Precision genome surgery

Alfred Pingoud & George H Silva

A redesign of zinc-finger nucleases reduces off-target cleavage.

Zinc finger nucleases (ZFNs) hold great potential for gene therapy as they allow one to cleave DNA at a chosen target sequence. Unfortunately, however, they also cleave prolifically at off-target sites, resulting in unacceptable toxicity. Two papers in this issue, by Miller *et al.*[1] and Szczepek *et al.*[2], show that off-target cleavage can be greatly reduced by rationally redesigning the ZFN dimer interface to inhibit homodimerization.

The goal of gene therapy is to repair genetic defects without otherwise modifying the genome. One of the most promising strategies for gene correction is homologous recombination. In this approach, the correct DNA sequence is supplied *in trans* and is integrated into the genome by an endogenous mechanism of site-specific recombination. Ideally, the deficient gene is replaced with a functional copy in its natural context while leaving the rest of the genome untouched. Homologous recombination can also be used for gene inactivation, insertion or deletion.

A major obstacle in the development of this approach has been efficiency. Natural recombination between an introduced DNA and the homologous chromosomal target occurs at a low frequency of ~10^{-6}. Remarkably, however, a double-strand break at or near the target sequence increases the frequency of homologous recombination by several orders of magnitude. To facilitate such double-strand break–induced recombination, engineered nucleases, chimeric restriction endonucleases, homing endonucleases and 'chemical' nucleases have been created and

