

Gene targeting using zinc finger nucleases

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The ability to achieve site-specific manipulation of the mammalian genome has widespread implications for basic and applied research. Gene targeting is a process in which a DNA molecule introduced into a cell replaces the corresponding chromosomal segment by homologous recombination, and thus presents a precise way to manipulate the genome. In the past, the application of gene targeting to mammalian cells has been limited by its low efficiency. Zinc finger nucleases (ZFNs) show promise in improving the efficiency of gene targeting by introducing DNA double-strand breaks in target genes, which then stimulate the cell's endogenous homologous recombination machinery. Recent results have shown that ZFNs can be used to create targeting frequencies of up to 20% in a human disease-causing gene. Future work will be needed to translate these *in vitro* findings to *in vivo* applications and to determine whether zinc finger nucleases create undesired genomic instability.

Knowledge of the complex interplay between the genome, the physiologic processes it governs and the environment with which it interacts has increased. Gene targeting has provided an important research tool for probing this complex interplay and for manipulating the genome. In gene targeting, an exogenously introduced DNA fragment replaces an endogenous segment of DNA by homologous recombination (Box 1). This process was reported in yeast more than 25 years ago and variations on this technique are now commonly used for evaluating gene function in that organism^{1–3}.

Gene targeting also has been demonstrated in mouse cells, and when it is applied to embryonic stem (ES) cells, it has enabled the production of mutant mice, both for studying gene function and for creating models of human genetic diseases^{4,5}. Although positive selection for the integrated gene is sufficient to recover the desired yeast cells, elegant and powerful selections for the transgene and against nontargeted integration had to be devised for gene targeting in mouse cells where most of the transgenes integrate at inappropriate sites in the genome; the advances have made this technique nearly routine^{6,7}. Thousands of transgenic mice and ES cell lines with precise genomic alterations have been created; their characterization has increased our understanding of mammalian physiology and the pathogenesis of numerous human diseases. Nonetheless, many applications of gene targeting are hindered by its inherently low frequency and the need for selection in culture before incorporation into whole animals.

In addition to its experimental applications, gene targeting could be useful in gene therapy. Human monogenic diseases, such as sickle

cell disease, hemophilia, cystic fibrosis and Huntington disease, are potentially ideal targets for genome-based therapies. Finding a cure for such diseases would eliminate billions of dollars in healthcare costs, not to mention immeasurable family and societal costs. Current approaches to gene therapy rely largely on methods that add back a normal copy of the defective gene, typically using a viral vector as carrier. Despite limitations of this approach—immunological reaction to the virus, long-term silencing of the therapeutic gene and insertional mutagenesis—and some well-publicized setbacks⁸, several promising advances have been reported^{9,10}.

An alternative to gene addition would be gene correction through gene targeting, which allows correction of the mutation *in situ* leaving the rest of the genome unperturbed. This strategy has several advantages over gene addition procedures, including the following: the risk of mutations arising from random insertion is reduced because the approach aims to incorporate exogenous DNA at a predetermined site in the chromosome; the exogenous DNA does not have to include a complete protein coding sequence or separate signals to ensure its expression because the donor is simply correcting a mutation in an endogenous locus; and inappropriate tissue specificity, timing, level and duration of expression are not issues because the targeted gene remains under normal, endogenous controls. Thus, if targeted correction could be accomplished with high efficiency and without significant side effects, normal function should be restored.

