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Custom-Designed Molecular Scissors for Site-Specific Manipulation of the Plant and Mammalian Genomes

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Summary

Zinc finger nucleases (ZFNs) are custom-designed molecular scissors, engineered to cut at specific DNA sequences. ZFNs combine the zinc finger proteins (ZFPs) with the nonspecific cleavage domain of the FokI restriction enzyme. The DNA-binding specificity of ZFNs can be easily altered experimentally. This easy manipulation of the ZFN recognition specificity enables one to deliver a targeted double-strand break (DSB) to a genome. The targeted DSB stimulates local gene targeting by several orders of magnitude at that specific cut site via homologous recombination (HR). Thus, ZFNs have become an important experimental tool to make site-specific and permanent alterations to genomes of not only plants and mammals but also of many other organisms. Engineering of custom ZFNs involves many steps. The first step is to identify a ZFN site at or near the chosen chromosomal target within the genome to which ZFNs will bind and cut. The second step is to design and/or select various ZFP combinations that will bind to the chosen target site with high specificity and affinity. The DNA coding sequence for the designed ZFPs are then assembled by polymerase chain reaction (PCR) using oligonucleotides. The third step is to fuse the ZFP constructs to the FokI cleavage domain. The ZFNs are then expressed as proteins by using the rabbit reticulocyte in vitro transcription/translation system and the protein products assayed for their DNA cleavage specificity.

Keywords

Gene therapy; Nonviral vectors; Zinc finger nucleases; Gene targeting; Genome engineering; Site-specific modification; Targeted mutagenesis; Gene correction; Homologous recombination; Nonhomologous end joining

1. Introduction

Cells use the universal process of homologous recombination (HR) to maintain their genomic integrity, particularly in the repair of a double-strand break (DSB), which otherwise would be lethal. DSB repair of a damaged chromosome by HR is a highly accurate form of repair, which works via the copy and paste mechanism, using the homologous DNA segment from the undamaged chromosomal partner as a template. Gene targeting—the process of replacing a gene by HR—uses an extrachromosomal fragment of donor DNA and invokes the cell's HR for sequence exchange. Gene targeting is not a very efficient process in plant and mammalian cells; approximately only one in a million cells provided with excess donor sequence undergo the desired gene modification (1–3). However, when a defined chromosomal break is introduced, HR is induced at that site in a large fraction of cells in a population (3). There is then a need to create custom molecular scissors for precision genome surgery to greatly enhance site-specific manipulation of plant and mammalian cells, including human cells.

Zinc finger nucleases (ZFNs) that combine the nonspecific cleavage domain (N) of FokI endonuclease with zinc finger proteins (ZFPs) offer a general way to deliver a site-specific DSB to the genome, and stimulate local HR in cells by several orders of magnitude. The Cys₂His₂ ZF motifs bind DNA by inserting an α -helix into the major groove of the double helix (4,5). Each finger primarily binds to a triplet within the DNA substrate. Key amino acids at positions -1, 2, 3, and 6 relative to the start of the α -helix contribute most of the sequence-specific interactions to the ZF motifs (4,5). These amino acids can be changed while maintaining the remaining amino acids as a consensus backbone to generate ZFPs with different sequence specificities (6,7). The ZFP also has the additional advantage that greater specificity can be achieved by adding more ZF motifs (a maximum of six ZF domains) to the ZFPs (8–10). Thus, ZF DNA-binding motifs, because of their modular nature and modular structure, offer an attractive framework for designing ZFNs with tailor-made sequence specificities (1,11,12). Several 3- and 4-finger ZFPs, each recognizing a 9- or 12-bp sequence, respectively, have been fused to the nonspecific cleavage domain of FokI to form ZFNs. The cleavage specificity of ZFNs correlates directly with the binding specificity of the corresponding ZFPs that are used to make them (13,14). Binding of two 3- or 4-finger ZFN monomers (each recognizing a 9- or 12-bp inverted site) is necessary because dimerization of the FokI cleavage domain is required to produce a DSB. Thus, a pair of ZFNs effectively has an 18- or 24-bp recognition site, which is long enough to specify a unique genomic location in plants and mammals. Reports from several labs including ours have shown that 3- and 4-finger ZFNs find and cleave their chromosomal target in cells; and, as expected, they induce local HR to repair the DSB. In the absence of homology-directed repair via HR (for example, if both alleles of a gene are damaged), cells repair the DSB by simple ligation via non-homologous end joining (NHEJ); repair by NHEJ is mutagenic. Thus, custom ZFNs can be used with and without homologous donor DNA sequences to induce “directed” mutations, by HR and NHEJ, respectively (1,2). Designer ZFNs have become valuable molecular scissors to perform precision genome surgery for various biological and biomedical applications. The ability to target a DSB to a specific genomic locus and stimulate HR by several orders of magnitude at that local site has great potential not only in genome engineering that is targeted manipulation of the mammalian (15,16) and plant genomes (17,18), but also to treat human diseases as a form of gene therapy.

