Abstract

Zinc finger nuclease (ZFN) is an artificial restriction enzyme that causes DNA double-strand breaks (DSB) on a specific locus of genome sequences, and is useful for genome editing by error-prone non-homologous-end-joining (NHEJ) or homologous recombination with exogenous DNA [1,2]. ZFN is reported to function in various cells including embryos and to be able to generate vertebrates, plants and insects with specific genetic modifications [3–13].

ZFNs consist of C2H2 zinc finger (ZF) domains and a FokI-derived DNA endonuclease domain. Paired ZFNs bind to the plus and minus strands of the target locus with 5–6 bp gaps [14] and digest DNA by dimerized nucleases [15]. A ZF domain binds to a specific DNA triplet through its 7-amino-acid DNA-recognition helix and a different combination of 7 amino acids will bind to a different DNA triplet. By combining multiple ZF domains with different recognition helices, ZFNs are able to bind specifically to arbitrarily chosen DNA target sites [16,17]. The relationship between the amino acid sequence of the DNA recognition helix and the target DNA triplets has been widely investigated [14,16–19], and website tools for designing the appropriate ZF against endogenous DNA sequences have been developed [20–23]. However, the lack of activity against endogenous target sites have been recently pointed out in ZFNs designed by these websites. It is difficult to design an exclusive ZFN, which functions with selectivity at each unique target site. Therefore, it is beneficial to be able to design several ZFN pairs against several sites within one target gene. This makes developing an easy and efficient ZFN construction method very important.

At present, two major construction methods are being commonly used: one is the overlapping of synthetic long oligonucleotides by PCR [16] and the other is the assembly of ZF modules from a prepared ZF-coding plasmid library through consecutive restriction and ligation reactions [24,25]. In the former method, an approximately 300 bp DNA sequence coding 3 ZF DNA-binding domains is divided into several synthetic long (a few dozen bp) oligonucleotide fragments, which have overlap sequences with the adjacent fragments in both ends, and these fragments are combined by PCR using the overlap sequences. Carroll et al. formulated a method to construct ZFNs consisting of 3 ZFs by overlapping 4 or more synthetic long oligonucleotides (60 bp<). They reported that the design, construction and cloning could be completed within about two weeks if all steps went smoothly, and expression and testing could be completed in an additional week [16]. Osborn et al. improved this method to shorten the construction period from design to testing to 1 week by constructing a specific expression vector consisting of nucleases and bind it with the prepared ZFs by enzymatic recombination [26]. However, the number of ZFs in
Figure 1. Experimental schemes for vector construction. (A) Composition of the platform vector used for ZFN construction. (B) Construction procedures for ZFN vectors by OLTA. In this figure, construction of a DNA-binding domain composed of 4 ZFs is shown as an example. Three partial ZF fragments were synthesized by the 1st PCR with the primer sets shown in Table 2 and they were combined by overlap extension PCR (2nd PCR) and amplified by 3rd PCR; then the DNA-binding domain was joined with the platform vector by TA cloning. (C) A scheme showing constructs of in vitro transcribed ZFN mRNA and in vivo translated ZFN protein, and ZFN binding with target DNA; Gli3 target site is shown in this figure for example.

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Table 1. ZFN target sequences and ZF alignment of each ZFN.

| Gene | Target Sequence | ZF1 | ZF2 | ZF3 | ZF4 | ZF5 | ZF6 |
|------|----------------|-----|-----|-----|-----|-----|-----|
| Gli3 | Left GAG CTG TGG GCC | GCC | TGG | CTG | GAG |
|      | Right GAC TGA GAG GAA | GAA | GAG | TGA | GAC |
| Rosa26 | Left AGA AAG ACT GGA GTT GCA | GCA | GTT | GGA | ACT | AAG | AGA |
|       | Right TGG GCG GGA GTC | GTC | GGA | GCG | TGG |
| Il2rg | Left GCA GCC TGA GGT TGG | TGG | GGT | TGA | GCG | GCA |
|       | Right GGT ATG AGA AGG GGG | GGG | AGG | AGA | ATG | GGT |
| Cdkn1b | Left GCG GGT GTG GAC | GAC | GTC | GTG | GCG |
|       | Right GAG GAA GAT GTC | GTC | GAT | GAA | GAG |
and a DNA sequencer (Applied Biosystems) according to the manufacturer’s instructions, and is referred to hereafter as the “platform vector”. The sequence of the platform vector is shown in Figure S1. Before TA cloning, the platform vector was digested with PvuII and BstZ17I, and dTTP was added to the 3′ end of digested sites using terminal transferase. The treated platform vector was purified by agarose gel electrophoresis, extracted using a gel extraction kit, and stored at −20°C until use.