What limits the frequency of gene targeting? Experiments in model systems have demonstrated clearly that manipulations of the donor DNA have very modest effects, but activation of the chromosomal target can improve the frequency by several orders of magnitude. Both in yeast and in mammalian cells, making a double-strand break (DSB) in the target effectively increases its interaction with an exogenous donor DNA. This is easily understood: an intact segment of chromosome does not benefit by interacting with another DNA; but

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Box 1 Homologous recombination

The maintenance of genomic integrity requires cells to repair DNA damage with high fidelity^{48,49}. One of the most dangerous DNA lesions that cells encounter are DNA double-strand breaks (DSBs) because every DSB can potentially lead to cell death or to oncogenic mutations. Fortunately, cells have redundant mechanisms to repair DSBs, among them homologous recombination. Homologous recombination, which has been reviewed elsewhere^{50,51} is basically a 'copy and paste' mechanism. This process uses an undamaged homologous segment of DNA, usually the sister-chromatid, as a template from which to copy the information across the break (Fig. 1a). Because it recovers a normal copy of the damaged DNA, homologous recombination is the most accurate form of DSB repair.

An alternative pathway of DSB repair is nonhomologous end joining, which joins ends without regard for homology and often results in small, localized deletions and/or insertions. A broken end

may also become joined to a completely unrelated site resulting in a chromosomal translocation (Fig. 1b)

In addition to repairing accidental DSBs, cells also use homologous recombination to create regulated genomic rearrangements. During meiosis, mating type switching in yeast, and the generation of immunoglobulin and T-cell receptor diversity in certain species, the rearrangements are created by homologous recombination⁵². In controlled rearrangements, a specific nuclease creates an intentional DSB and the DSB is repaired using a DNA template other than the sister chromatid. In zinc finger nuclease-mediated gene targeting, the goal is to mimic these natural rearrangements by creating a gene-specific DSB to activate the cell's endogenous homologous recombination machinery while simultaneously providing a DNA repair donor to introduce the desired genetic changes.

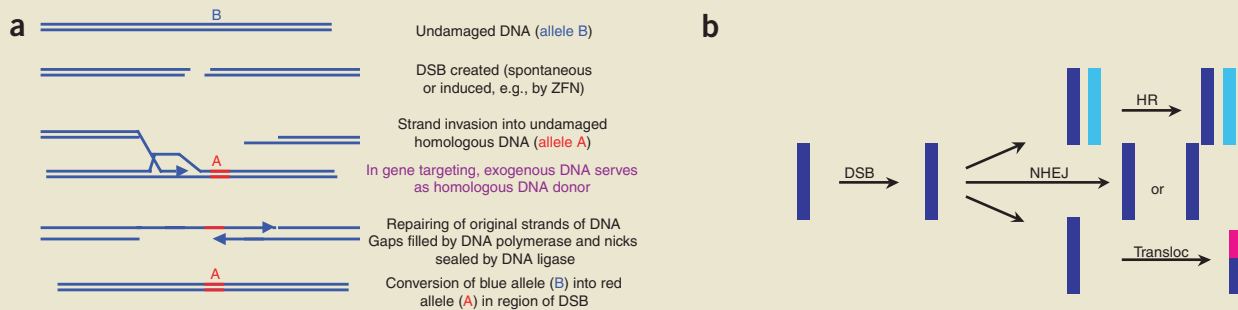


Figure 1 Double-strand break repair. **(a)** Synthesis-dependent DSB repair. This model, one of several proposed mechanisms, illustrates the essential features of DSB repair. A DSB is created in gene 'B,' which is then processed to form free 3' single-strand tails. The homologous recombination machinery uses the free 3' ends to invade a homologous donor. In the normal repair of a DSB, the donor is usually the sister chromatid, which is identical to the damaged allele. In gene targeting, the donor is an extrachromosomal fragment of DNA. After strand invasion, primed DNA synthesis occurs to generate an intact strand using the invading DNA as a template. The process is completed by annealing the new strand of DNA to its original partner and using that new DNA as a template for DNA synthesis. Allele 'B' is converted into allele 'A' whereas allele 'A' is unchanged. **(b)** Three outcomes of DSB repair. Dark and light blue lines represent related chromosomes that allow repair by homologous recombination. Nonhomologous end joining (NHEJ) often repairs a DSB cleanly, in a nonmutagenic fashion, but it can also result in a mutation creating a novel sequence at the junction (shown as a checkered box). The red chromosome is unrelated to the blue one, so the joining event causes a translocation.

a DSB is potentially lethal damage that must be repaired, and one pathway of repair is by homologous recombination with a closely related sequence (Box 1 and Fig. 1).