Routine and facile production of custom ZFNs and their rapid characterization for sequence-specific DNA binding and cleavage properties in vitro is a prerequisite for ZFN-mediated gene targeting (19,20). We describe here the protocols that are needed to design (Fig. 1) and rapidly generate custom ZFNs (Figs. 2 and 3), and the protocol to rapidly characterize their properties by cell-free ZFN cleavage assays using the rabbit reticulocyte in vitro transcription/translation (IVTT) system. The cognate sites for the engineered ZFNs are encoded in a plasmid, which is then used as a substrate to monitor sequence-specific cleavage activity of the custom ZFNs (Fig. 4).

2. Materials

2.1. PCR Generation of ZFP

1. User-designed overlapping synthetic oligonucleotides and end primers.
2. Amplitaq Gold (ABI).
3. 10 mM dNTP Mix (Invitrogen).
4. 1% Agarose gel.
5. Polymerase chain reaction (PCR) machine.

6. 1-kb DNA ladder (Invitrogen).

2.2. Cloning of PCR Fragments into a pUC18 Plasmid

1. Amplified PCR fragments (*see Subheading 3.2*).
2. BamHI-cut dephosphorylated pUC18 plasmid DNA.
3. T4 DNA ligase (Invitrogen or NEB).
4. Electroporation cuvettes (Bio-Rad).
5. Competent DH5 α cells (Invitrogen).
6. SOC medium and LB medium.
7. Standard LB plates.
8. Carbenicillin (Sigma).
9. X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside): 40 mg/ml stock solution stored at -20°C .
10. IPTG (isopropylthio- β -D-galactoside): 1 M stock solution stored at -20°C .
11. Plasmid Mini purification kit (Qiagen).
12. Gel purification kit (Qiagen).
13. PCR purification kit (Qiagen).
14. Restriction Enzyme BamHI (NEB).

2.3. Conversion of ZFPS into ZFNS

1. pET15b:ZFN plasmid construct containing the FokI cleavage domain (available from our lab on request).
2. Plasmids containing various ZFP designs (*see Subheading 3.2*).
3. Restriction enzymes NdeI and SpeI (NEB).
4. T4 DNA ligase (Invitrogen or NEB).
5. 100 mM DTT (Invitrogen).
6. 100 mM ATP (Invitrogen).
7. RR1 electro-competent cells.

2.4. Cell-Free ZFNS Cleavage Assay Using the IVTT System

1. pUC18 plasmid containing the ZFN binding sites for use as substrates.
2. pET15b plasmid containing ZFN constructs.
3. TnTT7 Quick-Coupled Transcription/Translation kit (Promega).
4. 5 mM ZnCl₂.
5. Separating buffer (4 $\times$): 1.5 M Tris-HCl, pH 8.7, 0.4% sodium dodecyl sulfate (SDS). Store at room temperature.
6. Stacking buffer (4 $\times$): 0.5 M Tris-HCl, pH 6.8, 0.4% SDS. Store at room temperature.
7. Thirty percent acrylamide/bis solution (37.5:1 with 2.6% C) (Bio-Rad).

8. *N,N,N,N'*-Tetramethyl-ethylenediamine (TEMED) (Bio-Rad).
9. 10× Tris-glycine-SDS running buffer (Bio-Rad).
10. 10× Tris-glycine transfer buffer (Bio-Rad).
11. Ammonium persulfate: Prepare 10% solution in water and immediately freeze in single use (200 µl) aliquots at −20°C.
12. Water-saturated isobutanol. Shake equal volumes of water and isobutanol in a glass bottle and allow it to separate. Use the top layer. Store at room temperature.
13. Prestained molecular weight markers (Invitrogen or NEB).
14. Supported nitrocellulose membrane from Hybond ECL, chromatography paper from Whatman.
15. Tris-buffered saline with Tween (TBS-T): Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 250 mM Tris-HCl, pH 7.4, 1% Tween-20. Dilute 100 ml with 900 ml water for use.
16. Blocking buffer: 5% (w/v) nonfat dry milk in TBS-T.
17. FokI polyclonal antibody (available from our lab).
18. FokI restriction enzyme (NEB).
19. Secondary antibody: Antirabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences).
20. Enhanced chemiluminescent (ECL) Western blotting detection reagents (Amersham Biosciences) and Bio-Max ML film (Kodak).

3. Methods

Engineering custom-designed ZFNs for an endogenous chromosomal gene target in mammalian cells entails the following steps: (1) Identify target sequences of the form (NNC)₃ or 4...(GNN)₃ or 4 separated by 5 or 6 bp within the gene of interest, which make for excellent targets. (2) Design or select ZFPs that recognize a chosen target site. (3) Convert the engineered ZFPs into ZFNs. (4) Characterize rapidly their *in vitro* cleavage specificity, which is essential before any cell culture studies can be performed using the designed ZFNs.