Construction of ZF-template Vector

The zinc finger domain of mouse early-growth-response protein 1 (Egr1) has been well studied of its function and configuration [32–34], and has been used for ZFN in previous reports [16,26]. In the present study, the partial sequence (104 bp) from the first to Gli3 Cdkn1b was obtained from published full-length cDNA sequences. These target sequences were shown in Table 1, and an outline of ZFN vector construction is shown in Figure 1B. All PCR were performed using the primer sets shown in Table 2 on the condition of Table 3. The 3rd PCR products were cloned into the PvuII/BstZ17I digested and 3′-end of digested sites using terminal transferase. The treated platform vector was purified by agarose gel electrophoresis, extracted using a gel extraction kit, and stored at −20°C until use.

Table 2. Primer sets for 1st PCR.

| Target DNA triplet | DNA-recognition helix sequence | Forward Primer* (NDN)** | Reverse Primer* (NDN)** |
|-------------------|--------------------------------|--------------------------|-------------------------|
| GAA               | GAG RSDNLAR                    | 5′-GAG RSDNLAR           | 5′-GAG RSDNLAR           |
| GAC               | GAT TSGHLR                     | 5′-GAT TSGHLR            | 5′-GAT TSGHLR            |
| GAG               | GGG RSDNLAR                    | 5′-GGG RSDNLAR           | 5′-GGG RSDNLAR           |
| GAT               | GGT TSGHLR                     | 5′-GGT TSGHLR            | 5′-GGT TSGHLR            |
| GCA               | GCC DCRDLAR                    | 5′-GCC DCRDLAR           | 5′-GCC DCRDLAR           |
| GCC               | GTC DPGALVR                    | 5′-GTC DPGALVR           | 5′-GTC DPGALVR           |
| GGC               | GTT TSGSLVR                    | 5′-GTT TSGSLVR           | 5′-GTT TSGSLVR           |
| GGG               | GTG RSDLSR                     | 5′-GTG RSDLSR            | 5′-GTG RSDLSR            |
| GGT               | GAG RSDNLAR                    | 5′-GAG RSDNLAR           | 5′-GAG RSDNLAR           |
| GTC               | GAG RSDNLAR                    | 5′-GAG RSDNLAR           | 5′-GAG RSDNLAR           |
| GTG               | GAG RSDNLAR                    | 5′-GAG RSDNLAR           | 5′-GAG RSDNLAR           |
| GCT               | GAG RSDNLAR                    | 5′-GAG RSDNLAR           | 5′-GAG RSDNLAR           |
| GGT               | GAG RSDNLAR                    | 5′-GAG RSDNLAR           | 5′-GAG RSDNLAR           |
| ACT               | CAA GTG TAC                      | 5′-CAA GTG TAC           | 5′-CAA GTG TAC           |
| AGA               | CAG TAC                        | 5′-CAG TAC              | 5′-CAG TAC              |
| AGG               | CAG TAC                        | 5′-CAG TAC              | 5′-CAG TAC              |
| ATG               | CAG TAC                        | 5′-CAG TAC              | 5′-CAG TAC              |
| CGA               | CAG TAC                        | 5′-CAG TAC              | 5′-CAG TAC              |
| CCA               | CAG TAC                        | 5′-CAG TAC              | 5′-CAG TAC              |
| CTG               | CAG TAC                        | 5′-CAG TAC              | 5′-CAG TAC              |
| TGA               | CAG TAC                        | 5′-CAG TAC              | 5′-CAG TAC              |
| TGG               | CAG TAC                        | 5′-CAG TAC              | 5′-CAG TAC              |

*The capital letters and small letters show same and different nucleotides compared to the zinc-finger-template vector.

**Number of different nucleotides compared to the zinc-finger-template vector.

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Construction of ZF Vector by Overlap Extension PCR and TA-cloning (OLTA)

Target sequence and DNA-recognition helices of ZFN against Rosa26 were the same as those reported previously [28], and those against Il2rg were drawn from the report in rat [29] with a change of ZF2 DNA-recognition helices in the left-ZFN from TAG to GCG according to the mouse sequence (GCA GCG TGA GGT TG). Target sites of Gli3 and Cdkn1b were determined by searching the GNN triplet or reported high quality triplet [16] from their sequences. These target sequences were shown in Table 1, and an outline of ZFN vector construction is shown in Figure 1B. All PCR were performed using the primer sets shown in Table 2 on the condition of Table 3. The 3rd PCR products were cloned into the PvuII/BstZ17I digested and 3′-end of digested sites using terminal transferase. The left-side ZFs and the right-side ZFs were cloned into the KEN platform vector and the EL platform vector, respectively, referred to hereafter as the left-ZFN vector and right-ZFN vector, respectively. After transformation of competent cells with these vectors and plating on LB plates, the colonies were subjected to colony PCR using T3-promoter primer as a forward primer and the reverse primer of 3rd PCR to confirm the correct insertion and direction of PCR products. The vectors that have the correct insertion size with true direction of PCR product were sequenced as described above.

