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Sandwiched zinc-finger nucleases demonstrating higher homologous recombination rates than conventional zinc-finger nucleases in mammalian cells

Tomoaki Mori, Koichi Mori, Takamasa Tobimatsu, Takashi Sera *

Department of Applied Chemistry and Biotechnology, Graduate School of Natural Science and Technology, Okayama University, Tsushima-Naka, Kita-ku, Okayama 700-8530, Japan

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ABSTRACT

We previously reported that our sandwiched zinc-finger nucleases (ZFNs), in which a DNA cleavage domain is inserted between two artificial zinc-finger proteins, cleave their target DNA much more efficiently than conventional ZFNs in vitro. In the present study, we compared DNA cleaving efficiencies of a sandwiched ZFN with those of its corresponding conventional ZFN in mammalian cells. Using a plasmid-based single-strand annealing reporter assay in HEK293 cells, we confirmed that the sandwiched ZFN induced homologous recombination more efficiently than the conventional ZFN; reporter activation by the sandwiched ZFN was more than eight times that of the conventional one. Western blot analysis showed that the sandwiched ZFN was expressed less frequently than the conventional ZFN, indicating that the greater DNA-cleaving activity of the sandwiched ZFN was not due to higher expression of the sandwiched ZFN. Furthermore, an MTT assay demonstrated that the sandwiched ZFN did not have any significant cytotoxicity under the DNA-cleavage conditions. Thus, because our sandwiched ZFN cleaved more efficiently than its corresponding conventional ZFN in HEK293 cells as well as in vitro, sandwiched ZFNs are expected to serve as an effective molecular tool for genome editing in living cells.

Artificial endonucleases have been developed by protein-engineering techniques including fusion of a DNA-binding protein to a DNA-cleaving enzyme and alteration of target specificities of existing restriction enzymes.[1–4] Among them, zinc-finger nucleases (ZFNs) have been demonstrated to efficiently cleave their target DNA in vitro and in vivo, allowing manipulation of genetic information.[5,6] One concern about in vivo applications is that off-target cleavage may cause cytotoxicity because ZFNs can form a homodimer as well as a heterodimer via their FokI catalytic domains. To reduce off-target cleavage, two groups have engineered a FokI-dimer interface so that the engineered ZFN preferentially formed a heterodimer.[7,8] However, because conventional ZFNs bind to both dsDNA substrates and cleavage products with equal affinities, they do not cleave target DNA with multiple turnovers differently from native restriction endonucleases.

To solve this problem, we previously developed sandwiched ZFNs[9–11] as novel ZFNs. The sandwiched ZFNs uniquely harbor a single-chain FokI dimer (designated scFokI[12]) sandwiched between two artificial zinc-finger proteins (AZPs[13]). Due to the unique molecular structure, our sandwiched ZFNs cleave target DNA with multiple turnovers like native restriction endonucleases. Because sandwiched ZFNs cleave the regions between two AZP-binding sites, dissociation constants of sandwiched ZFNs for DNA cleavage products are significantly increased. Therefore, sandwiched ZFNs are able to revisit and cleave their DNA substrates again, leading to multiple-turnover DNA cleavage (see Ref. 9 for more detailed explanation). Actually, sandwiched ZFNs demonstrated higher DNA-cleavage rates than conventional ZFNs in vitro.[9–11] For example, a sandwiched ZFN cleaved a 100-fold excess of its target plasmid completely. However, under the same condition, multiple-turnover cleavage by the corresponding conventional ZFN was not observed; only very faint DNA bands corresponding to a cleavage product were detected in the reactions with the conventional ZFN.[10]

In the present study, we examined whether our sandwiched ZFN cleaved target DNA more efficiently than its corresponding conventional ZFN in mammalian cells as well as in vitro. To this end, we used a plasmid-based single-strand annealing (SSA) reporter assay in HEK293 cells. Briefly, a gene encoding luciferase was divided into two segments, separated by a stop codon and a ZFN target site (Fig. 1A). Both segments contained a homologous 881-bp region in the direct repeat orientation. A ZFN-induced double-strand break between the segments allows efficient homologous recombination via SSA, resulting in the reconstitution of an active luciferase gene. The luciferase activity measured by an SSA reporter assay is therefore proportional to DNA-cleavage activity of a ZFN. In both the sandwiched and conventional ZFNs, two het-

* Corresponding author. Tel./fax: +81 86 251 8194.
E-mail address: sera@cc.okayama-u.ac.jp (T. Sera).

