extinguished over time, as is sometimes observed with virally delivered genes. A corrective gene fragment is unlikely to cause mutations by inserting into random sites in the genome, as has been experienced with viral

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gene delivery, although other unintended adverse consequences can arise (see below).

Until recently, the prospects for editing genes at their natural location seemed distant due to the very low efficiency of the required homologous recombination (HR) events. Zinc-finger nucleases (ZFNs) enhance this efficiency by making a break in the target site (Figure 1). The ability to modify the DNA-binding specificity of the ZFNs makes them applicable to targeting essentially any desired gene. The requirement for two ZFNs directed to neighboring sequences enhances their specificity, since both must recognize their portion of the target.

A ZFN-induced break can be used for gene editing in two different modes (Figure 1)[1]: HR or nonhomologous end joining (NHEJ). NHEJ is often inaccurate, creating localized insertions and deletions, which may be sufficient for cases in which inactivating a gene is the goal. To achieve the type of sequence replacement that is usually considered gene targeting, a donor DNA that is largely homologous to the target, but carries the specific desired sequence change, is delivered along with the ZFNs. When HR uses this donor as a template, the change is incorporated at the target.
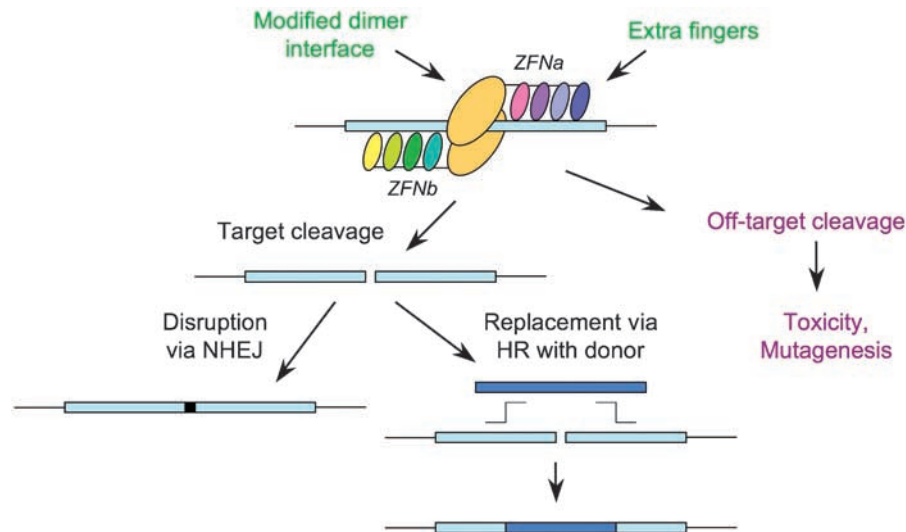
Figure 1 Zinc-finger nuclease (ZFN) binding and activity. The basic arrangement of ZFNs relative to their target DNA (light blue bar) is shown in color. Each ZFN (ZFNa, ZFNb) consists of a set of four zinc fingers, shown as smaller ovals, each in a different color to indicate that each binds a different DNA triplet. Each ZF set is linked to a cleavage domain (larger ovals), which must dimerize to cut DNA. Cleavage of the intended target gene can lead to disruption of its coding sequence by inaccurate repair through nonhomologous end joining (NHEJ). When a homologous donor DNA (dark blue bar) is introduced along with the ZFNs, it can be incorporated at the target by homologous recombination (HR). Cleavage at unintended, off-target sites can be toxic to cells and lead to off-target mutations (purple). Some of the approaches to limiting such deleterious effects are described in the text and include modification of the cleavage domain dimer interface and introducing additional fingers to enhance specificity (green).