Jasin and colleagues pioneered the use of highly specific DNA cleavage to investigate the stimulation of homologous recombination by DSBs in mammalian cells, making use of the yeast enzyme I-SceI encoded by *SceI*^{11,12}. *SceI* is a member of the homing endonuclease family of genes, so-called because they catalyze their own duplication into alleles by creating site-specific DSBs, which then initiate their own transfer by homologous recombination; it cuts DNA at an 18 base pair (bp)-long recognition site. When a *SceI* recognition site is inserted into a target gene and *SceI* is expressed in the cell, homologous recombination and gene targeting are stimulated by over 1,000-fold^{13,14}. The stimulation of gene targeting by *SceI* has been accomplished in several cell lines, including mouse ES cells, indicating that DSB-induced homologous recombination is a universal cellular phenomenon^{15–18}. Under optimized conditions, targeting rates of 3–5% have been achieved using a reporter gene¹⁹.

This work highlights the power of a DSB in stimulating gene targeting to levels that would be experimentally and therapeutically useful. To harness the stimulatory power of DSBs requires a method for creating

site-specific DSBs in endogenous genes. There are several ways to achieve this using modified triplex-forming oligonucleotides²⁰, modified polyamides^{21,22}, modified peptide-nucleic acids²³, modified homing endonucleases or zinc finger nucleases (ZFNs) (Box 2). Some success has been achieved targeting genes with modified homing endonucleases and this is covered in Box 3. In this review we focus on recent progress made with ZFNs. ZFNs are artificial fusion proteins that link a zinc finger DNA binding domain to a nonspecific nuclease domain. Results in model organisms indicate that ZFNs will be effective in producing designed mutations for genetic studies, and the first studies in human cells encourage pursuit of ZFNs for potential use in human gene therapy.

Development of ZFNs

ZFNs (originally termed chimeric restriction enzymes) were first developed by Chandrasegaran and coworkers. (The history of the initial development of ZFNs and other hybrid nucleases has been reviewed elsewhere^{24–26}.) They hypothesized that they could create novel sequence specificities by fusing the nonsequence-specific cleavage domain of the *FokI* type II restriction endonuclease (Fn domain) to a new DNA-binding domain. First with a *Drosophila melanogaster* homeobox domain, then with a zinc finger-DNA binding domain and finally with the yeast

Box 2 Zinc finger basics

The protein modules known as C_2H_2 zinc fingers (ZFs), originally discovered by Klug and coworkers in 1986 (ref. 53), are found in the DNA-binding domain of the most abundant family of transcription factors in most eukaryotic genomes. The human genome contains at least 4,000 such domains in over 700 proteins, which represents ~2% of human genes^{41,47}. As illustrated in **Figure 2**, each finger is composed of 30 amino-acids, folds into a $\beta\beta\alpha$ configuration, coordinates one Zn^{2+} atom using two cysteines and two histidine residues, and contacts primarily 3 bps of DNA⁵⁴. Two critical features of the structure are that each finger binds its 3-bp target site independently and that each nucleotide seemed to be contacted by a single amino acid side chain projecting from one end of the α -helix into the major groove of the DNA. From these features, two predictions were made. The first is that by combining individual zinc fingers with

different triplet targets, the overall binding specificity of the zinc finger protein could be changed. The second is that by altering individual amino acid residues in the α -helix, the specificity for an individual finger could be altered. These critical predictions have been substantiated in some contexts by a number of different labs and are reviewed elsewhere^{32,55-57}. Individual fingers have been designed to recognize many of the 64 different target triplets, but the greatest success has been in designing zinc fingers to recognize 5'-GNN-3' triplets (where N represents any of the four bases)^{58,59}. Although zinc finger recognition codes have been proposed^{32,55,60}, no code currently exists that consistently results in zinc fingers with high affinity binding. Improving the specificity of ZF binding, such as by increasing the number of fingers or by constructing multifinger proteins using two-finger units, remains an active area of research^{33,41,46,57,61-63}.