3.1. Protocol to Identify ZFN Targets Within the Gene of Interest

1. Search for ZFN binding targets within the desired gene. Inverted sequences of the form (NNC)₃ or 4...(GNN)₃ or 4 separated by 5 or 6 bp make for excellent targets. The efficiency of ZFN-mediated gene targeting falls off rapidly with increasing spacer length beyond 6 bp. Furthermore, for inverted ZFN target sites with greater than 6-bp separation, a 15-amino acid (Gly₄Ser)₃ linker needs to be inserted between the ZFP DNA-binding domain and the FokI cleavage domain for effective double-strand cleavage (21). The ZFN target sequences could be within a hundred base pairs away from the mutation site for gene conversion (*see* Notes 1 and 2).

¹Inverted sequences of the form (NNC)₃ or 4...(GNN)₃ or 4 separated by 5 or 6 bp make for the best ZFN target sites. The ZF motif designs for GNN and ANN triplets are the best studied and well characterized; and therefore, they are highly preferred over the ZFN sites containing CNN and TNN triplets.

²Whenever possible, select 4-finger ZFN sites, because they are expected to be more sequence specific and likely to show higher affinity for their cognate sites compared with the 3-finger sites, everything else being equal. The 4-finger ZFNs are also likely to be more selective in binding to their targets and less toxic to cells.

2. The Barbas lab has posted a website (<http://zincfingertools.org>) (22) that could be used to search for target sites in the desired gene. A similar program is available at the Zinc Finger Consortium website (<http://www.zincfingers.org>). An example of the ZFN target sequences within the mouse tyrosinase gene (mTYR) is shown in Fig. 1.

3.2. Protocol to Create Genes that Code for the Desired ZFPs

1. Our ZFPs were designed based on the previously described zinc-finger-framework consensus sequence derived from 131 ZF sequence motifs (6). We designed 3-finger ZFPs that recognize a specific 9-bp sequence within the chosen genes as follows: (1) by using the consensus framework backbone sequence for each and every finger within the ZFPs of the three invariant amino acid backbone oligos (BBO1, BBO2, and BBO3); (2) by varying the contact residues at positions -1, +1, +2, +3, +4, +5, and +6 of the α -helix within each ZF motif of the ZFPs using three specificity-determining oligos (SDO1, SDO2, and SDO3); the amino acid residues that confer DNA binding site specificity to each ZF motif were chosen from previously available DNA triplet recognition data for ZFPs in the literature (23–26) and wherever possible taking into account the positional data of each ZF motif in the context of its neighboring fingers (27) (*see* Note 3).
2. The overlapping oligonucleotide assembly strategy was used to construct the 3-finger ZFPs (Fig. 2). They were first assembled by Klenow reaction using the BBOs and SDOs. The assembled 3-finger ZFPs were then amplified by PCR using the forward primer (flanked by NdeI/BamHI site) and reverse primer (flanked by SpeI/BamHI site) to facilitate cloning of the engineered ZFPs into the desired target plasmid.

Component	Amount (ml)	Final concentration
0.83 ml of each 2 μ M oligo	5.0	0.2 μ M
1.25 mM dNTPs	1.0	0.125 mM
5 $\times$ Second-strand buffer	2.0	1 $\times$
100 mM DTT	1.0	10 mM
5,000 U/ml Klenow	1.0	0.5 U/ μ l

3. Set up the Klenow reaction for each of the ZFPs with the following:

The Klenow reaction is carried out at 37°C for 30 min and then at room temperature for another 30 min.

Component	Amount (μ l)	Final concentration
Klenow reaction product	2.5	

³The binding of the ZF motif to its cognate triplet occurs in the context of neighboring fingers, which could affect the specificity and affinity of the ZFPs. Therefore, designing and tethering of ZF motifs to form a ZFP, and hence linking the ZFP to the FokI cleavage domain to form the ZFN, for a chosen target site do not always yield ZFNs with the desired sequence specificity and affinity. More often than not, one needs to optimize the sequence-specific binding and affinity of the designed ZFPs by tinkering with ZF motif designs within a designed ZFP by substituting with other ZF motif designs that are available for a particular triplet, as was done by Urnov et al. (16).

Component	Amount (μl)	Final concentration
10 mM dNTPs	2.0	200 mM
10 μM Forward primer (NdeI)	2.5	250 nM
10 μM Reverse primer (SpeI)	2.5	250 nM
10× PCR buffer	10.0	1×
10 U/μl Amplitaq Gold Enzyme	1.0	0.1 U/μl
dH ₂ O	79.5	

- Set up the PCR using the product from the Klenow reaction as the template:
The PCR is run for 30 cycles of amplification, where each cycle is programmed for 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min.
- PCR-amplified ZFPs are run on 1% agarose gel to ensure the correct size for the DNA fragment. The PCR product is then purified by using a Qiagen PCR purification kit.

