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—Original Article—

Finding of a highly efficient ZFN pair for *Aqpep* gene functioning in murine zygotes

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Abstract. The generation efficiencies of mutation-induced mice when using engineered zinc-finger nucleases (ZFNs) have been generally 10 to 20% of obtained pups in previous studies. The discovery of high-affinity DNA-binding modules can contribute to the generation of various kinds of novel artificial chromatin-targeting tools, such as zinc-finger acetyltransferases, zinc-finger histone kinases and so on, as well as improvement of reported zinc-finger recombinases and zinc-finger methyltransferases. Here, we report a novel ZFN pair that has a highly efficient mutation-induction ability in murine zygotes. The ZFN pair induced mutations in all obtained mice in the target locus, exon 17 of aminopeptidase Q gene, and almost all of the pups had biallelic mutations. This high efficiency was also shown in the plasmid DNA transfected in a cultured human cell line. The induced mutations were inherited normally in the next generation. The zinc-finger modules of this ZFN pair are expected to contribute to the development of novel ZF-attached chromatin-targeting tools.

Key words: Artificial endonuclease, Genome-modified animals, Zinc-finger nuclease (ZFN)

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Engineered zinc-finger nucleases (ZFNs), which consist of a DNA-binding module of multiple C2H2 zinc-finger motifs and a FokI-derived DNA endonuclease unit, can induce a DNA double-strand break (DSB) in any target locus in diverse cell types and living organisms [1–12]. In mice, the induction of ZFNs into zygotes can generate genome-modified mice inexpensively and rapidly, and so far, diverse gene-modified mice have been generated by ZFNs [1–3, 12]. Engineered DNA-binding modules can also be used for a variety of artificial chromatin-targeting proteins by attaching enzymes other than DNA endonucleases. Engineered zinc-finger DNA-binding modules (ZF-binding modules) have already been applied to, for example, zinc-finger recombinases and zinc-finger methyltransferases [13–18]. A recent report on a novel construction method for ZFNs, by which arbitrary ZFNs can be synthesized rapidly, repeatedly and inexpensively [12], suggests a wider application of ZF-binding modules to artificial chromatin-targeting proteins in various research fields.

To generate an artificial chromatin-targeting protein, an enzyme is attached to a DNA-binding module through an appropriate amino acid linker. Several improvements in various areas, such as the length and configuration of the linker and modifications of the enzyme, are considered necessary to obtain suitable molecules [17, 19]. In these trial-and-error processes, the presence of high-affinity DNA-binding modules should be advantageous in obtaining an efficient architecture. Therefore, the discovery of high-affinity ZF-binding modules can contribute to the generation of various kinds of novel artificial chromatin-targeting tools, such as ZF acetyltransferases, ZF deacethylases, ZF histone kinases, ZF phosphatases and so on, as well as the improvement of reported zinc-finger recombinases and zinc-finger methyltransferases. However, it is difficult to design a highly efficient ZFN pair *in cellulo* using the information from *in vitro* affinity assays. Previously, the binding of zinc-finger motifs to various triplet sets with high affinity by *in vitro* screening was reported [21–27], but not all ZF-binding modules, which consist of high-affinity zinc-finger motifs, have a high affinity for target DNA sequences *in cellulo*, because of the chromatin state [27–29] or the "context-dependent effect" of the flanking DNA sequences [20]. The mutation-generating efficiencies of regular ZFNs are usually less than 25% of obtained pups [12], and the highest efficiency for a reported ZFN has never exceeded 70%.

In the present study, we report a novel ZFN pair with a highly efficient mutation-induction ability in murine zygotes. We constructed a ZFN set for exon 17 of the mouse 4833403I15Rik gene, known as aminopeptidase Q (Aqpep), and found that all of the obtained pups, which were derived from zygotes injected with the ZFN mRNA, exhibited induced mutations on the target locus. These induced mutations were inherited in the next generation. In addition, the high affinity of the present ZF-binding module pair was compared with that of a previously reported ZF-binding module pair having a high affinity score by a single-strand annealing (SSA) assay using partial eGFP-coding plasmid DNA in order to clarify the "context-dependent effect" of the DNA sequence.

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Materials and Methods

Ethics statement

All animal care and experiments conformed to the Guidelines for Animal Experiments of ahe University of Tokyo and were approved by the Animal Research Committee of the University of Tokyo.