In vitro Synthesis of mRNA

For the in vitro synthesis of mRNAs, constructed left-ZFN and right-ZFN vectors were linearized by XhoI and transcribed in vitro with T3-RNA-polymerase (Promega) in the presence of m7G(5′)ppp(5′)G to synthesize capped RNA transcripts. The RNA transcripts were precipitated with absolute ethanol, washed and resuspended in RNase-free water (GIBCO). The mRNA concentration was adjusted to 20 μg/ml or 200 μg/ml and the
left- and right-ZFN mRNAs were mixed as 1:1. The RNA solutions were stored at −80°C until use.

Microinjection of mRNA into Zygote

Following the guidelines for animal experiments at The University of Tokyo, sexually immature female C57BL/6NCr mice (4–5 weeks old) were superovulated by intraperitoneal injection of 7.5 IU eCG followed by 7.5 IU hCG at an interval of 48 h, and mated overnight with C57BL/6NCr male mice that were more than 10 weeks old. Zygotes were collected after 20 h of hCG injection by oviductal flashing, and pronuclei-formed zygotes were put into the M2 medium. Microinjection was performed using microinjector (Narishige) equipped with microscope. Approximately 4 μl of RNA solution were injected into the cytoplasm of each zygote by continuous pneumatic pressure. After injection, all zygotes were cultured in M16 medium for 24 h and subjected for following experiments.

### Table 3. Overlap-extension PCR conditions.

| PCR mixture                                      | PCR conditions          |
|--------------------------------------------------|-------------------------|
| **1st PCR**                                      |                         |
| 5 μl of 10X Ex Taq Buffer                       | 95°C, 2 min             |
| 4 μl of dNTP Mixture (2.5 mM each)              | 95°C, 15 sec X 40 cycles|
| 2.5 μl of 10 μM Forward primer                  | 54°C, 15 sec            |
| 2.5 μl of 10 μM Reverse primer                  | 72°C, 15 sec            |
| 1 μl of 100 μg/ml Template vector               | 72°C, 5 min             |
| 0.5 μl of 5 U/μl Ex Taq                        |                         |
| 34.5 μl of Ultra pure water                     |                         |
| **2nd PCR**                                     |                         |
| 5 μl of 10X Ex Taq Buffer                       | 95°C, 2 min             |
| 4 μl of dNTP Mixture (2.5 mM each)              | 94°C, 30 sec X 15 cycles|
| 0.5 μl of 5 U/μl Ex Taq                        | 68°C, 30 sec            |
| 1st PCR amplicons (10 μg/ml each)               | 68°C, 5 min             |
| 0.5 μl each of ZF1-ZF2 and ZF3-ZF4 amplicons and 1.5 μl of ZF2-3 amplicon (4ZF) |                     |
| or 0.5 μl each of ZF1-ZF2 and ZF4-ZF5 amplicons and 1.5 μl each of ZF2-3 and ZF3-4 amplicons (5ZF) |                |
| or 0.5 μl each of ZF1-ZF2, ZF2-ZF3, ZF4-ZF5 and ZF5-ZF6 amplicons and 1.5 μl of ZF3-4 amplicon (6ZF) |     |
| Adjust to 50 μl with Ultra pure water           |                         |
| **3rd PCR**                                     |                         |
| 5 μl of 10X Ex Taq Buffer                       | 95°C, 2 min             |
| 4 μl of dNTP Mixture (2.5 mM each)              | 94°C, 30 sec X 25 cycles|
| 2.5 μl of 10 μM ZF1 Forward primer             | 68°C, 30 sec            |
| 2.5 μl of 10 μM ZF4 (4ZF), ZF5 (5ZF) or ZF6 (6ZF) Reverse primer | 68°C, 5 min |
| 0.5–1 μl of 2nd PCR reacted mixture            |                         |
| 0.5 μl of 5 U/μl Ex Taq                        |                         |
| Adjust to 50 μl with Ultra pure water           |                         |

### Table 4. Genomic PCR conditions.

| PCR mixture                                      | Reaction conditions          |
|--------------------------------------------------|------------------------------|
| 2 μl of 10X Ex Taq Buffer                       | 95°C, 5 min                  |
| 2.4 μl of dNTP Mixture (2.5 mM each)            | 94°C, 30 sec X 40 cycles for 2-cell embryo or X 35 cycles for tale DNA |
| 1.5 μl of 10 μM Forward primer                  | 57°C, 30 sec                 |
| 1.5 μl of 10 μM Reverse primer                  | 72°C, 40 sec                 |
| 10 μl of embryo lysate or 1 μl of tale DNA solution | 72°C, 5 min                  |
| 0.6 μl of 5 U/ml Ex Taq                        | 12 μl of UltraPure Water     |
Table 5. Primer sets for genomic PCR.

