Creation and Biological Application of Intelligent Zinc Finger Proteins

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Zinc finger proteins are abundant in the human proteome and are involved in various functions. Given the biological importance of zinc finger proteins, many artificial zinc finger proteins have been created to improve their function and biological applications. Here, we review previous studies on the redesign and application of artificial zinc finger proteins, focusing on our experimental results. We also discuss the potential biological applications of artificial zinc finger proteins. This review will provide relevant information to researchers working in or interested in the field of engineered zinc finger proteins, which may provide new therapeutic approaches to various diseases.

Key words : Artificial zinc finger, Redesign, Metalloprotein, Zinc finger nuclease

1. Introduction

Zinc finger (ZF) proteins are DNA-binding motifs present in the DNA-binding domains of many transcription factors. Genome analysis of humans and mice has revealed that approximately 10% of proteins encoding genetic information in the whole genome sequence contain ZF motifs, which are universal.[1] In recent years, artificially designed ZF proteins have also attracted attention as a new biological tool, including for gene editing. Each ZF motif consists of approximately 30 amino acids, which are classified as Cys_2His_2, Cys_3His, Cys_4, and Cys_6, according to the amino acid residues (ligands) coordinating to the Zn(II). Among them, the Cys_2His_2-type ZF is a typical structural motif.[2] The two Cys and two His residues coordinate with the Zn(II) in a tetrahedral manner, forming a secondary structure with an inverted parallel β-sheet at the amino group end and α-helix (recognition helix) at the carboxyl group end. In addition, the secondary structural components are compactly folded to form a typical spherical motif (ββα structure), which endows the structure with DNA-binding ability (Fig. 1).

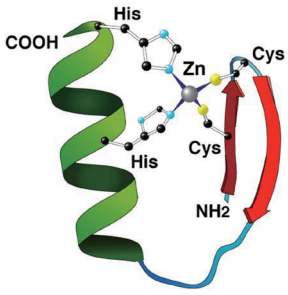

Fig. 1. Schematic structure of single Cys_2His_2-type zinc finger domain displaying ββα fold.

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DNA recognition by Cys_2His_2-type ZFs exhibits the following characteristics: (1) one finger unit recognizes three to four bases (triplet or quadruplet); (2) multiple fingers are typically connected in series via a linker to form a tandem sequence and selectively bind to contiguous DNA sequences; (3) DNA binding with a ZF monomer fashion is possible; (4) asymmetric sequences are recognized. [3) ,4)] The unique structure and DNA-binding recognition ability of ZF proteins are useful for designing artificial ZF proteins with novel functions and are considered an extremely attractive framework for artificial protein design.[4)] Our research has focused on designing functional artificial proteins using ZF motifs. Here, we describe our and other previous studies on artificial ZFs and the prospects for future research.

2. Modification of DNA Recognition Ability of ZFs by Domain Exchange

The binding of ZFs to DNA is generally achieved through the interaction of the α-helix region with the major groove of DNA. In the Cys_2His_2-type ZF, the amino acid residues 1, 2, 3, and 6 at the N-terminus on the α-helix interact with DNA bases to selectively recognize specific DNA sequences.[5)] Mutation of amino acid residues directly involved in sequence-selective DNA binding is an effective approach to modify the DNA recognition mode of wild-type ZF. One of the most effective methods is the phage display method, in which a ZF library is constructed by randomizing amino acid residues involved in DNA recognition, after which ZF proteins that can bind to the target DNA sequence are selected.[6)] Barbas et al. successfully constructed a ZF domain library using a phage display that can accommodate approximately 75% of the 64 sequences that combine all triplet sequences, enabling customization of the artificial ZF proteins that can accommodate the recognition of most DNA sequences.[7)]

The Sp1 ZF binds to the GC sequence; however, in the case of the CF2-II ZF, it binds selectively to the AT sequence. First, circular dichroism spectra were measured to investigate the secondary structure of Sp1HM, which showed a negative Cotton effect around λ= 206 and 222 nm and almost the identical spectra as wild-type Sp1, indicating that the ββα structure is retained in Sp1HM as in the wild-type. Next, the DNA-binding behavior was examined using a gel shift assay. The results showed that Sp1HM bound to DNA containing AT sequences at K_d = 1.3 nM but did not bind to DNA containing GC sequences, which are the binding sequences of wild-type Sp1. These results indicate that the redesigned Sp1HM binds to AT sequences with high affinity and selectivity, with successful modification achieved by domain exchange.