New ZFNs have been derived for new genomic targets

Although the potential of ZFNs for targeting a wide variety of sequences has been emphasized, until 2 years ago, very few genuine chromosomal loci had been successfully attacked, including only one human gene. Now the list is longer. Porteus[2] reported 3-finger ZFNs for four new 9-bp sequences from human genes (one site in each β-globin and the interleukin-2 receptor common gamma chain (IL-2Rγ, mutated in X-linked severe combined immunodeficiency, X-SCID), and two sites in the CD8A cell-surface antigen gene). Although these were tested with synthetic target constructs, not at the endogenous loci themselves, the results add to the success achieved with ZFNs. The group at Sangamo Biosciences, which had previously reported high frequencies of targeting at the *IL-2Rγ* locus, produced new ZFN pairs for the hamster dihydrofolate reductase (*DHFR*) gene[3] and the human chemokine receptor *CCR5* gene.[4,5] The latter serves as a co-receptor for entry of HIV-1 into T cells; it has been shown to be dispensable in humans and thus is an attractive target for gene disruption. Each ZFN contained four, or in one case five, zinc fingers, which can potentially increase the affinity and specificity for the target compared to 3-finger modules. A large-scale collaboration of laboratories in the Zinc Finger Consortium (http://www.zincfingers.org) derived 3-finger ZFN pairs for three human genes (*VEGF-A*, *HoxB13* and *CFTR*) and one plant gene.[6] Two papers reported the first applications of ZFNs to gene disruption in zebrafish. One used a pair of 3-finger proteins to target a vascular endothelial growth factor receptor (*kdr* gene),[7] and the other made pairs of 4-finger proteins that successfully cleaved the *no tail* and *golden* genes.[8]

Table 1 Genomic sequences targeted with ZFNs

Organism/cells	Gene name	Reference
Human T cells	CCR5	Perez et al.[5]
CHO cells	DHFR	Santiago et al.[3]
Various human cells	IL-2Rγ	Lombardo et al.[4]
		Moehle et al.[9]
HEK293 cells	VEGF-A	Maeder et al.[6]
HEK293 cells	HoxB13	Maeder et al.[6]
HEK293 cells	CFTR	Maeder et al.[6]
Tobacco	SuRA	Maeder et al.[6]
Zebrafish	kdr	Meng et al.[7]
Zebrafish	golden	Doyon et al.[8]
Zebrafish	ntl	Doyon et al.[8]
Drosophila	ry	Beumer et al.[10]
Drosophila	y	Beumer et al.[10]
Drosophila	bw	Beumer et al.[10]
Caenorhabditis elegans	Nw	Morton et al.[11]

In some cases, the most recent reference is given, rather than the original one.

Published reports have described successful ZFN-induced targeting of 14 genes in various different organisms at their endogenous chromosomal locations: 6 in mammalian cells, 3 in zebrafish, 3 in *Drosophila*, 1 each in nematodes and in plant cells (Table 1). The mammalian and plant cell experiments were performed with cultured cells, the zebrafish experiments were done by injection of very early embryos, whereas the *Drosophila* and nematode experiments were conducted in whole organisms. Undoubtedly more are on their way to publication. This demonstrates the generality of the ZFN approach and encourages future applications to new genes and organisms.

In a variation of standard ZFN construction, Minczuk et al.[12] targeted a specific mutant sequence in human mitochondrial DNA with a single ZFN that carries two cleavage domains linked in the same protein. The success in delivering ZFNs to this organelle and in lowering the ratio of mutant to wild-type genomes points the way to future applications for mitochondrial manipulation. The strategy of linking two cleavage domains is not likely to be widely adopted, however, as the protein may be constitutively active as a nuclease. One of the beneficial features of the usual two-protein ZFN scheme (Figure 1) is that the cleavage reagent is assembled at the target and is not active otherwise.

Additional methods of delivering ZFNs to human cells have been explored

In the studies cited above and in others, many different types of cells were used, and the efficiency of gene targeting varied widely. Some of this variation may be due to inherent target accessibility, but much is undoubtedly dependent on how the ZFNs and donor DNA are delivered to cells. Some cultured cells take up exogenous DNA quite efficiently, and ZFN-induced events have been reported at frequencies ranging from a few tenths of a percent up to 20% of all targets. Viral vectors may be more effective with other cell types, and they can potentially be designed to favor particular populations. Lombardo et al.[4] used an integration-defective lentiviral vector to deliver ZFNs directed at the human IL-2Rγ gene. They also produced a combination vector that carried one ZFN and one copy of the donor DNA, which reduced the number of separate viruses that had to infect the same cell. They achieved targeting frequencies up to 39% in K562 cells, but lower efficiencies in other cell types. Encouragingly, they saw some targeting of the CCR5 locus in primary human stem cells (0.1%) and in two human embryonic stem cell lines (13%).

Perez et al.[5] employed an adenoviral vector to deliver ZFNs for the CCR5 gene and achieved remarkably high frequencies of gene disruption: up to 80% of all targets in a cell line with multiple CCR5 expression cassettes and approximately 50% in primary CD4+ T cells. In this case, both ZFNs were delivered in a single, bicistronic vector, with their expression driven by a CMV promoter. In a number of cases, both alleles of the target gene have been modified simultaneously in individual cells.[3,5,9] Although this is not necessary in the case of correction of an inactive gene, it is quite important when the goal is gene inactivation, as in the case of CCR5. Other viral vectors can certainly be used and may be chosen on the basis of the cell or tissue being addressed.