| Target gene | Primer sequence          |
|-------------|--------------------------|
| Rosa26      | Forward  5'-ACGTTCCGACTTGAGTTCG  |
|             | Reverse  5'-ATATCCCGGACCGCATCAC  |
| Gli3        | Forward  5'-GTACTTAAGGAGCTGAAACTCA  |
|             | Reverse  5'-ACTAAAGTCTGCCCACCTATAACAC  |
| If2g        | Forward  5'-ATGACTAATACGAAAGGTGCAAG  |
|             | Reverse  5'-TAGGAAAGGTTCAGAAGCCAAAAG  |
| Cdkn1b      | Forward  5'-TCCAGTACACTTGATCCTGGAAG  |
|             | Reverse  5'-CTGTTAGAAGACTGGCCAAG  |

Genomic PCR of Single Embryo

For genome DNA collection, an individual 2-cell embryo was put in 10 µl of Ex Taq buffer (RR001B, TaKaRa), digested with 1 µg/µl of Proteinase K at 60°C for 30 min and heat-inactivated at 95°C for 10 min. The embryo lysate solutions were subjected to PCR on the condition of Table 4 using the primer sets in Table 5. The PCR products were purified by agarose gel electrophoresis, then extracted and sequenced as described above.

Immunoblotting

The micro-western blotting was used for the immunoblotting of the zygotes as described in a previous report [35]. Forty zygotes injected with 200 ng/µl mRNA solutions as described above, and subjected to PCR using the primer sets shown in Table 3. The purified PCR products were incubated at 95°C for 10 min, then cooled to 85°C at −2°C/sec and to 25°C at −0.5°C/sec for annealing of intact and mutated DNA strands. The re-annealed products were incubated with 2 U of T7 endonuclease I at 37°C for 3 h, then subjected to agarose gel electrophoresis.

Embryo Transfer and Genotyping of Pups

Two-cell embryos injected with 20 µg/ml ZFN mRNA solutions were transferred into the oviductal ampullas (10–17 embryos per oviduct) of 8-week-old female ICR mouse mated the previous night by vasectomy mated ICR males. After birth, approximately 1 mm of tail tips were obtained from the 4-day-old pups. Genome DNA was extracted from the tail tips and subjected to PCR on the condition of Table 4 using the primers shown in Table 5. PCR products were purified by agarose gel electrophoresis, and the extracted fragments were directly sequenced as described above.

Results

Construction of ZFNs by OLTA

At first, we examined whether the intended ZF could be produced efficiently by overlap PCR utilizing DNA-recognition helices as overlap regions. Short PCR primers consisting of a 21-bp DNA-recognition helix reported previously [16] and an 8-bp ZF common region were designed (Table 2) and partial ZF fragments extending from the DNA-recognition helix to the next DNA-recognition helix, were synthesized by 1st PCR using ZF-template vector and a forward primer (Fw) of one DNA-recognition helix and a reverse primer (Rv) of the next DNA-recognition helix. For the production of Gli3 left-ZFN, for example, GCC-Fw and TGG-Rv, TGG-Fw and CTG-Rv, and CTG-Fw and GAG-Rv were used as primer sets and 5 partial ZF fragments of ZF1-ZF2, ZF2-ZF3 and ZF3-ZF4 were synthesized. As shown in Figure 2A, 1st PCR, all partial ZF fragments were successfully synthesized and extracted from the PCR primer sets shown in Table 2. The 1st PCR products were purified by agarose gel electrophoresis and extracted, and then the 3–5 partial ZF fragments (equivalent to 4–6 ZFs) were subjected to a 2nd PCR without a PCR primer (overlap extension PCR) in order to elongate the PCR products. A 3rd PCR was performed using diluted whole 2nd PCR products without purification and extraction, and PCR primers for both ends, for example GCC-Fw and GAG-Rv for Gli3 left-ZFN. Although no obvious band was observed from the 2nd PCR products (Figure 2A, 2nd PCR), the electrophoresis of the 3rd PCR products showed ladder bands including one at the intended molecular weight (Figure 2A, 3rd PCR). The putative intended molecules extracted from the correct bands of the gels were ligated with platform vectors by TA-cloning; then the ligated vectors were mixed with competent cells and plated. These processes were competed within one day as shown in Table 6. On the following day, colony PCR was performed using T3-promoter primer as forward primer and the reverse primer of 3rd PCR, and each four colonies showing the correct molecular weight and correct direction were selected from each plate. As shown in Figure 2B, 1 to 4 of the 4 colonies had the intended ZF sequences after sequencing without any reference to the number of ZFs in each array. These results show that the vectors of ZFNs composed of 4–6 ZFs can be produced efficiently by the combination of overlap PCR, utilizing DNA-recognition helices as overlap regions, and TA-cloning.
Functional Analysis of the Constructed ZFNs