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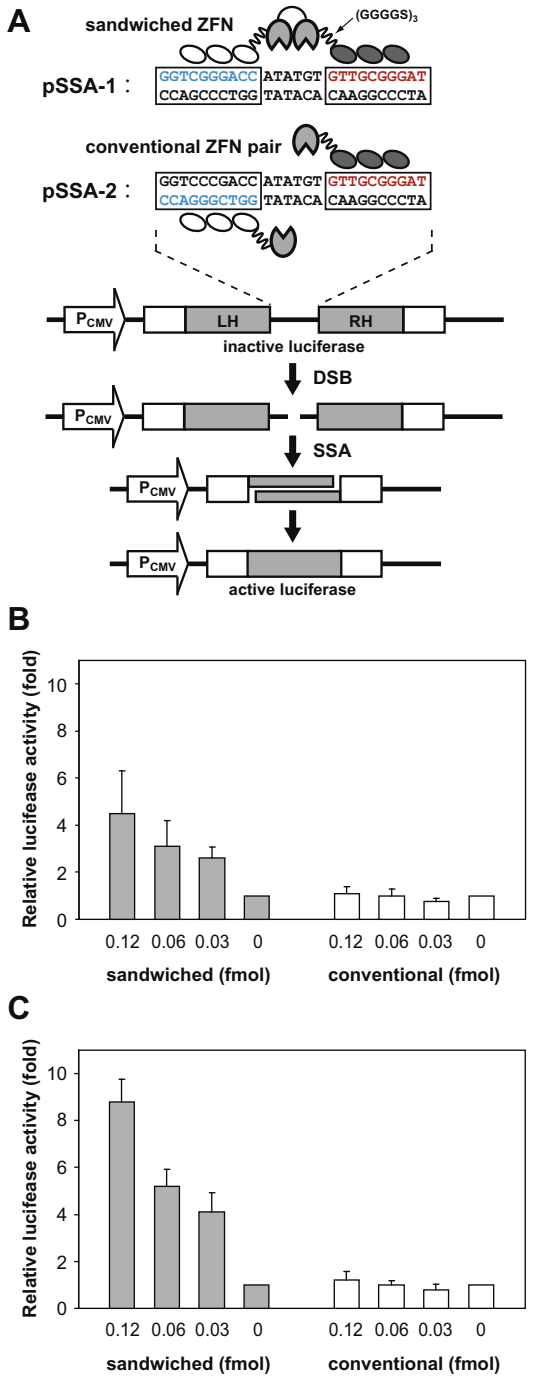

Figure 1. DNA cleavage rates of sandwiched and conventional ZFNs in HEK293 cells by SSA assay. (A) Plasmid-based SSA assay. Plasmids used for the SSA assays and SSA reaction mechanism are illustrated. Single-strand resection of the DNA ends allows the left (LH) and right (RH) homology regions of the split luciferase gene to anneal and recombine to form an active luciferase gene. The sense strands of DNA sequences recognized by each ZFN are shown in color. Relative luciferase activities three (B) or six (C) days after transfection of sandwiched or conventional ZFN-expression plasmid. Amounts of sandwiched or conventional ZFN-expression plasmid used are indicated below each lane. The data represent an average of three independent experiments, and the SD is shown.

erogeneous AZPs, which recognize two 10-bp targets of 5′-GGTCGGGACC-3′ and 5′-GTTGCGGGAT-3′, respectively, were used as the DNA-binding domains. However, the target sites of these ZFNs are different (Fig. 1A): two AZP-binding sites in the target of the sandwiched ZFN reside in the same DNA strand, but each AZP-binding site in that of the conventional ZFN resides in a different DNA strand. Accordingly, the pSSA-1 reporter plasmid was used for the sandwiched ZFN and the pSSA-2 one for the conventional ZFN, as shown in Figure 1A.