Several types of editing have been achieved, including large insertions

As noted earlier, ZFN-targeted modifications can be introduced by NHEJ or by HR with a designed donor DNA. Inactivation of the DHFR[3] and CCR5[5] genes relied simply on NHEJ after ZFN cleavage. This was also the case for the genes targeted by Maeder et al.[6] The new mutations were mostly small insertions and deletions, with some larger deletions. They were detected in both cases by use of the CelI nuclease, which only cleaves when mismatches are present in heteroduplexes formed between the wild-type and mutant PCR products.

For the correction of mutant disease genes, efficient use of a donor DNA as a template for repair will be required. The Sangamo group addressed the issue of how large an insertion in the donor can be incorporated, without sacrificing efficiency.[9] They found that modest insertions of about 1 kb were copied into the IL-2Rγ target at an efficiency of ∼5% when flanked by 750 bp of homology on either side. Importantly, a 7.8-kb insert was incorporated at the same rate, and the three genes it carried were all expressed. One issue for future investigation is how these frequencies might be affected by longer and shorter flanking homologies.

Lombardo et al.[4] demonstrated a useful application of such insertions for practical gene therapy. A significant concern is how close the ZFN cleavage site must be to the sequence that requires modification. During double-strand break repair in mammalian cells, donor sequences are incorporated only up to about 100 bp on either side of the break. This means that new ZFN pairs would have to be derived for each different mutation in a particular gene. Lombardo et al. placed a complete downstream portion of the IL-2Rγ cDNA between the 750-bp homology arms and showed that it was incorporated at the same frequency as other insertions (∼5%). This means that any mutation downstream of the ZFN site can be 'corrected' in this manner (Figure 2). Although the resulting gene structure is not normal, correction has occurred at the normal locus, and regulatory features should be intact.

Modifications of the cleavage domain reduce toxicity

The zinc-finger sets used in ZFNs are rarely completely specific, and some induce significant toxicity. In at least two cases, the toxicity has been demonstrated to be due to excessive cleavage, presumably at unintended sites.[10,13] It is also possible that simple binding of single ZFNs to genomic sites without cleavage could disrupt normal gene expression or other cellular processes. Off-target cleavage is not surprising, as the ZFNs are being asked to discriminate in favor of a single target within an entire genome of potential sites. Several groups use assays for cytotoxicity based on the survival of ZFN-modified cells.[2,4,14,15] The extent of off-target cleavage can be estimated by staining cells for repair foci that are induced by DNA damage using antibodies against proteins recruited to such foci, like γH2AX and 53BP1.[5,13–16] In some cases, tens of foci are seen, where a maximum of two is expected if only the designed target is cut on both alleles.

Although improving specificity by redesigning the ZFs is often possible, three groups modified the cleavage domain instead.[13,15,16] Toxicity is often a property of a single ZFN that forms homodimers at off-target sites. Therefore, these groups made complementary substitutions in the dimer interface that prevented homodimer formation, while still permitting the heterodimers that cleave the desired target. The modifications introduced by each group were somewhat different, but all
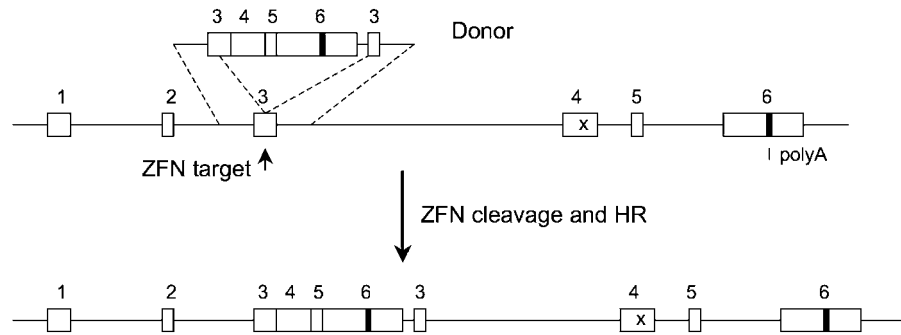
Figure 2 Scheme for correcting gene sequences downstream of the ZFN cleavage site. A hypothetical target gene is illustrated with six exons (boxes 1–6). The sixth exon has a polyA addition sequence that defines the end of the mRNA from this gene. The specific target for ZFN cleavage is envisioned in exon 3. A donor DNA carries sequences of exon 3 and all downstream exons, but without downstream introns. The ends of the donor have homology to the left and right sides of the ZFN target, as illustrated with dashed lines. After cleavage by the ZFNs and HR using those homologies, the target will have the structure illustrated at the bottom. As long as the sequences in the donor are all normal, any mutations in exons 3–6 will be corrected in the eventual mRNA. For example, a mutation in exon 4 of the target (shown as an x) would not be present in the mRNA after incorporation of the donor as shown. Generalized from Lombardo et al.[4]