Construction of plasmid DNAs

The left and right ZFN plasmids were constructed by the overlap extension PCR and TA cloning (OLTA) method according to a previous report [12]. Constructed DNA sequences and predicted amino acid sequences are shown in Supplementary Figs. 1 and 2 (online only). For construction of SSA reporter plasmid DNA, two overlapped fragments of eGFP ORF (1 to 601 and 120 to 710) were cloned by PCR using primers (EGxxFP Fw1 and Rv1 and EGxxFP Fw2 and Rv2) shown in Supplementary Table 1 (online only). Each PCR amplicon was joined at the BamHI site and inserted into the EcoRI site of a pCAGGS vector. The forward and reverse target oligonucleotides shown in Supplementary Table 1 (Aqpep insert and Rosa26 insert) were annealed and ligated at the BsmBI site of the plasmid vectors. These vectors were sequenced using a commercial sequencing kit (Applied Biosystems, Foster City, CA, USA) and a DNA sequencer (Applied Biosystems) according to the manufacturer's instructions.

In vitro synthesis of RNA, microinjection and generation of genome-modified mice

ZFN mRNAs were synthesized *in vitro* according to a previous report [12]. Ten micrograms/milliliter of left and right ZFN mRNAs were mixed at a ratio of 1:1, and the mRNA solution (about 4 pl) was microinjected into the cytoplasm of C57BL/6NCr zygotes obtained by natural mating and oviduct flushing. After microinjection, the zygotes were cultured in M16 medium for 24 h, and 10 to 12 embryos that reached the 2-cell stage were transferred into each oviduct of 0.5 dpc pseudopregnant ICR female mice. Pups were obtained by natural childbirth, and their genomic DNAs were extracted from their tails. Mutation induction in the pups was evaluated by direct sequencing of genomic PCR products using each of the 4 forward and 4 reverse primers shown in Supplementary Table 1 as appropriate.

SSA assay using eGFP reporter plasmids

SSA assays using eGFP reporter plasmid DNAs were performed according to previous studies [22, 30] with some modifications. Briefly, 250 ng of reporter plasmid vector and 200 ng of left and right ZFN plasmid vectors were transfected into 1×10^5 of HEK293 cells with Lipofectamine LTX according to the manufacturer's protocol. Forty-eight hours after transfection, confluently grown cells were fixed with 3.5% PFA in PBS, and then the fluorescent signals were observed using a confocal laser scanning microscope. Three rounds of experiments were performed, and obtained fluorescent signals were measured by ImageJ and analyzed statistically by Student's *t*-test.

Results

First, left and right ZFN plasmids on exon 17 of mouse Aqpep were constructed by the OLTA method (Fig. 1, Table 1) [12]. The mRNAs of the ZFN pair were injected into C57BL/6NCr mouse zygotes, and after these embryos were transferred, 16 live pups were obtained. The mutation induction in the obtained pups was assessed by the direct sequencing of genomic PCR products, which revealed that all of the pups had diverse mutation patterns (+4 to –2350) in the target locus (Fig. 2A). Three of the obtained pups were monoallelic and showed a wild-type allele with a mutation allele by PCR direct sequencing, but the other 13 pups showed only mutation alleles that indicated biallelic mutations.

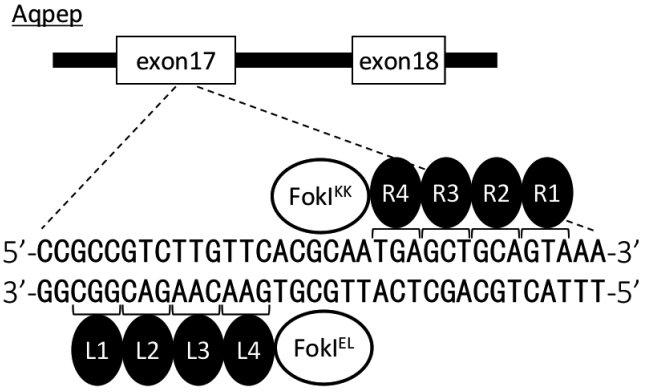

Fig. 1. Schematics of constructed ZFNs and the target DNA sequence. L1–4 and R1–4 indicate each zinc-finger motif.

One male having 5 base deletions and 16 base deletions in each allele, respectively, and one female having the same 2 base deletions in both alleles were mated, and the inheritances of the mutation allele were assessed in the next generation. The F0 mutations were naturally inherited in the next generation, but one unexpected mutation pattern was observed in one F1 pup, indicating mosaicism in germ cells of the male (Fig. 2B). There was no apparent phenotype in F0 and F1 pups.

The binding of zinc-finger proteins to their target DNA is known to be affected by their chromatin states, such as DNA CpG methylation and histone modifications [27–29]. Therefore, in order to clarify whether the high activity level of the present ZFN pair depends on the high affinity of the ZF-binding modules for the target sequences or not, we compared the DSB-induction activity of the present and previous ZFNs for Rosa26 [12], which showed mutation induction in 10–20% of pups [12], by an SSA assay using reporter plasmids with no chromatin modifications for the targets. No signal was observed in the cells transfected with only reporter plasmids (Fig. 3). In contrast, the cells transfected with Aqpep reporter plasmids and the Aqpep-ZFN pair showed significantly higher levels of signaling than the cells transfected with the Rosa26 reporter plasmid and the Rosa26-ZFN pair (Fig. 3). These results suggest that the Aqpep-ZFN pair has a high affinity for the target DNA sequence.