In order to evaluate the site-directed nuclease activity of the constructed ZFNs, ZFN mRNA sets against four different genome loci, *Rosa26*, *Gli3*, *Il2rg* and *Cdkn1b*, were injected into zygotes and the induction of mutations on the target loci were observed after 24 h of injection. First, PCR for the target loci was performed using 10 embryos, and the PCR products were denatured, re-annealed and treated with T7 endonuclease I, which digests the mismatched base pair. As a result, short fragments caused by mismatch digestion were observed in the ZFN mRNA-injected group. The construction and expression of ZFNs are shown in Figure 2. The construction and expression of ZFNs. (A) Three-step PCR for the construction of DNA-binding domain of ZFs. PCR products for each PCR step for 6-finger ZF (left-ZF of *Rosa26*), 5-finger ZF (left-ZF of *Il2rg*), and 4-finger ZF (left-ZF of *Gli3*) are shown. Arrows indicate the intended base pairs of PCR products. (B) Construction efficiencies of left- and right-ZFNs for 4 target loci. (C, D) The expression of constructed ZFN in mouse zygotes. ZFN mRNA at 200 μg/ml (100 μg/ml each for right- and left-ZFNs) were injected into the cytoplasm of mouse zygotes and after 4 h, ZFN protein expression was confirmed by immunoblotting (C) and immunocytochemistry (D) with anti-Flag antibody. Alpha-tubulin immunoblotting is shown as the internal control, and DNA was stained by propidium iodide.

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groups of all target loci (Figure 3), suggesting the digestion of the target loci by the constructed ZFNs. Then, these PCR products were directly sequenced using each forward primer. Microinjection of 200 μg/ml ZFN mRNA against Gli3 and Rosa26 resulted in target-site mutations in higher efficiency than microinjection of 20 μg/ml ZFN mRNA (Table 7), however even in the case of 20 μg/ml ZFN mRNA injection, mutated embryos were present at target loci for all ZFNs (Table 7). These results indicate that all of the constructed ZFNs could function as site-directed endonucleases.

Generation of Site-directed Mutated mice Using the Constructed ZFN

Finally, we examined the toxicity of constructed ZFNs and whether the constructed ZFN vectors were useful for the generation of site-directed mutated mice. About 80% of the embryos injected with water or 20 μg/ml of Gli3, Rosa26 or Il2rg ZFN mRNA developed to become blastocysts (Table 8). However, the embryos injected with Cdkn1b ZFN mRNA developed normally up to the 2-cell stage, but many of them stopped thereafter (Table 8). Agreeing with these results, when the embryos injected with 20 μg/ml of ZFN mRNA were transferred into the oviducts of recipient mice at the 2-cell stage, site-directed mutated mice were obtained in every case other than Cdkn1b ZFN-mRNA injection (Table 9). These results indicate that all of the constructed ZFNs could function as site-directed endonucleases.

Discussion

Although ZFN is a useful tool for site-directed genome modification, the development of useful construction methods that are easy, inexpensive and repeatedly usable for multiple kinds of ZFN should contribute to the further widespread use of this technology. In this study, we established a novel construction method named “OLTA”, in which the intended DNA-binding domains, composed of 4 to 6 ZFs, were synthesized by overlap extension PCR of partial ZF fragments and joined with a nuclease vector by TA cloning. Using this method, we succeeded in constructing beneficial ZFN vectors in a low-cost manner in a short period of time. All ZFNs constructed by OLTA in the present study functioned as site-directed nucleases, and a genetically modified mouse was successfully generated using the constructed ZFN.

The most common construction method for ZFN thus far has been the assembly of ZF modules from a prepared ZF vector library [24,25] or the overlapping of synthetic long oligonucleotides by PCR [16,26]. In the reported overlap-PCR method for ZFN construction, a DNA-binding domain of ZFN was divided into several synthetic long (60 bp) oligonucleotide fragments having overlap sequences in both ends, and these fragments were combined by PCR utilizing the overlap sequences. In this method, each DNA-recognition helix was coded at various positions in each fragment. In order to combine the multiple fragments into a correct order by PCR at once, the overlap sequences of each fragment were selected from different sites of ZF domain. Therefore an oligonucleotide coding one DNA-recognition helix could be used only for a specific position, and as a result, new long oligonucleotides were required each time for changing the ZF position. In contrast, OLTA amplifies common ZF framework by PCR using short (30 bp) primers consisting of a 21-bp DNA-recognition helix and an 8-bp ZF common region, and these partial ZF fragments extending from the DNA-recognition helix to the next DNA-recognition helix are combined by PCR utilizing the overlapped DNA-recognition helix sequences. Therefore, once prepared, the primers corresponding to each DNA triplet can be used repeatedly for the construction of other ZFN vectors without position limitation, in every case of the present study, partial ZF