succeeded in eliminating detectable consequences of off-target cleavage. In some cases, this reduced the efficacy of target cleavage, but often not severely. The modified cleavage domains were employed very effectively in several recent examples of ZFN targeting.[3–5,8] The reduction achieved in off-target effects is particularly important for applications to human gene therapy, where unintended consequences are not tolerated.

New methods allow screening of new ZFNs before use in human cells

When a new target gene is selected, new ZF sets must be designed for sequences in that locus. Each finger binds primarily to three base pairs of DNA, and there are 64 possible 3-bp sequences. Libraries are available of ZFs that have been selected to recognize many of these triplets, and they can be assembled conveniently into new ZFNs on the assumption that each finger acts as an independent module.[17–19] Taking this approach ignores possible effects of the protein and DNA contexts on recognition that could be important for success. Maeder et al.[6] address the issue of context by selecting 3-finger ZF sets for particular 9-bp DNA sequences from pools of partially randomized ZF coding sequences Their bacterial two-hybrid assay is based on activation of transcription in bacteria, dependent on the binding of the ZFs to a target oligonucleotide placed upstream of the reporter gene. A similar bacterial reporter system based on a single hybrid protein was used in the production of ZF sets to target the zebrafish kdr gene.[7]

The bacterial assays select for high-affinity ZF sets in a cellular setting. The context of recognition is substantially different, however, from that found in eukaryotic cell chromatin, which will certainly influence ZF binding. It is also possible that the requirements for optimal cleavage by a pair of ZFNs do not simply reflect binding capabilities of each ZF set, but depend on subtle features that are specific to the pair. Doyon et al.[8] addressed these points with an assay in (eukaryotic) yeast cells that depends on ZFN cleavage per se and the reconstruction of a gene that produces a readily scored phenotype. The synthetic target in the reporter gene can be arranged to carry sites for either a single ZFN or a heterologous pair. There is still some concern that yeast chromatin is somewhat different from that of higher eukaryotes and that the natural chromatin structure of an endogenous gene will not be accurately reflected in a synthetic substrate. The ZFs and ZFNs that perform well in the bacterial and yeast assays have succeeded on their genomic substrates in several cases, so these approaches produce strong candidates for further use.

Prospects

The most likely initial applications of ZFN-mediated gene targeting will be in the modification of stem cells ex vivo. Perez et al.[5] clearly had this in mind when they mutated the CCR5 gene in CD4+ T cells and produced an HIV-resistant population. This pair of ZFNs can be used to treat bone marrow cells from patients who are HIV positive. Mutation of even a subset of such cells would allow reconstitution of the T-cell arm of the immune system with resistant cells upon retransplantation. A Phase I trial of this type is currently under review. To be effective, both CCR5 alleles would have to be inactivated in the same cell. As noted earlier, this has been achieved in model experiments, and it seems likely that the frequency can be enhanced, perhaps by repeated ZFN application. A similar scenario can be envisioned for treatment of X-SCID by ex vivo gene correction with ZFNs and a donor DNA that carries the normal IL-2Rγ gene sequence.[4]

The latter scheme is similar in concept to that used in earlier gene therapy trials in which the therapeutic IL-2Rγ gene was introduced on a retroviral vector. Those trials produced clinical benefit in most patients, but induced leukemia in some, due to insertional mutagenesis by the viral genome. As noted above, the off-target effects produced by ZFNs are of a different type, but could still be mutagenic at critical loci. As with viral delivery, it is very difficult to anticipate exactly what problems may arise, as very low frequency events can be amplified to deleterious levels by selection based on a growth advantage.