Component	Amount (μl)	Final concentration
ZFP DNA fragment	10.0	1 μg/μl
10× NEB Buffer 2	2.0	1×
20,000 U/ml BamHI	2.0	1.0 U/μl
dH ₂ O	6.0	

- The purified DNA fragment is subjected to digestion with BamHI:
The digestion reaction is performed at 37°C for 12–16 h.
- The BamHI-digested DNA fragment is run on 1% gel and the 300-bp ZFP DNA fragment is gel purified using a Qiagen gel purification kit.

Component	Amount (μl)	Final concentration
BamHI-digested DNA	2.0	0.2 μg/μl
BamHI-cut pUC18 plasmid	1.0	0.1 μg/μl
1 U/μl T4 DNA ligase	1.0	0.05 U/μl
5× Ligase buffer	4.0	1×
dH ₂ O	12.0	

- The purified fragment is then ligated to the BamHI-cut and dephosphorylated pUC18 plasmid:
The ligation reaction is incubated at 16°C overnight. The ligation product is extracted once with an equal volume of phenol-chloroform and twice with chloroform. The DNA is then precipitated with 100% ethanol at –80°C for 1 h. The precipitate is spun down in a microcentrifuge and washed with 70% ethanol. The pellet is air-dried and reconstituted in 10 μl of dH₂O.
- One microliter of the purified ligation product is used to electroporate DH5α cells, the SOC medium is added immediately, and the cells are incubated at 37°C for 1 h to express the antibiotic resistance gene. LB plates containing 100 μg/ml of

carbenicillin are spread with 40 μ l of 40 mg/ml X-gal and 40 μ l of 100 mM IPTG and incubated at 37°C until the cells are ready to be plated. The cells are then plated on the LB plates and incubated at 37°C overnight.

10. The next day, 12 white colonies are picked and grown in LB medium containing 100 μ g/ml of carbenicillin overnight. The following day, plasmid purification is done using Qiagen tip20. The plasmids are digested with BamHI and the products are run on 1% agarose gel to ensure the size of the ZFP fragment. The plasmids containing the ZFP fragments are also sequenced.

3.3. Protocol to Convert ZFPS into ZFNS

The pUC18 plasmid DNA containing the ZFPs coding sequence is digested with NdeI/SpeI to release the DNA fragment encoding the ZFPs, which are then ligated into the NdeI/SpeI-cleaved pET15b:ZFN vector (27), thereby replacing the existing ZFPs with the newly created ZFPs. These constructs link the designed consensus framework based ZFPs to the C-terminal 196 amino acids of FokI restriction enzyme, which constitutes the FokI cleavage domain. The ZFN fusions are of the form $\text{NH}_3^+ \text{--ZF1--ZF2--ZF3--FokI (N)--CO}_2^-$. When the separations between the inverted ZFN target sites are 5 or 6 bp, which are optimal for efficient cleavage, no linker is included between the ZFPs and the FokI cleavage domain; however, for ZFN target sites with greater than 6-bp separation, the ZFP is connected to the FokI cleavage domain through a $(\text{Gly}_4\text{Ser})_3$ linker. Furthermore, during the initial cloning of the engineered ZFNs into the bacterial cells, clones carrying the ZFN constructs are made more viable by increasing the constitutive levels of the DNA ligase within these cells (13,14) (*see* Note 4).

Component	Amount (μ l)	Final concentration
pUC18 plasmids containing ZFPs	10.0	1.0 μ g/ μ l
10 $\times$ NEB Buffer 2	2.0	1 $\times$
10 $\times$ BSA	2.0	1 $\times$
20,000 U/ml NdeI	1.0	1.0 U/ μ l
10,000 U/ml SpeI	2.0	1.0 U/ μ l
dH ₂ O	3.0	

1. The pUC18 plasmids, those that are determined to be error-free by sequencing, are subjected to digestion with NdeI and SpeI. The reaction is performed at 37°C overnight. The digested products are run on 1% agarose gel and the 300 bp ZFP fragments are gel purified using a Qiagen gel purification kit.

⁴The requirement for dimerization of the FokI cleavage domain restricts cleavage by a pair of ZFNs to long sequences, but it also introduces a potential problem, because this dimer interaction does not select for the heterodimer species, which could lead to unwanted off-target cleavage elsewhere in the genome of cells. Two different homodimers could result from the two individual ZFNs with different sequence specificities, and, more often than not, they do occur. The homodimers, although they are not relevant for gene modification, potentially could and often do affect the safety and efficacy of ZFN-mediated gene targeting of cells. Two recent articles have addressed this issue of ZFN cytotoxicity by redesigning the ZFN dimer interface to inhibit homodimerization, thereby, greatly reduce the off-target cleavage by ZFNs, and hence their cytotoxicity (40,41). We strongly recommend using these improved FokI cleavage domains in pairs of ZFNs to reduce cytotoxicity.