Discussion

In the present study, we found incidentally a novel ZFN pair that could induce an indel mutation with extremely high efficiency in a target locus in mouse zygotes. Recently, the CRISPR/Cas system, another kind of engineered endonuclease system, consisting of

Table 1. Amino acid sequences of DNA recognition sites in each zinc-finger motif used in the ZFNs

	DNA recognition sequence	Target triplet DNA
L1	DRSHLTR	GGC
L2	DRSNLTR	GAC
L3	QSGNLTE	CAA
L4	QSGNLAR	GAA
R1	QSSSLVR	GTA
R2	QSGDLTR	GCA
R3	QSSDLTR	GCT
R4	QAGHLAS	TGA

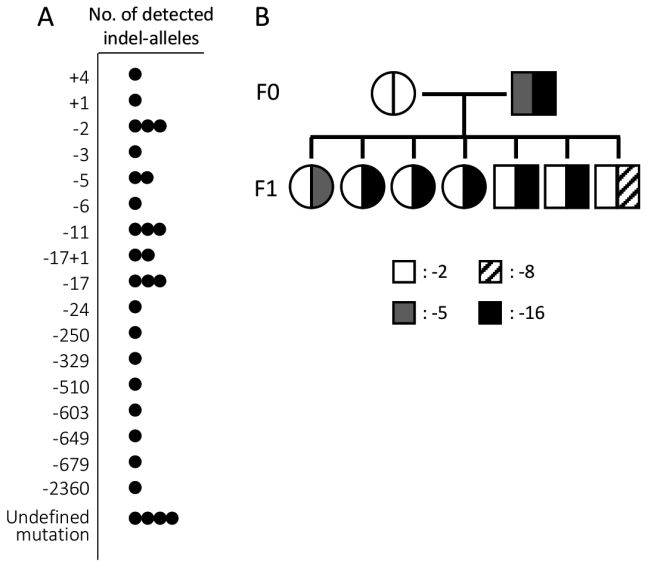

Fig. 2. Generation of mutant mice and their inheritance. (A) Mutation patterns of mutated alleles from 16 obtained pups. The target loci of the pups were amplified by genomic PCR, and the PCR products were sequenced directly. Three pups were monoallelic mutants, and the other 13 were biallelic mutants. Four mutated alleles were not defined by the present PCR analysis. The vertical row indicates the number of inserted (+) or deleted (–) bases in each allele, and the horizontal row indicates the number of alleles having the indicated mutations. (B) Inheritance analysis of the mutations. A female having 2 base deletions in both alleles and a male having 5 base deletions and 16 base deletions in each allele were used as F0 mice. F0 mutations were naturally inherited in all F1 pups, but one unexpected mutation was observed in one pup.

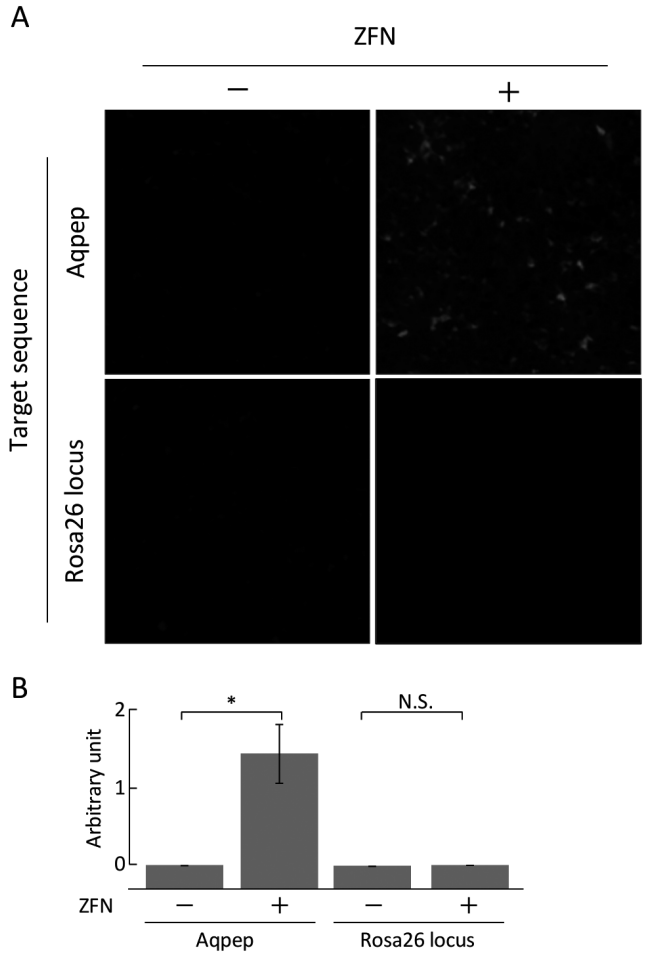

Fig. 3. SSA assay of the Aqpep-ZFN and Rosa 26-ZFN pairs. (A) One of the eGFP reporter plasmids was transfected with or without the corresponding ZFN plasmid into HEK293 cells, and then the fluorescent signals were observed after 48 h. (B) Three rounds of experiments were performed, and the fluorescent signals were analyzed by ImageJ. The results are shown as the mean + SD. * Significant difference ($P < 0.05$). N.S., no significant difference.