Gal4 DNA-binding domain, they demonstrated that cutting could be redirected in the chimeras^{24,27,28}. ZFNs (**Fig. 2**) consist of an N-terminal zinc finger DNA-binding domain, a variable peptide linker and a C-terminal Fn domain (**Box 2**). Whereas initial *in vitro* work suggested that a ZFN could cleave DNA at a monomeric copy of its recognition site²⁹, subsequent studies, demonstrated that ZFNs cleave as dimers^{30,31}.

Using a *Xenopus laevis* oocyte system, Bibikova *et al.*³¹ showed that the most efficient cleavage and recombination was obtained when the binding sites were inversely oriented and separated by six nucleotides, and when there was no intentional linker between the zinc finger and nuclease domains. Taken together, these experiments showed that ZFNs not only could create DSBs on naked DNA templates *in vitro*, but also create DSBs in a cell, thereby activating substrates for homologous recombination.

The *in vitro* and *X. laevis* studies were done using zinc finger DNA binding domains with known recognition sites. The appeal of ZFNs, however, is that the zinc finger DNA binding domain could be modified to recognize novel target sequences, including those in endogenous genes³²⁻³⁴ (**Box 2**).

ZFNs in model organisms

The first genomic locus to be targeted successfully with designed ZFNs was the yellow gene of the fruit fly *D. melanogaster*³⁵. Bibikova

*et al.*³⁵ produced a pair of three-finger ZFNs for a sequence within this gene based on the fact that fingers had been identified at that time that would bind all DNA triplets of the form 5'-GNN-3'. Given the requirements for ZFN cleavage, they selected a site with the form, 5'-NNC NNC NNC NNNNNN GNN GNN GNN-3' (5'-(NNC)₃N₆(GNN)₃-3'). Two zinc finger nucleases were assembled to recognize one 9-bp site each. Heat-shock induction of these two proteins from integrated transgenes in fly larvae led to both targeted mutagenesis following cleavage³⁵ and, in the presence of a marked donor DNA, targeted gene replacement by homologous recombination³⁶. These alterations were stably passed through the germ line and, in initial studies, represented a few percent of all the chromosomal targets. Recent experiments have extended this procedure to two additional *D. melanogaster* loci, and targeting frequencies up to 25% have been achieved (*D.C. et al.*, unpublished data).

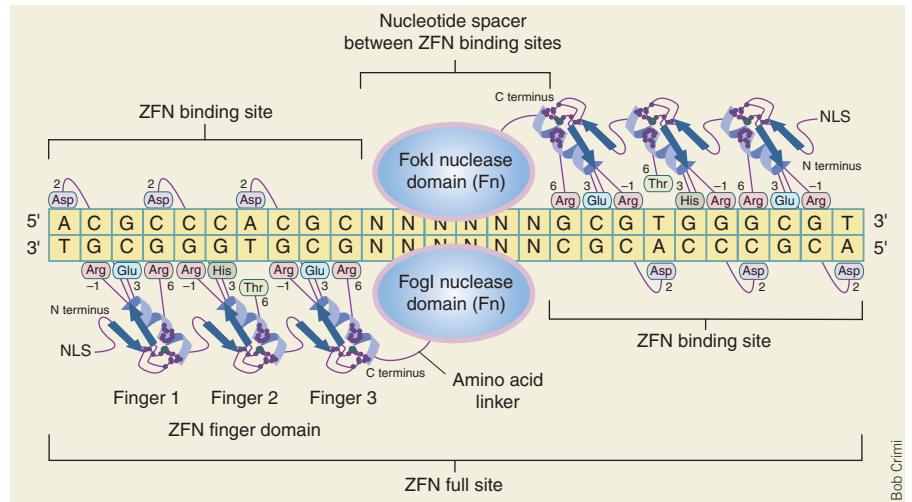
This approach should be applicable to essentially all model organisms, although unique experimental conditions will likely have to be established for each organism. Evidence to support this broad applicability comes from experiments showing that ZFNs create targeted mutations in the plant *Arabidopsis thaliana*³⁷. These studies indicate that ZFNs will be powerful tools for making directed modifications in experimental organisms for functional studies and for creating models of human genetic diseases.