HEK293 cells were co-transfected with a ZFN expression plasmid and its SSA reporter plasmid. Luciferase activity was measured at three and six days post-transfection. As shown in Figure 1B, three days after transfection, the sandwiched ZFN activated a luciferase gene much more effectively than the conventional ZFN at all equimolar amounts of transfected ZFN-expression plasmids. For example, in the transfection with 0.12 fmol of a ZFN-expression plasmid, the sandwiched ZFN activated a luciferase gene 4.5 times, while the conventional ZFN activated a luciferase gene only 1.1 times. Furthermore, six days after transfection, the sandwiched ZFN showed greater DNA-cleavage rates than that of the conventional ZFN in HEK293 cells as well (Fig. 1C). The sandwiched ZFN enhanced luciferase gene expression 8.8 times when 0.12 fmol of the expression plasmid was transfected. In contrast, the conventional ZFN enhanced the gene expression only 1.2 times under the same conditions. To further confirm that sandwiched ZFNs have greater abilities of homologous recombination than conventional ZFNs, we additionally constructed two different sandwiched ZFNs (designated 'sandwiched ZFN(2)' and 'sandwiched ZFN(3)'), which targeted 5′-GGGGAGCAGGATATGTTAGGGAGCCC-3′ and 5′-GGAGAAGGACATATGTGTGGGCTTGT-3′ harboring unique AZP-binding sites (underlined), respectively, and their corresponding conventional ZFNs (see Supplementary Fig. S1). We then performed SSA reporter assays as described above. As shown in Supplementary Figure S1, these two sandwiched ZFNs demonstrated higher recombination rates than conventional ZFNs as well.

Western blot analysis of sandwiched and conventional ZFNs revealed that the greater DNA cleavage by the sandwiched ZFN was not caused by higher expression of the sandwiched ZFN in HEK293 cells. Because conventional ZFNs must form a dimer for DNA cleavage at one site, the ratio of signal intensity of the sandwiched ZFN observed in Western blot analysis to that of the conventional ZFN is 1 to 2 when an equal amount of sandwiched ZFN is expressed. As shown in Figure 2, the band intensity of sandwiched ZFN is lower than half of that of the conventional ZFN at both three and six days after transfection. Namely, the expression level of the sandwiched ZFN was lower than that of the conventional ZFN, indicating that the greater DNA-cleaving activity of the sandwiched ZFN was not due to higher expression of the sandwiched ZFN. We also obtained the same or similar results regarding sandwiched ZFN(2) and sandwiched ZFN(3) (see Supplementary Fig. S2).

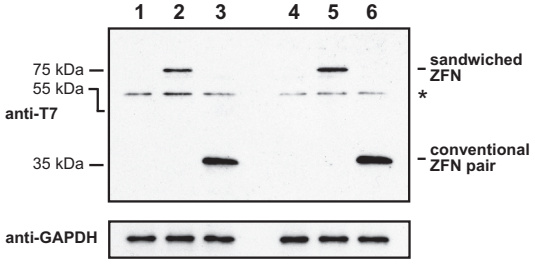

Figure 2. Immunoblots of ZFN derivatives in cell lysates. The molecular weights of sandwiched and conventional ZFNs are 75.8 and 39.2 kDa, respectively. Lane 1, sample from cells (3 days post-transfection) transfected with pcDNA3.1 as a control; lane 2, sample from cells (3 days post-transfection) transfected with sandwiched ZFN; lane 3, sample from cells (3 days post-transfection) transfected with conventional ZFN; lane 4, sample from cells (6 days post-transfection) transfected with pcDNA3.1 as a control; lane 5, sample from cells (6 days post-transfection) transfected with sandwiched ZFN; lane 6, sample from cells (6 days post-transfection) transfected with conventional ZFN. The antibody used for each panel is indicated on the left of each panel. The additional bands indicated with an asterisk in the upper panel are artifacts caused by cross-reactivity of an anti-T7 antibody.