Table 6. Time schedule for OLTA.

| Procedure                          | Time required (h) |
|------------------------------------|-------------------|
| Day 1                              |                   |
| 1st PCR                            | 1                 |
| Gel purification                   | 1                 |
| 2nd PCR                            | 1                 |
| 3rd PCR                            | 1                 |
| Gel purification                   | 1.5               |
| Ligation                           | 0.5–1             |
| Transformation, Plating            | 0.5               |
| Day 2                              |                   |
| Colony PCR                         | 2                 |
| Gel electrophoresis                | 1                 |
| Culture of candidate colonies      | O/N*              |
| Day 3                              |                   |
| Plasmid DNA isolation              | 1                 |
| Sequencing reaction                | 6                 |

*Overnight.

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Figure 3. Functional analysis of the constructed ZFNs in preimplantation mouse embryos. Each of ten 2-cell embryos injected with 20 μg/ml of ZFN mRNAs (10 μg/ml each for right- and left-ZFNs) were subjected to T7 endonuclease I assay. Arrows indicate the mismatch-digestion fragments derived from ZFN-induced genome mutation at the target loci. The other fragments digested at the probeable SNP sites are indicated by arrowheads.

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fragments were successfully synthesized precisely by the 1st PCR using each set of primers shown in Table 2 (Figure 2A). This result indicates that partial ZF fragments including various types of DNA recognition helices can be synthesized by the present PCR-primer conditions; the number of nucleotide differences with the template vector are 11 or less and 8 bp of 3'-end are completely complementary. Although only 21 kinds of DNA-recognition helices were synthesized in this study, more than 46 other kinds of DNA-recognition helices specific for various DNA triplets have been reported to date [16,17]. All of these helices can be expected to be synthesized by OLTA, because their primer sets satisfy the above-mentioned PCR-primer conditions. Therefore, OLTA should be considered as a versatile and powerful method for ZFN construction.

It is highly conceivable that the numerical difference in ZFs have affected the ZFN recruiting efficiency for the correct position and mutation rates of endogenous target genome loci. The method of ZF-module assembly has the merit to combine ZFNs without number limitation at least in principle. However, this method using each set of primers shown in Table 2 (Figure 2A). This result indicates that partial ZF fragments including various types of DNA recognition helices can be synthesized by the present PCR-primer conditions; the number of nucleotide differences with the template vector are 11 or less and 8 bp of 3'-end are completely complementary. Although only 21 kinds of DNA-recognition helices were synthesized in this study, more than 46 other kinds of DNA-recognition helices specific for various DNA triplets have been reported to date [16,17]. All of these helices can be expected to be synthesized by OLTA, because their primer sets satisfy the above-mentioned PCR-primer conditions. Therefore, OLTA should be considered as a versatile and powerful method for ZFN construction.

Table 7. ZFN-induced site-directed mutations in mouse 2-cell embryos.

| Target gene | ZFN concentration (μg/ml) | No. of embryos | Sequence of target site* | Modification (S, D, I)** | Number of embryos |
|-------------|--------------------------|----------------|------------------------|-------------------------|------------------|
|             | Examined Mutated         |                |                        |                         |                  |
| Gli3        | 200                      | 16 3           | AGGCCCACTCTCTAGGCCGAAG| WT                      | 13               |
|             |                          |                | AGGCCCACTCTCTAGGCCGAAG| 1 S                    | 1                |
|             |                          |                | AGGCCCACTCTCTAGGCCGAAG| 3 D                    | 1                |
|             |                          |                | AGG-------------      | 142 D                   | 1                |
|             | 20 32 3                  |                | AGGCCCACTCTCTAGGCCGAAG| 1 S                    | 29               |
|             |                          |                | AGGCCCACTCTCTAGGCCGAAG| 1 D                    | 1                |
|             |                          |                | AGGCCCACTCTCTAGGCCGAAG| 16D                    | 1                |
|             |                          |                | AGGCCCACTCTCTAGGCCGAAG| 3 D, 2 S               | 1                |
| Rosa26      | 200                      | 9 9 9          | CTGCACTCCAGTCTCTATGAA| WT                      | 0                |
|             |                          |                | CTGCACTCCAGTCTCTATGAA| 3 D                    | 4                |
|             |                          |                | CTGCACTCCAGTCTCTATGAA| 12 D                   | 1                |
|             |                          |                | CTGCACTCCAGTCTCTATGAA| 16 D                   | 1                |
|             |                          |                | CTGCACTCCAGTCTCTATGAA| 23 D                   | 1                |
|             |                          |                | CTGCACTCCAGTCTCTATGAA| 122 D                  | 1                |
|             |                          |                | ---------------------| 146 D                   | 1                |
|             | 20 31 5                  |                | CTGCACTCCAGTCTCTATGAA| WT                      | 26               |
|             |                          |                | CTGCACTCCAGTCTCTATGAA| 2 D                    | 1                |
|             |                          |                | CTGCACTCCAGTCTCTATGAA| 3 D                    | 1                |
|             |                          |                | CTGCACTCCAGTCTCTATGAA| 3 D                    | 2                |
|             |                          |                | CTGCACTCCAGTCTCTATGAA| 7 D                    | 1                |
| Il2rg       | 20                       | 30 4           | ACCAACCCTACGTCGATAGCTA| WT                      | 26               |
|             |                          |                | ACCAACCCTACGTCGATAGCTA| 3 D                    | 1                |
|             |                          |                | ACCAACCCTACGTCGATAGCTA| 4 D                    | 1                |
|             |                          |                | ACCAACCCTACGTCGATAGCTA| 9 D                    | 1                |
|             |                          |                | ACCAACCCTACGTCGATAGCTA| 16 I                   | 1                |
| Cdkn1b      | 20                       | 39 3           | GGTCACACCCGGCCGGAGAGAATGCTCA| WT                  | 36               |
|             |                          |                | GGTCACACCCGGCCGGAGAGAATGCTCA| 1 D                  | 1                |
|             |                          |                | GGTCACACCCGGCCGGAGAGAATGCTCA| 2 D                    | 1                |
|             |                          |                | GGTCACACCCGGCCGGAGAGAATGCTCA| 2 S                    | 1                |