Next, we investigated the effect of exchanging the β-hairpin sites of two different types of ZF proteins on the DNA-binding ability. As described above, the α-helix portion is involved in DNA recognition of ZF proteins by directly binding to the major groove of DNA. Therefore, it is thought that the β-hairpin portion is only involved in tertiary structure formation and is not directly involved in DNA binding. Therefore, modification of the β-hairpin portion when redesigning ZFs has not been widely examined. Thus, we focused on the β-hairpin portion of the Sp1 ZF and GLI ZF and created mutants in which the β-hairpin portion was replaced to investigate the role of the β-hairpin portion in ZF DNA binding.[8)] Interestingly, binding to the GC box was stronger in Sp1(zf23)BG, a mutant of Sp1 with the β-hairpin portion of GLI, than in the ZF of wild-type Sp1. In contrast, the GLI mutant GLI(zf45)BS, which contained the β-hairpin portion of Sp1, completely lost its binding affinity to DNA. Therefore, the β-hairpin portion of the ZF may play a major role in DNA binding, and it is possible to change the DNA affinity and sequence selectivity of ZF proteins by rational redesign using domain exchange to design functional artificial ZF proteins.

3. Redesign of Linker Site of ZFs

ZF proteins are typically connected in a tandem manner using a linker consisting of the five amino acids "TGEK(R)P" with two to three conserved finger domains, thereby allowing sequence-selective binding to contiguous DNA sequences.

In general, a ZF protein must recognize and bind to more than 18 bases to locate one specific site in the vast genome sequence. We attempted to expand the DNA base recognition region of ZF proteins by linking the ZF domains of Sp1 (Sp1ZF3).[9] Two or three units of Sp1ZF3 were linked using the conserved linker "TGEKP" to produce the multi-ZF proteins Sp1ZF6 and Sp1ZF9 containing six and nine ZF motifs, respectively (Fig. 2). As mentioned above, Sp1ZF3 binds to nine base pairs enriched in GC bases, known as GC boxes. The DNA-binding behavior of Sp1ZF6 and Sp1ZF9 was examined in a gel shift assay, which confirmed that Sp1ZF6 and Sp1ZF9 selectively bind to 18 and 27 base pairs, corresponding to 2 and 3 GC boxes, respectively. Therefore, multi-fingered by tandem-like binding of ZF units with conserved linkers can bind to longer DNA sequences, indicating that redesigning linker sites effectively extends the contiguous DNA recognition region of ZFs.

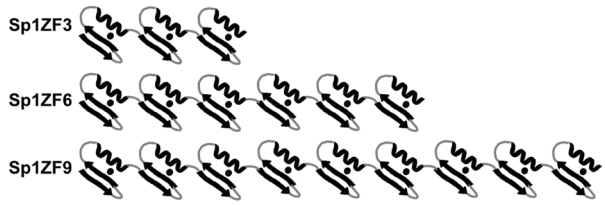
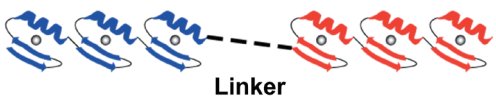


Fig. 2. A schematic of engineered multiplex ZF proteins based on Sp1 ZFs, Sp1ZF6 and Sp1ZF9, with two and three Sp1 ZFs, respectively, connected by a consensus linker.

Next, artificial ZF proteins that can recognize discontinuous DNA sequences were redesigned by connecting Sp1ZF3 to each other with a longer and more flexible glycine linker.[10] Sp1ZF6$(Gly)_7$ and Sp1ZF6$(Gly)_{10}$ with 7 and 10 glycine residues, respectively, were created, and their binding behavior to discontinuous DNA sequences was examined (Fig. 3). The results showed that these 6-ZF proteins could bind to two target sequences, "2GC(10)," which are 10 base pairs apart, corresponding to one helical turn of DNA. Furthermore, the 6-ZF protein induced a curvature between two target DNA sequences by binding to DNA, and the magnitude of the distortion was larger for Sp1ZF6$(Gly)_7$ than for Sp1ZF6$(Gly)_{10}$ and depended on the linker length. Structural changes of DNA are very important in the transcription process, and artificial ZF proteins created by this redesigning method that can bend DNA may be useful in regulating transcription in cells.