For this reason, an area of intense interest will involve continuing efforts to improve the specificity and safety of ZFNs by learning to detect off-target events and to prevent them. Staining for repair foci gives some indication of the extent of excess cleavage, but very rare events could still cause problems. Some groups have examined candidate secondary cleavage sites that were identified by sequence-searching or *in vitro* binding and found very low levels of ZFN-induced mutation.[5,7,8] These may not be the genomic sites most at risk in living cells, however, due to chromatin structure or other features of the nuclear environment. A more general method to identify secondary targets would be to capture broken ends from cells expressing the ZFNs and to sequence the flanking DNA. This might be possible through the use of ligation-mediated PCR and high-throughput sequencing, for example, using the 454 or Solexa sequencing platforms.

The engineering of the cleavage domain to prevent homodimerization, described above, has been very effective in reducing off-target cleavage. In addition, it should be possible to limit the timing and duration of ZFN expression to reduce the opportunities for untoward effects. Because ZFs do not discriminate perfectly, greater specificity may be achieved with pairs of 4-finger ZFNs that recognize 24-bp targets, compared with 3-finger proteins directed at 18-bp sequences. There is some danger, however, that the additional fingers will lead to cleavage at more off-target sequences, as a subset of fingers may be sufficient to direct binding. One study[15] has shown greater cytotoxicity with a particular pair of 4-finger ZFNs; however, the 3- and 4-finger proteins were not directed to the same genomic target, and neither pair carried the modified cleavage domains. Clearly additional work in this area is needed.

A related issue is that both mutagenesis through NHEJ and gene replacement by HR are stimulated by the same break in the target. In some cases where HR is desired, a background of new mutations is tolerable. For example, when correcting a mutant *IL-2Rγ* gene, inducing new mutations at this target will not matter, as long as sufficient numbers receive the correction. In other situations target mutagenesis may be more problematic. It may be possible to minimize this by transiently disabling components of the NHEJ pathway, or by artificially stimulating HR.

In the more distant future, ZFN-based modifications could be combined with other stem cell therapies, particularly when *in vitro* culture and expansion are possible. The frequency of targeted modification that must be achieved will differ in different situations, but the recent reports of both NHEJ and HR events in the range of 10% (and greater) of treated cells should be sufficient when expansion and selection will occur after retransplantation, or in cases in which this degree of correction is adequate, as it will be for some secreted proteins. Delivery of ZFNs and corrective donor DNAs to specific tissues *in vivo* presents more of a challenge, particularly when multiple organs may be affected by a disease. The same sorts of considerations regarding delivery will apply as in other types of gene therapy, and progress in the development of viral vectors can be adopted for ZFNs. The construction of dual expression vectors for pairs of ZFNs[5] and of adenoviral and integration-defective lentiviral vectors[4] have already proved effective *in vitro*.

A possible limitation of the ZFN approach is the dependence on specific cellular pathways of DNA repair. If the HR pathway is relatively inactive in a particular target tissue, gene correction will be correspondingly more difficult. Because the ZFNs are typically expressed only transiently and would not be present after *ex vivo* treatment, it is unlikely that they would cause an immune reaction. Just as in gene addition strategies, however, the correction of a non-functional disease gene will lead to the synthesis of a normal protein, but one that has not been present during the previous life of the patient and thus may induce an immune response.

The gene therapist who wants to target a new gene will find continuing assistance in the design, production and testing of new ZFN pairs. The recent efforts by the Zinc Finger Consortium are an example of how things are likely to proceed.[6] In addition, Sangamo has entered an agreement with Sigma-Aldrich for the production and sale of ZF proteins, including ZFNs. This should make the technology more widely available. While there is no guarantee of success with new ZF designs, and failures are rarely reported in the literature,[20] both modular assembly and selection have produced functional ZFNs. Compared with other approaches to enhance gene targeting, ZFNs show greater ease and flexibility in design for new targets than homing endonucleases.[21] Unlike site-specific insertion mediated by the ΦC31 integrase,[22] ZFN-mediated events modify the target gene in its natural location.

The ZFN field is still in its early stages. As more experience is obtained in more laboratories, we will gain better understanding of the parameters that are most critical and the procedures that are most effective. We are fortunate that many of the investigators who have taken an interest in the technology are focused on applications to human disease, as this will undoubtedly speed the development of useful therapies. In addition, lessons learned from model organisms will contribute to our understanding of ZFN capabilities and limitations. This is an exciting time for the field, as we discover what can be achieved toward the amelioration of human genetic diseases and at what cost.

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