Box 3 Modified homing endonucleases

Homing endonucleases, such as I-SceI of yeast, are natural genetic elements that catalyze their own duplication into recipient alleles by creating site-specific DSBs that initiate their own genetic transfer by homologous recombination⁶⁴⁻⁶⁶. A key feature of these enzymes is that they create DSBs at recognition sites that are 14- to 40-bp long⁶⁴. A second key feature is that their expression in mammalian cells does not cause overt cytotoxicity or seem to cause gross chromosomal rearrangements¹³. This appealing attribute is at least in part due to their site specificity. Although several hundred different homing endonucleases with different recognition sites have been identified, the major limitation to using them in gene targeting is that most mammalian genes do not have recognition sites for them. One strategy, therefore, is to use protein engineering to modify homing endonucleases to recognize target sites in mammalian genes.

This line of experimentation is still in its infancy but several investigators have made progress using structure-based protein engineering. In this work, chimeric homing endonucleases have been made with novel recognition sites, and *in vitro* modifications have been made that alter the target site specificity⁶⁷⁻⁷⁰. This work suggests that these enzymes can be modified to recognize new recognition sites. Currently, however, no modified homing endonuclease has been made that recognizes a sequence from an endogenous mammalian gene. Moreover, because one of their attractions is their lack of cytotoxicity, it also remains to be seen whether modified versions retain that characteristic or if by changing the site-specificity one also loosens their site specificity, thereby creating additional DSBs at undesired sites and consequent cytotoxicity.

Figure 2 ZFN homodimer binding to DNA. Shown is a three-finger zinc finger linked to the Fn domain through a flexible peptide linker. At the N-terminus of each ZFN resides a nuclear localization signal (NLS). The Fn domain is linked to the C-terminal finger (in this case finger 3) of the zinc finger domain. For most efficient cleavage there is no amino acid linker between the zinc finger domain and the Fn domain. The binding sites are arranged in an inverted orientation so that one ZFN is making most of its major contacts with one strand of DNA, whereas the other ZFN is making most of its major contacts with the other strand of DNA. Between the two binding sites is a nucleotide spacer (NNN...), the sequence of which does not seem to be important. This figure is a modification of Figure 2c from Jantz *et al.*⁴⁷.



ZFNs in human somatic cells

The first demonstration that ZFNs could stimulate gene targeting in mammalian somatic cells came from Porteus (M.H.P.) and Baltimore¹⁹. In these experiments, recognition sites for known zinc finger DNA-binding domains were inserted into a green fluorescent protein (GFP) reporter gene, which was integrated as a single copy into the genome of the human embryonic kidney cell line (HEK293; Fig. 3). In this system, gene targeting is measured by the correction of an integrated mutant GFP target gene by a transfected donor plasmid and the resultant conversion of GFP-negative cells into GFP-positive cells. ZFNs were made using a three-finger zinc finger domain from

a natural zinc finger protein, Zif268 (Zif-ZFN) and a designed three-finger domain, QQR (QQR-ZFN)^{30,31}. The stimulation of targeting was most efficient when the ZFN recognition sites were oriented as inverted repeats separated by six nucleotides and the ZFN did not contain a linker between the zinc finger and the Fn domains, showing that the same rules applied to mammalian cells as had been found earlier with *X. laevis* oocytes^{19,30,31}. This work and the work of Bibikova *et al.* (2003) has been briefly reviewed elsewhere³⁸.