Next, we investigated the use of arginine, which is positively charged and has a highly sterically-hindered side chain, as a linker (Fig. 3).[11] The six-ZF protein Sp1ZF6$(Arg)_8$ was prepared by linking Sp1ZF3 with eight consecutive arginine-containing sequences (GRRRRRRRRQ) as a linker, and its DNA-binding properties were compared with those of Sp1ZF6$(Gly)_{10}$, which has the same number of amino acid residues and a flexible linker. Table 1 shows the DNA-binding affinity results. Sp1ZF6$(Gly)_{10}$ exhibited the same affinity for both target sequences, whereas Sp1ZF6$(Arg)_8$ showed the same affinity as Sp1ZF6$(Gly)_{10}$ for the discontinuous target sequence 2GC(10) but a more than 20-fold decrease in affinity for the continuous target sequence 2GC(0). However, a 20-fold decrease in affinity was observed for the consecutive target sequence 2GC(0). Possible reasons for reduced binding of Sp1ZF6$(Arg)_8$ to 2GC(0) include electrostatic and physical repulsion within the linker and inhibition of binding to the GC box sequence due to nonspecific binding of the arginine linker to DNA. Our results suggest that the degree of DNA binding selectivity and affinity can be controlled by appropriately redesigning the structure of the linker moiety.



Consensus	TGEKP	Sp1ZF6
Flexible	Sp1ZF6(Gly)$_n$	Polyglycin
Cationic	Sp1ZF6(GR)$_4$	Polyarginine
	Sp1ZF6(Arg)$_8$	
Functional	Sp1(P1)GLI	Metal-binding loop

Fig. 3. Schematic representation of artificial six-ZFs with various linker structures.

Next, we prepared an artificial nuclease molecule Sp1(P1G)GLI by attaching a functional linker, the cerium-binding peptide sequence P1 (DKDGDGYISAAE), to two different ZFs, Sp1(zf23) and GLI(zf45) (Figs. 3 and 4).[12] Most artificial restriction enzymes are prepared by combining a DNA-binding site with a cleavage site in parallel; this exonuclease type cleaves outside the DNA-binding site. In contrast, Sp1(P1G)GLI introduces a cleavage site peptide sequence between the two DNA-binding sites, the ZFs, so that it can cleave inside the target DNA sequence, resulting in an endonuclease type.

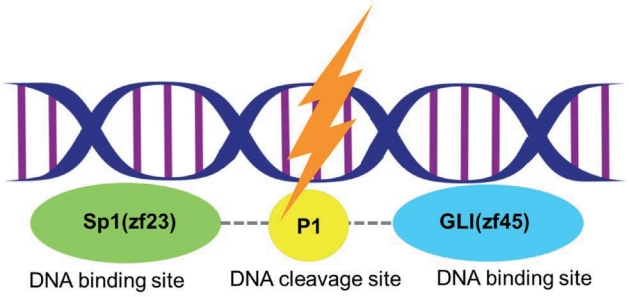


Fig. 4. Schematic diagram of endonuclease-type artificial six-ZF protein with DNA cleavage domain (P1) at the linker site.

DNA cleavage experiments using Sp1(P1G)GLI with the addition of tetravalent cerium ions revealed that it selectively cleaved the DNA region between the DNA-binding sites of the two fingers. Thus, by designing the linker part, the DNA-binding properties of ZF proteins can be changed, and artificial ZF proteins with new functions can be created. Redesign of the linker part is an important target for artificial gene regulation

4. Functionalization of ZF Proteins by Redesign of Metal Coordination Sites

ZF proteins have a DNA-binding function but cannot catalyze biological reactions as observed for enzymes. However, as described above, ZF proteins provide a framework for redesigning functional metallopeptides because they have two precise molecular recognition capabilities: metal binding and DNA binding. Particularly, we are interested in redesigning the zinc ion coordination site to provide ZF proteins with new functions, such as catalysis. Andreini et al. categorized approximately 3000 ZF proteins encoded in the human genome according to their functions and zinc ion binding sequences.[13] The analysis revealed that approximately one-third of human ZF proteins are involved in transcription factors, and most ZF proteins are in this category. Focusing on the amino acid pattern of the zinc ion binding site, the most common coordination site pattern was the Cys_4 type, followed by the Cys_2His_2 type, accounting for approximately 70% of the total (Fig. 5). Unexpectedly, the $CysHis_3$ and His_4 type sequences were absent from human zinc proteins.