Component	Amount (μl)	Final concentration
NdeI/SpeI-digested ZFP DNA	2.0	0.1 μg/μl
NdeI/SpeI-cut pET15b:N plasmid	1.0	0.05 μg/μl
5× Ligase buffer	4.0	1×
T4 DNA ligase	1.0	0.05 U/μl
dH ₂ O	12.0	

- The gel-purified fragment is ligated to NdeI/SpeI-digested pET15b:N plasmid containing the FokI cleavage domain:

The ligation reaction is incubated at 16°C overnight. The ligation product is extracted once with an equal volume of phenol-chloroform, and twice with chloroform. The DNA is then precipitated with 100% ethanol and incubated at −80°C for 1 h. The precipitate is spun down in microcentrifuge, and the precipitate washed with 70% ethanol. The precipitate is air-dried and then resuspended in 10 μl of dH₂O.

- One microliter of the ligation product is electroporated into RR1 cells. During the initial cloning of the engineered ZFNs into the bacterial cells, clones carrying the ZFN constructs are made more viable by increasing the constitutive levels of the DNA ligase. The cells are plated on LB plates containing 100 μg/ml of carbenicillin and incubated at 37°C overnight. Twelve colonies are picked and grown overnight in LB medium containing carbenicillin. The next day, plasmid purification is done using Qiagen Tip20. Approximately 1 μg of plasmid is digested with NdeI and SpeI to confirm the presence of ZFPs in the recombinant plasmid. All of the above said steps are done to convert each pair of the ZFPs pair into corresponding ZFNs (Fig. 3).

3.4. Protocol for In Vitro Expression of ZFNS Using the IVTT System

The modified in vitro transcription-translation (IVTT) assay (28) was used to rapidly screen for the sequence specific cleavage by the engineered ZFNs. This protocol uses the rabbit reticulocyte IVTT system that yields a sufficient amount of the fusion protein product in the crude extract to study sequence-specific cleavage of the plasmid substrate encoding the ZFN target site. Each of the ZFNs is expressed from the pET15b:ZFN plasmids using the manufacturer's protocol. Assemble the reaction components in a 1.5-ml centrifuge tube with the following components:

Perform IVTT reactions for ZFN123 and ZFN456 in separate tubes. Incubate at 30°C for approximately 60 min. Analyze the reaction products by Western blot.

Component	Amount (μl)	Final concentration
TNT Quick Master Mix	40.0	
1 mM Methionine	1.0	0.2 mM
T7 TNT PCR enhancer	1.0	
5 mM ZnCl ₂	1.0	0.1 mM
1–2 μg pET15b:ZFN	2.5–5.0	0.02–0.04 μg/μl
dH ₂ O	4.5–2.0	

3.5. SDS-PAGE- Expressed ZFNS Using the IVTT System

1. Prepare a 1.5-mm thick, 10% gel by mixing 7.5 ml of 4× separating buffer with 10 ml acrylamide/bis solution, 12.5 ml water, 100 µl ammonium persulfate solution, and 20 µl TEMED. Pour the gel, leaving space for a stacking gel, and overlay with water-saturated isobutanol. This helps to smooth out the surface of the gel. The gel should polymerize in approximately 30 min.
2. Pour off the isobutanol and rinse the top of the gel twice with water.
3. Prepare the stacking gel by mixing 2.5 ml of 4× stacking buffer with 1.3 ml acrylamide/bis solution, 6.1 ml water, 50 µl ammonium persulfate solution, and 10 µl TEMED. Then pour the stacking gel and insert the comb. The stacking gel should polymerize in approximately 15–30 min.
4. Prepare the running buffer (Tris–glycine–SDS) by diluting 100 ml of the 10× running buffer with 900 ml of water in a measuring cylinder. Mix thoroughly.
5. Once the stacking gel has set, carefully remove the comb. Add the SDS running buffer to the upper and lower chambers of the gel unit and load 5–10 µl of each sample in a well. The samples are prepared by adding 2 µl of 5× SDS gel loading dye mixed with 8 µl of IVTT reaction product and heated at 60°C for 10 min. Include one well for prestained molecular weight marker and another well to run a positive control with the FokI enzyme.
6. Complete the assembly of the gel unit and connect to a power supply. The gel can be run during the day (in ~5 h) at 20 mA through the stacking gel and 30–40 mA through the separating gel.