A critical next step was to design ZFNs to recognize natural sequences and demonstrate that they could also stimulate targeting in mammalian cells as had been done in the *D. melanogaster* germ line³⁶. The prediction that sites of the form 5'-(GNN)₃-3' could be targeted in mammalian cells with assembled three-finger proteins was verified when ZFNs to several different targets were shown to stimulate gene targeting³⁹. In addition, this work showed that targeting with ZFN could be induced simultaneously at both the site of the break and at a distance of 400 bp from the break, demonstrating that a single pair of ZFNs can stimulate targeting in a relatively large region surrounding a DSB.

ZFN-mediated gene targeting of the SCID gene

Recently, a paper by Urnov *et al.*⁴⁰ (M.H.P. contributed to this work) has reported that designed ZFNs can cleave an endogenous human gene in cultured cells and lead to targeted gene replacement in up to 20% of the cells. The target was the gene for interleukin (IL)-2R γ , a cytokine receptor that is required for T-cell development and the establishment of a functional immune system⁴¹. Mutations in the human IL2RG gene (γ_c) are the most common cause of severe combined immunodeficiency (SCID) and, importantly, it has been shown to be an effective target for gene addition therapy. In the earlier experiments, a viral vector was used to deliver a normal IL-2R γ gene to bone marrow cells isolated from affected children^{8,9}. Returning these manipulated cells to their hosts resulted in restoration of immune competence. In three cases, however, T-cell leukemias arose owing to activation of an oncogene as a result of nearby integration of the therapeutic transgene¹⁰. This highlights the need for procedural adjustments, but the clinical success is very encouraging.

Urnov *et al.*⁴⁰ used a commercial archive of two-finger modules⁴² to create two four-finger ZFNs (γ_c -ZFN-L and γ_c -ZFN-R) targeted to exon 5 of the γ_c gene, a mutational hot spot on the gene. In addition to the novel source of zinc fingers, the binding sites in this case were separated by only 5 bp, which further expands the repertoire of sites that

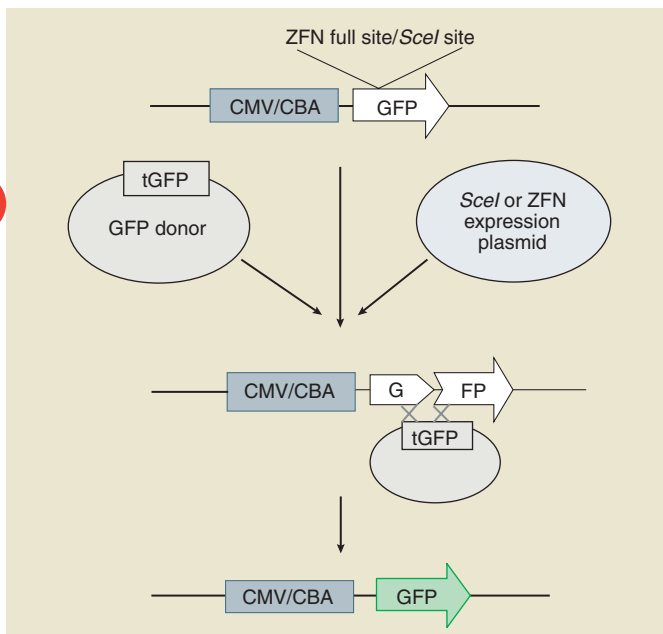


Figure 3 GFP gene-targeting reporter system. A mutated GFP gene, with an inserted in-frame stop codon and recognition sites for *Scel* and zinc finger nuclease, is integrated as a single copy into the genome of a cell (top line). The cell is transfected with a plasmid with a nonfunctional GFP gene (tGFP) and expression plasmids for either the ZFNs or *Scel*. The integrated GFP is repaired by gene targeting, and the cell becomes GFP positive. CMV/CBA, cytomegalovirus enhancer/chicken β -actin hybrid promoter; GFP, green fluorescent protein.