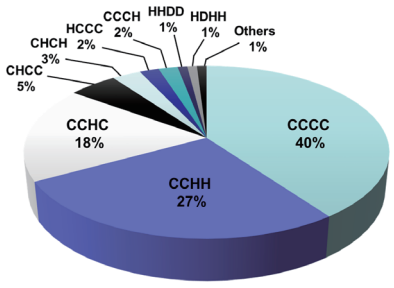


Fig. 5. Ligand-type distribution of metal-binding patterns detected in presumptive human zinc proteins; only four ligand patterns are shown, which cumulatively group approximately 83% of the proteins.

Considering that this sequence does not exist in nature, we predicted that the sequence could be applied for redesigning ZF protein zinc coordination sites. The two Cys residues involved in Zn(II) coordination of wild-type Sp1ZF were mutated to His to create His_4-type artificial ZF protein. (Fig. 6). [14] Circular dichroism and nuclear magnetic resonance spectroscopic analyses showed that each mutant bound to Zn(II) at the same energy as the wild-type, forming a typical ββα structure. Next, we prepared H4Sp1, which consists of three His_4-type ZF domains, and examined its DNA-binding behavior using a gel shift assay and DNase I footprinting. We found that H4Sp1 selectively bound to the GC box, which is the DNA-binding site of wild-type Sp1.

In addition, the hydrolysis reaction of ester compounds was investigated as a possible catalytic function of the non-natural $CysHis_3$ and His_4-type ZF mutants.[15), 16)] 4-Nitrophenyl acetate was used as a substrate for the hydrolysis reaction. The results showed that ZFs with naturally occurring zinc coordination sites did not promote ester hydrolysis, whereas both unnatural $CysHis_3$- and His_4-type ZFs promoted ester hydrolysis (Table. 1). Furthermore, H4Sp1 induced phosphate hydrolysis and DNA sequence-specific cleavage reactions under mild conditions, which are generally considered as difficult (Fig. 6).[17), 18)]

Thus, this successful example of a ZF protein with a catalytic function may explain why $CysHis_3$- and His_4-type zinc-binding sequences were eliminated as components of human proteins during natural evolution.

We further prepared CXHH-type ZF peptides (X = C, H, D, and E) by replacing cysteine (C) in the CCHH-type coordination site with other amino acid residues coordinating zinc, histidine (H), aspartic acid (D), and glutamic acid (E) and studied the effect of ligand substitution on both Zn(II)-binding and DNA-binding properties.[19)] The results showed that it is possible to redesign artificial ZF proteins with various DNA-binding affinities that respond to various concentrations of Zn(II) by substituting Zn(II)-coordinating ligands.

In summary, the redesign of the metal coordination of ZF proteins may enable the creation of artificial enzymes with new functions and the development of artificial transcriptional control systems.

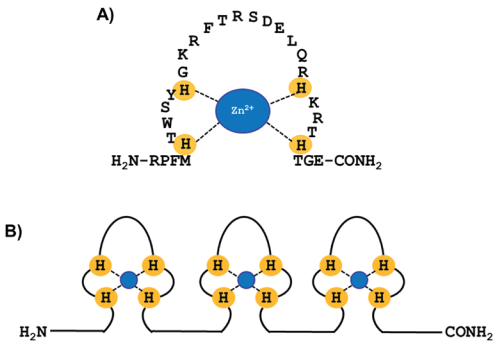


Fig. 6. Artificial H4-type ZF protein: (A) Sequence of the His_4-type ZF domain designed from the second finger of Sp1, (B) Artificial ZF protein H4Sp1 with all three finger domains of Sp1 converted to His_4-type ligand pattern.