3.6. Western Blot of ZFN Proteins Generated Using the IVTT System

1. The samples that have been separated by SDS-PAGE are transferred to supported nitrocellulose membranes by electrophoresis. A tray of transfer buffer is prepared that is large enough to lay out a transfer cassette with two pieces of foam and with two sheets of 3MM filter paper submerged on one side. A sheet of the nitrocellulose cut just larger than the size of the separating gel is laid on the surface of a separate tray of distilled water to allow the membrane to wet by capillary action. The membrane is then submerged in the setup buffer on top of the 3MM paper.
2. The SDS gel unit is disconnected from the power supply and disassembled. The stacking gel is removed and discarded and one corner cut from the separating gel to guide the loaded samples. The separating gel is then laid on top of the nitrocellulose membrane. Two further sheets of 3MM paper are wetted in the setup buffer and carefully laid on top of the gel, ensuring that no bubbles are trapped in the resulting sandwich. The second wet foam sheet is laid on top and the transfer cassette closed. The cassette is placed into the transfer tank such that the nitrocellulose membrane is between the gel and the anode. It is important to properly ensure this orientation or the proteins will be lost from the gel. The setup is placed in the cold room. The tank is covered and connected to the power supply. Transfers can be accomplished at 70 V for 2 h. Once the transfer is complete, the cassette is taken out of the tank and carefully disassembled, with the top sponge and sheets of 3MM paper removed. The gel is left in place on top of the nitrocellulose and these are laid on a glass plate so that the shape of the gel (including the cut corner for orientation) can be cut into the membrane using a razor blade. The gel and excess nitrocellulose can then be discarded. The colored molecular weight markers should be clearly visible on the membrane.

3. The nitrocellulose is then incubated in 50 ml blocking buffer (5% milk in TBS-T) for 1 h at room temperature on a rocking platform.
4. The blocking buffer is discarded and the membrane quickly rinsed before addition of a 1:2,000 dilution of the polyclonal FokI antibody in 5% milk for 1 h at room temperature on a rocking platform.
5. The primary antibody is then removed and the membrane washed three times for 5 min each with 50 ml TBS-T.
6. The secondary antibody is freshly prepared for each experiment as a 1:20,000-fold dilution in 5% milk and added to the membrane for 60 min at room temperature on a rocking platform.
7. The secondary antibody is discarded and the membrane washed six times for 10 min each with TBS-T.
8. During the final wash, 10-ml aliquots of each portion of the ECL reagent are warmed separately to room temperature and the remaining steps are done in a dark room. Once the final wash is removed from the blot, the ECL reagents are mixed together and then immediately added to the blot, which is then rocked gently by hand for 1 min to ensure even coverage.
9. The blot is removed from the ECL reagents, blotted with Kim-Wipes, and then placed between Saran wrap cut to appropriate size of the membrane. The Saran wrap containing the membrane is then placed in an X-ray film cassette with film for a suitable exposure time, typically a few minutes. An example of the results produced is shown in Fig. 4.

Component	Amount (μl)	Final concentration
Plasmid substrate (pUC18 encoding the targeted gene or ZFN sites)	2.0	0.005–0.01 μg/μl
10× NEB buffer 4	20.0	1×
ZFN123 IVTT extract	2.5	
ZFN456 IVTT extract	2.5	
dH ₂ O	173.0	

3.7. Protocol for the Cell-Free ZFNS Cleavage Assay Using the IVTT Extract

1. The chosen ZFN target sites are cloned into pUC18 plasmid, which serves as the substrate (14,21) for ZFN digestion. The ZFN digestion reaction is set as follows:

The control for the reaction is set up with the IVTT reaction product obtained without adding for ZFN expression plasmid. The reaction is incubated at 30°C for 1 h.
2. The digest is extracted with phenol-chloroform and then precipitated with ethanol; the precipitate is air-dried and resuspended in 100 μl of autoclaved water. The digest is analyzed using a 1% agarose gel. Similarly, reactions using other restriction enzymes (AatII or ScaI or XmnI, respectively) can also be performed.

Component	Amount (μl)	Final concentration
ZFN-digested substrate plasmid	10.0	0.5 μg/μl
5,000 U/ml SspI	1.0	0.25 U/μl
10× NEB buffer 2	2.0	1×
dH ₂ O	7.0	

The digest is performed at 37°C overnight. One microliter of RNase (5 mg/ml) is then added to the reaction mix and incubated for another 60 min at 37°C. The digest is analyzed using a 1% agarose gel. Similarly, reactions using other restriction enzymes (AatII or ScaI or XmnI, respectively) can also be performed. An example of this gel profile is shown in Fig. 4 (for trouble shooting, *see* Note 5).