guideRNA, a DNA-binding RNA module, and Cas9, a specific DNA endonuclease binding with guideRNA, was also found to be applicable for the generation of gene-modified animals [31–37]. The CRISPR/Cas system can induce indel mutations in target loci of almost all obtained pups by microinjection of guideRNA and Cas9 mRNA into zygotes in mice and rats [31–37]. The mutation generation efficiency of the ZFN pair found in the present study was comparable to that of the CRISPR/Cas system, showing the high possibility of ZFNs but in only some limited cases. We also showed the inheritance of the mutations induced by the present ZFN pair in the next generation and the presence of mosaicism in one of the pups. These characteristics are also the same as those reported for CRISPR/Cas systems.

Several methods to measure the binding affinity of a zing-finger motif to a DNA triplet *in vitro* have been reported [21–27]. These assays have reported zinc-finger motifs bound to specific DNA triplets with high affinity [21, 22]. However, even though these high-affinity zinc-finger motifs were joined, not all of the ZF-binding modules could bind to the target loci efficiently *in cellulo* [27]. This was attributed to the "context-dependent effect," which is the effect of other nucleotide sequences flanking the target triplet on the binding affinity of the zinc-finger motif [20]. This indicates the difficulty of designing ZFN pairs having high efficiency *in cellulo* by using the information from *in vitro* affinity assays. In fact, a previous study showed the number of quality parameters for each zinc-finger motif for DNA triplet binding [21, 22]. According to the score table, the

total numbers of quality parameters of the present ZFNs, 4 and 10 for the left and right ZF-binding modules, respectively, were not higher but were rather lower than those of our previous Rosa26-ZFNs (7 and 10 for the left and right ZF-binding modules, respectively), which induced mutations in 12.5 to 22.7% of generated pups. This shows clearly that it is necessary to predict the efficiency of ZF-binding modules *in cellulo* with consideration for elements other than the parameters obtained by an *in vitro* affinity assay of a DNA triplet.

The DNA-binding affinities of the modules are expected to be affected by epigenetic modifications such as DNA methylation and histone methylation, phosphorylation, acetylation and sumoylation as well as target DNA sequences. We addressed this issue in the present study with the SSA assay using vector DNAs of the target sequences and revealed that the present Aqpep-ZFN pair had much higher efficiency than the previous Rosa26-ZFN pair, suggesting that the high efficiency is due mainly to the affinity to the target DNA sequence. Although this result does not exclude the influence of epigenetic modifications on ZFN efficiency, the importance of the context-dependent effect of the DNA sequence for the high affinity of the ZF-binding modules was indicated.

The transcription activator-like effector nuclease (TALEN) is another kind of engineered endonuclease system having the same FokI endonuclease unit as ZFN, and it is also applied to the generation of gene-modified animals [38, 39]. The transcription activator-like effector, a DNA-binding module of TALEN, has been reported to have almost the same DNA-binding affinity as ZF-binding modules, but it is known to have no bothersome context effect [40]. However, each unit of the transcription activator-like effector consists of 33–35 amino acids that recognize only one base, whereas each zinc-finger motif consists of fewer than 30 amino acids and can recognize triplet bases; therefore a ZF-binding module can target a much longer DNA sequence than a transcription activator-like effector having the same molecular weight. In addition, it takes less labor to construct ZF-binding modules than transcription activator-like effector modules. Artificial chromatin-targeting enzymes other than endonucleases, such as methylase, recombinase and acetylase, have been reported using only zinc-finger motifs as DNA-binding modules [13–19]. Recently a novel construction method for ZFNs, by which arbitrary ZFNs can be synthesized rapidly, repeatedly and inexpensively, has been reported [12]. This method should accelerate the use of ZF-binding modules in a variety of fields. These enzymes are attached to the DNA-binding module through appropriate linker amino acids, and the length of a linker sequence should be crucial to the enzyme activity. Since the ZF-binding module pair we reported here is certain to bind to the target sequence, ZF-containing enzymes could be modified or improved without the need to consider DNA-binding affinity if the present ZF-binding module is used. This module is expected to contribute significantly to further development of artificial chromatin-targeting proteins.

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