Table 1 Potential applications of zinc finger nucleases

Experimental uses	Drug development	Therapeutics
Create knockout genes (cell lines, primary cells, transgenic animals)	Create humanized cell lines	Correction of genes in monogenic diseases (e.g., Huntington disease)
Create point mutations or small deletions in permanent or primary cell lines	Create cell lines for drug target validation	Inserting genes into precise (safe and permissive) locations for correcting complex mutations (hemophilia A) and introducing RNAi, for example
Improve efficiency of gene targeting in ES cells	Create cell lines for high-throughput screening for novel compounds	Altering alleles; for example, the CCR5 gene to create resistance to HIV.
Create targeted transgenics with insertions into precise genomic locations		Designer immunotherapeutics
Genome manipulation in model organisms currently without gene targeting mechanism (worms, zebrafish)		Modification of stem cells

ZFNs target. After showing that the γ_c ZFNs had a high affinity binding to their target sites *in vitro*, we then tested them using a GFP reporter system and found that they efficiently stimulated targeting. Subsequent protein engineering optimized binding of each ZFN to its cognate site, leading to a fivefold improvement in targeting in the GFP reporter system. Importantly, in K562 cells, a transformed human erythroleukemia cell line, the γ_c ZFNs stimulated mono-allelic targeting in 11% of cells and bi-allelic targeting in 6% of cells without selection.

Furthermore, successful targeting was achieved in cultured primary T cells and in established lines, and conversion both from normal to mutant and back to normal was demonstrated in successive experiments. These experiments gave clear proof of principle that ZFNs could be used as a powerful tool to create subtle and specific changes in the genome of human somatic cells and that ZFNs could be used to correct mutations that cause human disease.

Toxicity and other undesired effects

In early studies, it was noticed that expression of ZFNs could have cytotoxic effects. In *D. melanogaster* for example, one of the two ZFNs designed for the yellow gene proved to be lethal when overexpressed, although tolerable and effective levels of expression were readily found³⁵. In the initial experiments with human cells¹⁹ toxicity was demonstrated by loss of targeted GFP⁺ cells upon continued culture. The four-finger proteins constructed by Urnov *et al.*⁴⁰ for the human γ_c gene, in contrast, did not show toxicity. Using a relatively insensitive assay, they did not detect any gross chromosomal rearrangements, although this does not rule out the possibility of a low frequency of translocation.

With *D. melanogaster*, the lethality was demonstrated to be a consequence of excessive cleavage, as a point mutation in the nuclease active site restored full viability (K. Beumer and D.C., unpublished data). We assume that the toxicity in mammalian cells is also due to cleavage of nontarget sequences; when the number of 'off-target' DSBs becomes too great for that cell type, cell death ensues. An advantage of ZFNs is that dimerization of the nuclease domain is required for cleavage. Thus, a pair of three-finger ZFNs will usually have a unique 18-bp site at which it most efficiently cuts. Off-target cleavage is likely the result of two ZFNs binding at noncanonical sites, perhaps ones related in sequence to the desired target or may be the result of binding of a single ZFN with solution dimerization of the nuclease domain as can occur with natural FokI *in vitro*⁴³. Evidence from experiments with both cultured cells⁴⁰ and flies (D.C. *et al.*, unpublished observations) shows that it is possible to design ZFNs with sufficient specificity to reduce toxicity, but new approaches may also be required.

Another consequence of cleavage by ZFNs is the creation of break-induced sequence alterations through nonhomologous end joining

(**Box 1** and **Fig. 1**). Mutations of this sort can occur both at the desired target if the DSB is repaired by nonhomologous end-joining rather than by homologous recombination with the donor and at sites of off-target cleavage. In some instances the goal may be to alter the target sequence—for example, to knock out the activity of a particular gene—but in directed gene targeting procedures, particularly in gene therapy settings, it would be undesirable to create new mutations while correcting an existing one. Finally, it is known that DSBs are a source of oncogenic translocations^{44,45}. It is important, therefore, to determine if ZFNs are creating such translocations by the induction of DSBs and to develop assays to detect these rare but potentially dangerous events.