3.8. Applications of ZFN-Mediated Gene Targeting: Site-Specific and Permanent Modification of Plant and Mammalian Genomes, Including the Human Genome

Several laboratories have shown that designed 3- and 4-finger ZFNs find and cleave their chromosomal targets in a variety of cell substrates, which include frog oocytes (29), *Drosophila* (30,31), plant cells (17,18), *Caenorhabditis elegans* (32), and human cells (15,16,33); and, as expected, they induce local HR at the site of cleavage to repair the DSB. In the absence of HR (for example, if both alleles of a gene are damaged), cells repair the DSB by simple ligation with the addition or deletion of some sequence (via NHEJ). Therefore, repair via NHEJ is mutagenic by nature. NHEJ-mediated repair has been shown for DSBs induced by ZFNs in *Drosophila* (30,31) and *Arabidopsis* (18). Thus, to induce targeted gene modifications in a variety of organisms and cells, ZFNs can be used with or without a homologous template DNA sequence (involving HR and NHEJ, respectively). Urnov et al. (16) have used designed 4-finger ZFNs in human cells to target an endogenous target site within the IL2Rγ gene (which causes the human X-linked disease, severe combined immune deficiency [SCID]). They achieved highly efficient permanent modification of the IL2Rγ gene in the K562 cell line—a remarkable gene-modification efficiency of 18% of K562 cells had the desired gene replacement without additional cell selection, and one third of these were altered on both X chromosomes. They obtained similar results using primary human T lymphocytes. Thus, the designer ZFNs have become powerful molecular tools to deliver a targeted genomic DSB to cells and stimulate local HR in human cells with an exogenously provided DNA template (15–17,30,33–35). The high targeting efficiency attests to the power of the ZFN-evoked HR for site-specific engineering of the human genome and raises the possibility of developing ZFN-based strategies (1) for laboratory research of gene-modified human cells, to complement what can be done to study gene function in knock-out and knock-in models of mice and other species, (2) for targeted genome engineering of plant species, and (3) potentially for human therapeutics as a form of gene therapy in the future (1,16,33,35–38) (*see* Notes 4 and 6).

⁵The TnTT7 Quick-Coupled Transcription/Translation System also contains nonspecific nucleases that could degrade DNA. If present during ZFN digestion of plasmid substrate encoding the ZFN sites, they could degrade DNA fragments resulting from substrate cleavage. The transcribed/translated ZFN gene reaction mixture using IVTT will also contain messenger RNA (mRNA) and transfer RNAs (tRNAs), which could appear as background during agarose gel electrophoresis. RNaseA could help in their removal. ZFNs could also remain bound to substrate and to the product fragments resulting from ZFN cleavage, giving rise to gel-shifted bands during agarose gel electrophoresis. Incubation of the reaction mixture with proteinase K might be needed for the removal of ZFNs from the resulting product fragments.

⁶The pair of custom-designed ZFNs that target the mTYR gene are then cloned into a single pIRES plasmid at two different multiple cloning sites (MCS) downstream of the cytomegalovirus (CMV) promoter used in the co-transfection of mouse melanocytes along with the exogenous donor DNA during ZFN-evoked gene targeting experiments using cell cultures (Fig. 5). Alternatively, the pair of ZFNs with different sequence specificities could also be cloned individually into the pIRES plasmid and then both plasmids simultaneously introduced into the cells along with the donor DNA.

3.9. Summary

ZFN technology has shown great promise and has the obvious potential to have a tremendous impact on both biotechnology and genetic medicine. ZFN technology has been referred to as a “game changing innovation” specifically for agriculture because it is now possible to target native genes in plants using custom ZFNs with exceptional precision. ZFNs have made it possible to target a preselected site and enable site-specific transgene integration in plants. It is only a matter of time before the ZFN technology is used toward trait generation and trait stacking across different crop plants. Thus, the ZFN technology platform has significant potential to contribute to the development of precision traits and to enable efficient and reproducible generation of combinations or stacks of multiple traits by targeted insertion of new traits into plants.

ZFN-based strategies as a form of gene therapy, in particular by modifying human stem cells *ex vivo*, may provide a new paradigm in genetic medicine for treating monogenic human diseases (2,39) by correcting the causative genetic defect at its origins. Many of the difficulties associated with gene therapy are likely to be overcome if one could insert the corrected version of the mutation at the precise location of the genetic defect within the human genome. The current gene therapy vectors lack the requisite sequence specificity necessary for targeted correction of the defective site within the human genome. ZFN-based strategies for gene correction of human stem cells may provide a viable option to treat monogenic human diseases in the future. The ZFN technology is still at its infancy and it has the potential to fulfill its promise for human therapeutics, in particular of curing monogenic diseases over the next decade.