*Under lines indicate the target sites of ZFNs. Small letters and hyphen show the different and deleted nucleotides compared to the WT sequences, respectively.

**S: substitution, D: deletion, I: insertion.

Table 8. In vitro development of ZFN-injected mouse embryos.

| Target gene | No. (%) of embryos |
|-------------|-------------------|
|             | total 2-cell Blastocyst |
| Non-injected| 27 27 (100) 27 (100) |
| Water-injected| 40 38 (95) 32 (80) |
| Gli3        | 165 148 (89.7) 125 (75.8) |
| Rosa26      | 68 63 (92.6) 59 (86.8) |
| Il2rg       | 83 72 (86.7) 64 (77.1) |
| Cdkn1b      | 48 40 (83.3) 11 (22.9) |
Table 9. ZFN-induced site-directed mutations in new-born mouse.

| Target gene | 2-cell embryos transferred | Born | Mutated | Sequence of target site* | Modification (D, I)** | Number of embryos |
|-------------|---------------------------|------|---------|--------------------------|-----------------------|------------------|
| Gil3        | 95                        | 22   | 5       | AGGCCCAACAGCTCTACGGGAGTGAAGGAAGAAGGC | WT                     | 17               |
|             |                           |      |         | AGGCCCAACAGCTCTACGGGAGTGAAGGAAGAAGGC | 1 D                    | 1                |
|             |                           |      |         | AGGCCCAACAGCTCTACGGGAGTGAAGGAAGAAGGC | 1 D                    | 1                |
|             |                           |      |         | AGGCCCAACAGCTCTACGGGAGTGAAGGAAGAAGGC | 2 D                    | 1                |
|             |                           |      |         | AGGCCCAACAGCTCTACGGGAGTGAAGGAAGAAGGC | 5 D                    | 1                |
|             |                           |      |         | AGGCCCAACAGCTCTACGGGAGTGAAGGAAGAAGGC | 2 I                    | 1                |
| Rosa26      | 42                        | 7    | 1       | CTGAAATCAGATTCCCTCTACTAGAGATGCGGGAGAGTCT | WT                     | 6                |
|             |                           |      |         | CTGAAATCAGATTCCCTCTACTAGAGATGAGAGTCT | 5 D                    | 1                |
| Il2rg       | 40                        | 8    | 1       | ACCAACCTACAGCTCCTACCATAGGATGAGAAGGGGA | WT                     | 7                |
|             |                           |      |         | ACCAACCTACAGCTCCTACCATAGGATGAGAAGGGGA | 7 D                    | 1                |
| Cdkn1b      | 61                        | 0    | 0       | GTCGCCACACACCCGGGAGAAGGAAATGCTCA | WT                     | -                |

*Under lines indicate the target sites of ZFNs. Small letters and hyphen show the inserted and deleted nucleotides compared to the WT sequences, respectively.
**D: deletion, I: insertion.