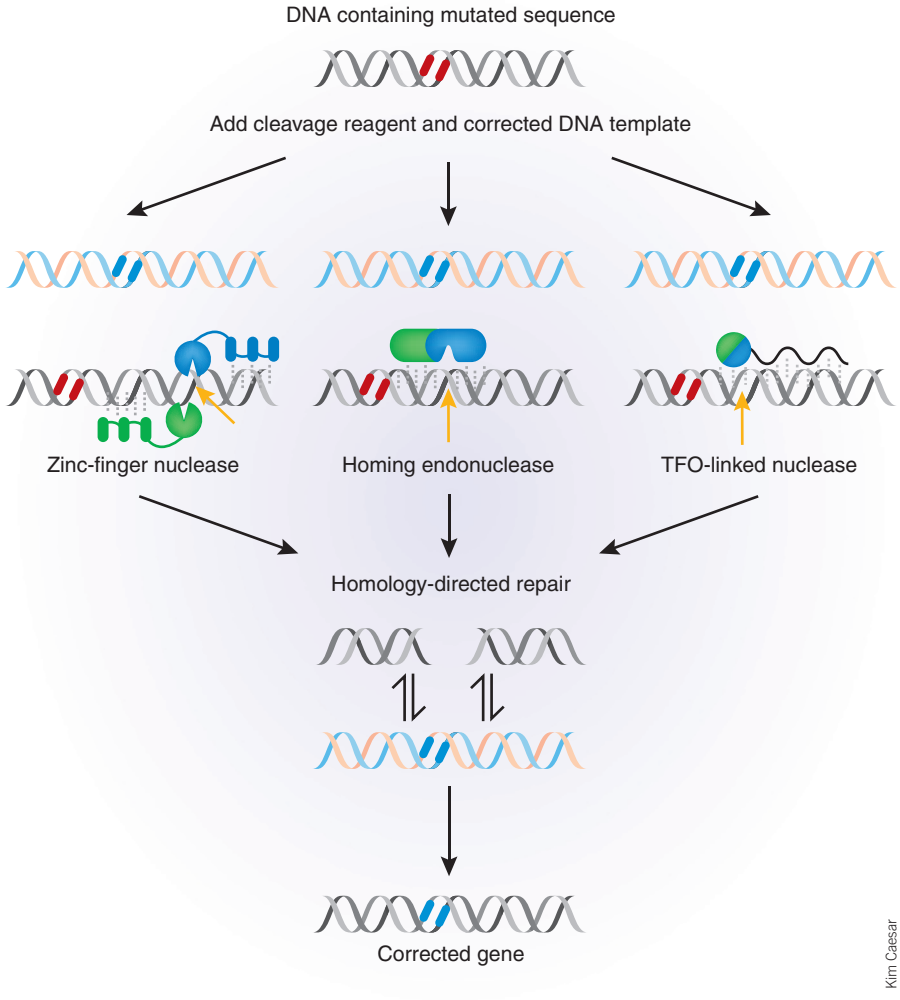

Figure 1 Three approaches for cleaving DNA to increase the efficiency of homologous recombination for gene correction. By exploiting the host's homology-directed repair mechanisms to fix double-strand breaks in DNA, precise changes can be made within a genome using highly specific nucleases targeted to a sequence of interest. (**a–c**) Cleavage can be effected by a heterodimeric zinc finger nuclease (**a**), a homing endonuclease (**b**) or a triple-helix forming oligo (TFO) linked to a 'chemical' nuclease or restriction enzyme (**c**). For efficient homology-directed repair, cleavage need only be within 100 base pairs of the sequence to be changed. Mutated DNA region shown in red, repaired DNA region in blue. Yellow arrow marks point of cleavage. Broken gray lines indicate specific protein-DNA or TFO-DNA interactions.

Alfred Pingoud and George H. Silva are at the Institut fuer Biochemie, Fachbereich Biologie und Chemie, Justus-Liebig-Universitaet, Heinrich-Buff-Ring 58, D-35392 Giessen, Germany.
e-mail: alfred.m.pingoud@chemie.bio.uni-giessen.de

successfully used in experimental systems[3] (**Fig. 1**). Although the efficiency of some of these methods is now high enough to contemplate certain clinical applications, other limitations remain, including a requirement that the cleavage site be uniquely present in the chromosomal target and toxicity due to off-target cleavage.

More than ten years ago, Chandrasegaran and colleagues reported the creation of the first ZFN by fusing zinc finger motifs to the cleavage domain of the *Fok*I endonuclease[4]. As each zinc finger is specific for a DNA base triplet, tethering multiple fingers in series allows for specific targeting of a wide variety of extended DNA sequences. To this end, extensive effort has been devoted to designing new fingers to recognize all 64 possible DNA triplets. ZFNs function as dimers; each of the two polypeptides consists of the *FokI* cleavage domain fused at its N-terminus to three or four zinc fingers that form the DNA-binding domain. A heterodimeric ZFN that recognizes asymmetric sequences of 18 to 24 base pairs is therefore in principle sufficiently specific to cleave a unique sequence in the human genome.

In fact, however, specificity is compromised by the mechanism of ZFN activity. DNA cleavage requires that the two subunits dimerize via the *Fok*I cleavage domain and that at least one subunit is bound to DNA.[5] Because dimerization occurs independently of DNA binding, heterodimerization (the anticipated case) and homodimerization of each of the two subunits (the side effect) give rise to at least three enzymes with different targets. Although it is unlikely that such large palindromes will exist in the genome as secondary targets, it is conceivable that a homodimeric ZFN of lower specificity could effect cleavage of pseudo-palindromic sequences. Furthermore, under certain conditions, DNA binding of only one subunit of a dimer (recognizing only 9 to 12 bases) can result in DNA cleavage, a situation thought responsible for the toxicity of ZFNs. Thus, two different ZFN subunits give rise to a total of seven possible 'cleavage-active' enzyme species (only one of which is desired).

To address these issues, Miller *et al.* and Szczepek *et al.* engineer variants of the *Fok*I (cleavage/dimerization) domain that exclusively favor heterodimer formation. By inspection of the *Fok*I crystal structure[6], both groups identify residues in two α-helices that mediate the subunit-subunit interaction and that, when exchanged, prevent homodimer formation. Simply stated, the authors generate an asymmetric interface.