Table1. DNA binding affinity of $Sp1ZF6(Gly)_{10}$ and $Sp1ZF6(Arg)_8$

Binding site	K_d (nM)	
	$Sp1ZF6(Gly)_{10}$	$Sp1ZF6(Arg)_8$
2GC(0)	0.96 ± 0.36	71 ± 7.7
2GC(10)	1.5 ± 0.14	3.1 ± 0.94

5. Genome Editing Technology Using Artificial ZF Proteins

Finally, we describe artificial ZF protein technology for genome editing. Genome editing is a technology that uses site-specific nucleases to modify target genes as desired. The major site-specific nucleases are ZF nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered

regularly interspaced short palindromic repeats (CRISPR)/Cas9. These technologies have a much wider range of applications compared to conventional genetic engineering and gene therapy and are being studied intensively.[20] In this section, we introduce ZFNs, which were the focus of the earliest research and development for genome editing.

As mentioned above, the phage display system allows the production of finger motifs that specifically recognize any three bases; by combining these motifs, it may be possible to create artificial ZFs that can bind to any base sequence. Some attempts have been made to "correct" and normalize mutated chromosomes using artificial ZFs for practical use in gene therapy.[21]

Sangamo Bioscience attempted to create an artificial chimeric protein, ZFN, by fusing the DNA cleavage domain of the restriction enzyme *Fok* I to a ZF protein for genome editing (Fig. 7). They successfully altered DNA sequences by inducing homologous recombination between chromosomes and extrachromosomal DNA at specific sites of DNA double-strand breaks on chromosomes by ZFNs. In fact, genome editing using a ZF protein designed to target a mutation site on the interleukin-2 receptor (IL2R) gene that causes X-linked severe combined immunodeficiency, an incurable human disease, resulted in a high frequency of DNA homologous recombination (15–20%) in mutant human cells. As a result, genetically repaired human cells were obtained without selection conditions, and the mutant gene was successfully recombined into the normal sequence.

Since then, numerous studies have been conducted using ZFN-based genome editing,[22), 23)] and this genome editing system is now commercially available and highly versatile. Although CRISPR/Cas9 is currently the mainstream method for genome editing,[24)] ZFNs, which still have high sequence selectivity and diverse functions, are expected to be applied for gene analysis and editing and as novel gene therapy.

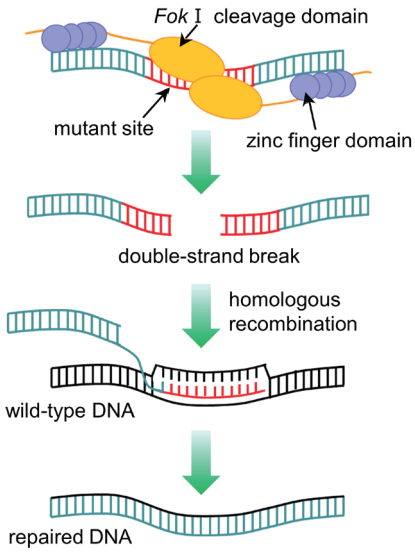


Fig. 7. ZFN is an artificial restriction enzyme consisting of ZF, the DNA recognition and binding domain, and *Fok* I, the DNA cleavage domain, which act in pairs, with *Fok* I forming a homodimer to induce double-stranded DNA breaks (DSB), followed by gene editing by homologous recombination using oligo DNA as the template *Fok* I.

6. Outlook

The findings from numerous studies have led to a comprehensive understanding of the human genome, and the expression of its genes can now be artificially controlled. ZF proteins are involved in various functions that are fundamental to living organisms, such as transcription, development, and differentiation. They are important for further developing artificial ZFs created through redesign in various research areas, such as protein engineering and gene therapy. In addition, research on new functions, such as the interaction of ZF proteins with other biomolecules, is actively being conducted. Combining these studies with artificial protein design research may make it possible to construct more complex artificial biological systems and control more advanced biological functions.