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requires consecutive restriction and ligation reactions, which make this method complex and time consuming. In contrast, ZFN vectors can be synthesized within a single day by OLTA; the construction process is completed within three days even if the transformation and the sequencing of ZFN vectors are included as shown in Table 6. Furthermore, the fact that one month was sufficient for the generation of mice with site-directed mutations, from the construction of the ZFN to the obtaining of pups, indicates an extremely short-term generation of genome-modified animals is possible with OLTA. With regard to the overlapping of long oligonucleotides, ZFNs can be synthesized in a short term as with OLTA [26], but the reported numbers of ZFs in DNA-binding domains have been confined to 3. The past routine for the preparation of a 6-finger protein, for example, was to make 3-finger proteins by overlap PCR and then to ligate the two 3-finger proteins together into a 6-finger protein. In the case of OLTA, at least 5 partial ZF fragments were successfully joined by 2nd and 3rd PCRs. The vector construction efficiencies, 1/4 to 4/8 (25–100%) in OLTA (Figure 2B), were almost the same as those in the previous method of [27] reported previously. Thus, the OLTA method compensates adequately for the weak points of traditional construction methods.

In the present study, all four ZFN sets constructed by OLTA functioned as site-directed nucleases for genome DNA in mouse 2-cell embryos. The most likely explanation for the high ZFN activity on the endogenous target loci in the present study might be the presence of 4 to 6 ZFs in the present ZFN sets instead of 3 ZFs in the previous method. Previous reports studying the effects of ZF numbers on the target recognition efficiencies have shown enhancement of recognition efficiency by more than 4 ZFs [28,36,37]. Further, there are several reports about the direct-mutagenesis of mouse embryonic Rosa26 locus using ZFN that have different numbers of fingers. Meyer et al. generated site-directed mutated mice using ZFN sets that have 4- and 6-fingers for the target sequence the same as us, resulted that 22% of pups showed NHEJ-mediated mutation [4]. On the other hand, Hermann et al. reported that several 3-fingers of ZFN sets designed by OPEN generated only 7.4% of mutated pups [5]. In the present study, 4- and 6-finger ZFN sets against the Rosa26 locus generated 14.3% of mutated pups (Table 9), which efficiency is higher as well as Meyer’s report than Hermann’s. These results may support the hypothesis that the numbers of fingers increase the efficiencies of the mutation induction. Another reason might be the use of relatively high ZFN concentration for the evaluation of ZFN activities. It is well known that ZFNs have off-target effects, non-specific digestions of non-target sites, and this has become a general problem for ZFN experiments [38]. A previous report showed that off-target incidences increase depending on the concentration of ZFN [39]. The culture cells attacked by the off-target effects should be removed from the culture system by the induction of apoptosis even if their target loci were digested correctly. Therefore, the concentration of ZFNs was usually kept as low as possible to exert only the desired effect. On the other hand, we used mouse fertilized embryos that can develop to the 2-cell stage by the help of maternal factors even in the presence of off-target effects so as to evaluate the ZFN activity free of influence from off-target effects. In fact, although ZFNs injected at a concentration of 200 μg/ml showed higher mutation efficiency than those at 20 μg/ml, development stopped at the 2-cell stage and blastocysts were not observed. This failure of oocyes do develop further is most likely due to the off-target effect, by excessive ZFN expression.

The embryos injected with 20 μg/ml, all with the exception of Cdkn1b, ZFN mRNA successfully developed to pup that had mutations at the correct target loci. This result suggests that off-target effects can be evaded by using a 4 or more ZF-containing ZFN mRNA set at a concentration of 20 μg/ml. For the evasion of off-target toxicity of ZFN, another effective solution is thought to increase the number of ZFs in each array and elevating the ZFN specificity. Comparison study using human cells revealed that 3-finger-ZFNs showed off-target cutting at 31 loci whereas 4-finger-ZFNs showed 9 loci [39]. Until now, only 3 to 6 ZF-containing ZFNs have been used for site-directed genome modification, and the efficiency of more than 6 ZF-containing ZFNs has never been reported. One reason for this may have been the difficulty of constructing a long ZFN using conventional methods. In contrast, in the present electrophoretic patterns of the 3rd PCR, bands of longer than 6 ZF were observed (Figure 2A), suggesting that the OLTA method can be adopted for the construction of ZFNs consisting of more than 6 ZFs—although some DNA-binding domains obtained by OLTA in the present study showed incorrect ZF order. It is necessary to examine how many ZFs can be
connected precisely by OLTA and confirm the correlation of ZF domains for transcription factors and nucleases. Nucleic Acids Res 34: W516–523.

Supporting Information

Figure S1 DNA and protein sequences of KK or EL mutation-induced Platform vector. Boxes showed the T3 promoter site and underline showed the XhoI site for linearization.

(DOC)

Figure S2 DNA sequence of a partial ZF sequence of template vector. Boxes showed the DNA recognition helices.

(DOC)

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Author Contributions

Conceived and designed the experiments: WF KN. Performed the experiments: WF KS. Analyzed the data: WF KK. Contributed reagents/materials/analysis tools: WF KK KS. Wrote the paper: WF KN.

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