Each group resolves the heterodimerization issue in slightly different ways, yet with similar success. Of particular interest for those involved in protein engineering are the subtle differences in methods between the two groups. Although the strategies are similar in principle (that is, re-engineering of electrostatic and hydrophobic interactions), there is little overlap in the residues mutated in the final designs. In both cases, the effects predicted by computational analyses are confirmed experimentally with *in vitro* DNA cleavage assays as well as with cell-based gene correction[1] or gene targeting[2] experiments.

As anticipated from their designs, *in vitro* analyses show that the heterodimeric ZFNs predominately cleave only the hetero-target while leaving the homo-targets virtually untouched. Both groups also examine the questions of gene-conversion efficiency, either via endogenous gene correction[1] or reporter gene rescue[2], and cytotoxicity in cells transfected with plasmids coding for ZFNs. The reported recombination frequencies of ~10% for heterodimeric ZFNs are on par with that of 'wild-type' ZNFs, indicating that the re-engineered interface has little effect on function. Although the recombination frequencies are not quite at the ~20% level of the 'gold-standard' endonuclease I-*Sce*I, the significant advance in these studies lies in the reduced cytotoxicity. Immunocytochemistry and flow cytometry analyses to measure the number of double-strand breaks induced in transfected cells show clearly that the heterodimeric ZFNs are up to ~tenfold less toxic than ZFNs having the wild-type *Fok*I cleavage domain.

> The work of Miller *et al.* and Szczepek *et al.* represents a considerable step forward in the generation of ZFNs as tools for gene therapy.

The work of Miller *et al.* and Szczepek *et al.* represents a considerable step forward in the generation of ZFNs as tools for gene therapy. By reducing the complexity of cleavage-active enzyme species, they eliminate much of the off-target cleavage thought to give rise to cytotoxicity. It must be emphasized, however, that the improved ZFNs can still cleave DNA at off-target sites, albeit with lower efficiency, when binding to DNA via only one subunit. Unlike the natural *Fok*I enzyme from which the nuclease domain is derived, ZFNs currently lack allosteric control over cleavage. It is perhaps here that the next step in ZFN development lies, reigning in the nuclease to finally harness the power of targeted DNA binding.

ZFNs are not the only 'meganucleases'[3] that are being developed for the purpose of gene targeting (**Fig. 1**). Homing endonucleases, which recognize extended sequences of up to ~30 base pairs, have been successfully re-engineered to target new sequences[7,8]. For one of these enzymes, I-*Cre*I, it has been shown that the protein-DNA interface can be extensively engineered to create novel endonucleases with tailored specificities[7]. It remains to be seen whether conjugates of triple-helix forming oligonucleotides (TFO)[9] can compete with ZFNs and homing endonucleases in targeting genes. Whereas ZFNs and TFO-conjugates can be more easily constructed to cleave a wide variety of different sequences, homing endonucleases have the advantage that their cleavage activity is tightly coupled to recognition, thus making erroneous cleavage less probable. Undoubtedly, the choice of technology will depend on further technical refinements and the requirements of the particular application at hand.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

1. Miller, J.C. *et al. Nat. Biotechnol.* **25**, 778–785 (2007).
2. Szczepek, M. *et al. Nat. Biotechnol.* **25**, 786–793 (2007).
3. Paques, F. & Duchateau, P. *Curr. Gene Ther.* **7**, 49–66 (2007)
4. Kim, Y.G., Cha, J. & Chandrasegaran, S. *Proc. Natl. Acad. Sci USA* **93**, 1156–1160 (1996)
5. Bitinaite, J., Wah, D.A., Aggarwal, A.K. & Schildkraut, I. *Natl. Acad. Sci USA* **95**, 10550–10575 (1996)
6. Wah, D.A., Hirsch, J.A., Dorner, L.F., Schildkraut, I. & Aggarwal, A.K. *Nature* **388**, 97–100 (1997)
7. Arnould, S. *et al. J. Mol. Biol.* **355**, 443–458 (2006)
8. Silva, G.H, Wende, W. Belfort, M. & Pingoud, A. *J. Mol. Biol.* **361**, 744–754 (2006)
9 Duca *et al. Nucl. Acids Res.* **34**, 1900–1911 (2006)