Future directions

The development of ZFNs to stimulate gene targeting by homologous recombination in mammalian somatic cells represents the synergistic fusion of two seemingly independent fields: the study of zinc finger domains and the study of homologous recombination. The results reported here provide cause for optimism that ZFN-mediated targeting will provide a useful experimental tool for manipulating the mammalian genome for many of the potential experimental applications highlighted in **Table 1**. Moreover, with further development, the ZFN strategy may be applied in the treatment of human genetic diseases as well as in other areas of biotechnology. Before such applications can be realized and ZFN approaches become widely adopted, however, several challenges remain: first, applicability of the ZFN approach needs to be broadened; second, the method for delivering ZFNs and repair substrate to cells requires optimization; and third, our understanding of the process of homologous recombination itself needs to be enhanced.

Efforts to broaden the applicability of the approach will require not only the design of ZFNs targeting a greater variety of gene targets but also the determination of the conditions for performing gene targeting in different cell types. Until now, ZFNs have been primarily applied to transformed mammalian cell lines that are relatively resistant to apoptotic stimuli. An important advance, therefore, will be to develop ways of using ZFNs in primary cells that are more sensitive to DNA damage.

The optimization of ZFN design must address two key, related issues: specificity and cytotoxicity. Addressing these issues will necessitate thorough analysis of the mechanisms of gene target recognition and binding for the zinc finger component. Simply assembling ZFNs for sites composed entirely of GNN triplets has its limitations, but may prove to be practical for many applications. In the case of ZFNs designed to target the γ_c gene⁴⁰, the method used to create the ZFN affected both the ratio of specificity to cytotoxicity and the ZFNs and the ability to

efficiently target sequences that contain non-GNN triplets. It is not clear how many zinc fingers are optimal for activity and precise targeting. Whereas four-finger γ_c ZFNs are less cytotoxic than three-finger ZFNs in transformed mammalian somatic cells, even these show potential 'off-target' effects when expressed alone at high levels using a relatively crude cytotoxicity assay (M.H.P, unpublished data).

Several lines of investigation may offer solutions to ZFN cytotoxicity issues. First, zinc fingers may be further refined to better discriminate between the gene target of choice and related off-target binding sites. This will require continued basic research into the nature of the recognition process—an area that has been studied by structural biologists for several decades and is not trivial. Second, further research is required to elucidate the optimal number of fingers in each ZFN. Future studies should determine whether increasing the number of fingers consistently results in improved ZFNs, both from a specificity and kinetic standpoint. Experiments thus far have demonstrated that moving from three to four fingers provides significant improvement, but it is not clear whether further increases may be beneficial^{46,47}. Third, efforts should focus on tighter control of the level and duration of expression of potentially toxic ZFNs. The loss of targeted cells with time in culture is likely a consequence of continued, although still transient, expression. Because the homologous recombination event is expected to occur rapidly after the target is cut, brief expression of ZFNs should be adequate. Finally, it may be possible to engineer other parts of the ZFNs—the peptide linker and the cleavage domain (including the possibility of alternative nuclease domains)—to optimize the ratio of target to off-target events.

The second main challenge for wide adoption of the ZFN approach will be to optimize the delivery method of the ZFNs and repair substrate to stem cells. Studies of ZFNs have been primarily performed using transfection techniques specific to cultured cell lines. Whether those techniques will work in primary cells or whether other methods, including the adaptation of viral delivery methods or direct microinjection as was done with *X. laevis* oocytes, will be better are important areas of future study.

A final challenge will be to increase our understanding of the process of homologous recombination itself in somatic stem cells as these are the cells that are most likely to generate long-term therapeutic benefits if targeted. All evidence suggests that homologous recombination is a universal process. However, studies are needed to analyze the rate of homologous recombination in somatic stem cells, particularly quiescent ones.

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