References

1) J. C. Venter, M. D. Adams, E. W. Myers, P. W. Li, R. J. Mural, G. G. Sutton, H. O. Smith, M. Yandell, C. A. Evans, R. A. Holt, J. D. Gocayne, P. Amanatides, R. M. Ballew, D. H. Huson, J. R. Wortman, Q. Zhang, C. D. Kodira, X. H. Zheng, L. Chen, M. Skupski, G. Subramanian, P. D. Thomas, J. Zhang, G. L. Gabor Miklos, C. Nelson, S. Broder, A. G. Clark, J. Nadeau, V. A. McKusick, N. Zinder, A. J. Levine, R. J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, V. Di Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A. E. Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T.J. Heiman, M.E. Higgins, R. R. Ji, Z. Ke, K. A. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G. V. Merkulov, N. Milshina, H. M. Moore, A.K. Naik, V. A. Narayan, B. Neelam, D. Nusskern, D. B. Rusch, S. Salzberg, W. Shao, B. Shue, J. Sun, Z. Wang, A. Wang, X. Wang, J. Wang, M. Wei, R. Wides, C. Xiao, C. Yan, A. Yao, J. Ye, M. Zhan, W. Zhang, H. Zhang, Q. Zhao, L. Zheng, F. Zhong, W. Zhong, S. Zhu, S. Zhao, D. Gilbert, S. Baumhueter, G. Spier, C. Carter, A. Cravchik, T. Woodage, F. Ali, H. An, A. Awe, D. Baldwin, H. Baden, M. Barnstead, I. Barrow, K. Beeson, D. Busam, A. Carver, A. Center, M.L. Cheng, L. Curry, S. Danaher, L. Davenport, R. Desilets, S. Dietz, K. Dodson, L. Doup, S. Ferriera, N. Garg, A. Gluecksmann, B. Hart, J. Haynes, C. Haynes, C. Heiner, S. Hladun, D. Hostin, J. Houck, T. Howland, C. Ibegwam, J. Johnson, F. Kalush, L. Kline, S. Koduru, A. Love, F. Mann, D. May, S. McCawley, T. McIntosh, I. McMullen, M. Moy, L. Moy, B. Murphy, K. Nelson, C. Pfannkoch, E. Pratts, V. Puri, H. Qureshi, M. Reardon, R. Rodriguez, Y.H. Rogers, D. Romblad, B. Ruhfel, R. Scott, C. Sitter, M. Smallwood, E. Stewart, R. Strong, E. Suh, R. Thomas, N.N. Tint, S. Tse, C. Vech, G. Wang, J. Wetter, S. Williams, M. Williams, S. Windsor, E. Winn-Deen, K. Wolfe, J. Zaveri, K. Zaveri, J. F. Abril, R. Guigó, M. J. Campbell, K. V. Sjolander, B. Karlak, A. Kejariwal, H. Mi, B. Lazareva, T. Hatton, A. Narechania, K. Diemer, A. Muruganujan, N. Guo, S. Sato, V. Bafna, S. Istrail, R. Lippert, R. Schwartz, B. Walenz, S. Yooseph, D. Allen, A. Basu, J. Baxendale, L. Blick, M. Caminha, J. Carnes-Stine, P. Caulk, Y.H. Chiang, M. Coyne, C. Dahlke, A. Mays, M. Dombroski, M. Donnelly, D. Ely, S. Esparham, C. Fosler, H. Gire, S. Glanowski, K. Glasser, A. Glodek, M. Gorokhov, K. Graham, B. Gropman, M. Harris, J. Heil, S. Henderson, J. Hoover, D. Jennings, C. Jordan, J. Jordan, J. Kasha, L. Kagan, C. Kraft, A. Levitsky, M. Lewis, X. Liu, J. Lopez, D. Ma, W. Majoros, J. McDaniel, S. Murphy, M. Newman, T. Nguyen, N. Nguyen, M. Nodell, S. Pan, J. Peck, M. Peterson, W. Rowe, R. Sanders, J. Scott, M. Simpson, T. Smith, A. Sprague, T. Stockwell, R. Turner, E. Venter, M. Wang, M. Wen, D. Wu, M. Wu, A. Xia, A. Zandieh, X. Zhu, "The Sequence of the Human Genome", *Science*, **291**, 1304 (2001)..