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mTYR (exon 1 sequence)

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2581  TTCCAGATCT CTGATGGCCA TTTTCCTCGA GCCTGTGCCT CCTCTAAGAA CTTGTTGGCA
2641  AAAGAATGCT GCCCACCCTG GATGGGTGAT GGGAGTCCCT GCGGCCAGCT TTCAGTCAGA
2701  GGTTCCTGCC AGGATATCCT TCTGTCCAGT GCACCATCTG GACCTCAGTT CCCCTTCAAA
2761  GGGTGGATG ACCGTGAGTC CTGGCCCTCT GTGTTTATA ATAGGACCTG CCAGTGCTCA
2821  GGCAACTTCA TGGGTTTCAA CTGCGGAAAC TGTAAGTTTG GATTGGGGG CCCAAATTGT
2881  ACAGAGAAGC GAGTCTTGAT TAGAAGAAAC ATTTTGTATT TGAGTGTCTC CGAAAAGAAT
2941  AAGTTCTTTT CTTACCTCAC TTTAGCAAAA CATACTATCA GCTCAGTCTA TGTCATCCCC
3001  ACAGGCACCT ATGGCCAAAT GAACAATGGG TCAACACCCA TGTTTAATGA TATCAACATC
3061  TACGACCTCT TTGTATGGAT GCATTACTAT GTGTCAAGGG ACACACTGCT TGGGGGCTCT

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Fig. 1.

Selection of ZFN target sites within the nucleotide sequences of mouse tyrosinase (mTYR) gene. The nucleotide sequence of the mTYR exon 1 is shown. The best targets are inverted sequences of the form (NNC)₃ or 4... (GNN)₃ or 4 separated by 5 or 6 bp. The ZFN designs for the chosen targets that have been constructed and characterized for their DNA binding and cleavage properties are *shaded*. Other potential target sites for ZFN designs are *boxed*. The site of point mutation within the tyrosinase gene responsible for transition from pigmented (*black*) to nonpigmented (albino) mice is shown in *bold*.

Fig. 2.

Assembly of 3-finger ZFPs by using PCR. **(a)** The genes for the ZFPs are first assembled using the overlapping BBOs and SDOs (60-mers) in a Klenow reaction, which is then amplified by PCR using the outside forward and reverse primers, which are flanked by unique restriction sites (NdeI and SpeI sites, respectively) to facilitate cloning. BBO1, BBO2, and BBO3 correspond to the consensus backbone oligonucleotides whereas SDO1, SDO2, and SDO3 correspond to specificity-determining oligonucleotides for ZF1, ZF2, and ZF3, respectively. **(b)** Scheme for assembling the 3-finger ZFPs via the oligonucleotide assembly strategy using the consensus framework residues and the chosen contact amino acid residues at positions -1, +1, +2, +3, +4, +5, and +6 of the α -helix, which confer specificity to each of the ZFs. The indicated top strand (*bold*) and bottom strand oligonucleotides overlap and will be assembled using PCR. The bottom strand oligonucleotides are depicted as having NNN, which code for the contact residues that confer specificity to each ZF.

Fig. 3. Converting ZFPs into ZFNs. The NdeI/SpeI-cut ZFPs are ligated into the pET15b:N, the plasmid containing the FokI cleavage domain to form pET15b:ZFN.

Fig. 4.

Cell-free ZFN cleavage assays using the IVTT system. **(a)** Western blot profile of the fusion proteins made using the in vitro transcription-translation (IVTT) system. This yields sufficient fusion protein for rapid characterization of the cleavage specificity of the custom-designed ZFNs. **(b)** Nucleotide sequences of the ZFN target sites (TS) for mTYR, the gene encoded in the plasmid substrates (pUC18: TS) for use in the cleavage reactions. **(c)** Schematic representation of the plasmid substrates (pUC18: TS) encoding the ZFN target site of the mTYR gene at the multiple cloning site of pUC18. Four unique restriction enzyme sites, namely AatII, SspI, and XmnI, within the plasmid substrates are indicated. The expected sizes of the fragments upon cleavage by ZFNs, followed by AatII, SspI, or XmnI, respectively, are shown. **(d)** Agarose gel profile of engineered ZFN cleavage of mTYR plasmid substrates. The plasmid substrate was digested by the ZFNs, followed by one of the restriction enzymes, namely AatII, SspI, or XmnI. The particular restriction enzyme used in the reactions after the corresponding ZFN digestion is indicated on *top of*

each lane. Plasmid substrates digested with the control IVTT product (which contained no ZFN plasmids), followed by one of the enzymes, AatII, SspI, or XmnI, respectively, for each are also shown. The 1-kb ladder marker is included in the gel profile.

Fig. 5.

Structure of pIRES: ZFNs. The pair of ZFNs that bind to the mTYR site, are cloned into a single pIRES plasmid at two different multiple cloning sites (MCS) downstream of the CMV promoter for use in the co-transfection of mouse melanocytes along with the exogenous donor DNA during ZFN-mediated gene targeting experiments.