2) A. Klug, J. W. Schwabe, "Zinc Fingers", *FASEB J.*, **9**, 597 (1995).

3) M. Dhanasekaran, S. Negi, Y. Sugiura, "Designer Zinc Finger Proteins: Tools for Creating Artificial DNA-Binding Functional Proteins", *Acc. Chem. Res.*, **39**, 45 (2006).

4) S. Negi, M. Imanishi, M. Matsumoto, Y. Sugiura, "New Redesigned Zinc-Finger Proteins: Design Strategy and Its Application", *Chem. Eur. J.*, **14**, 3236 (2008).

5) (a) N. P. Pavletich, C. O. Pabo, "Zinc Finger-DNA Recognition: Crystal Structure of a Zif268-DNA Complex at 2.1 Å", *Science*, **252**, 809 (1991). (b) M. Elrod-Erickson, M. A. Rould, L. Nekludova, C. O. Pabo, "Zif268 Protein-DNA Complex Refined at 1.6 A: a Model System for Understanding Zinc Finger-DNA Interactions", *Structure*, **4**, 1171 (1996).

6) (a) E. J. Rebar, C. O. Pabo, "Zinc Finger Phage: Affinity Selection of Fingers with New DNA-Binding Specificities", *Science*, **263**, 671 (1994). (b) Y. Choo, A. Klug, "Selection of DNA Binding Sites for Zinc Fingers Using Rationally Randomized DNA Reveals Coded Interactions", *Proc. Natl. Acad. Sci. U. S. A.*, **91**, 11168 (1994).

7) (a) D. J. Segal, B. Dreier, R. R. Beerli, C. F. Barbas III, "Toward Controlling Gene Expression at Will: Selection and Design of Zinc Finger Domains Recognizing Each of the 5′-GNN-3′ DNA Target Sequences", *Proc. Natl. Acad. Sci. U. S. A.*, **96**, 2758 (1999). (b) J. G. Mandell, C. F. Barbas III, "Zinc Finger Tools: Custom DNA-Binding Domains for Transcription Factors and Nucleases", *Nucleic Acids Res.*, **34**, W516 (2006).

8) Y. Shiraishi, M. Imanishi, T. Morisaki, Y. Sugiura, "Swapping of the β-Hairpin Region Between Sp1 and GLI Zinc Fingers: Significant Role of the β-Hairpin

Region in DNA Binding Properties of C_2H_2-type Zinc Finger Peptides", *Biochemistry*, **44**, 2523 (2005).

9) T. Kamiuchi, E. Abe, M. Imanishi, T. Kaji, M. Nagaoka, Y. Sugiura, "Artificial Nine Zinc-Finger Peptide with 30 Base Pair Binding Sites", *Biochemistry*, **37**, 13827 (1998).
10) M. Imanishi, Y. Hori, M. Nagaoka, Y. Sugiura, "DNA-Bending Finger: Artificial Design of 6-Zinc Finger Peptides with Polyglycine Linker and Induction of DNA Bending", *Biochemistry*, **39**, 4383 (2000).
11) M. Imanishi, W. Yan, T. Morisaki, Y. Sugiura, "An Artificial Six-Zinc Finger Peptide with Polyarginine Linker: Selective Binding to the Discontinuous DNA Sequences", *Biochem. Biophys. Res. Commun.*, **333**, 167 (2005).
12) T. Nakatsukasa, Y. Shiraishi, S. Negi, M. Imanishi, S. Futaki, Y. Sugiura, "Site-Specific DNA Cleavage by Artificial Zinc Finger-Type Nuclease with Cerium-Binding Peptide", *Biochem. Biophys. Res. Commun.*, **330**, 247 (2005).
13) C. Andreini, L. Banci, I. Bertini, A. Rosato, "Counting the Zinc-Proteins Encoded in the Human Genome", *J. Proteome Res.*, **5**, 196 (2006).
14) Y. Hori, K. Suzuki, Y. Okuno, M. Nagaoka, S. Futaki, Y. Sugiura, "Artificial Zinc Finger Peptide Containing a Novel His_4 Domain", *J. Am. Chem. Soc.*, **122**, 7648 (2000).
15) A. Nomura, Y. Sugiura, "Sequence-Selective and Hydrolytic Cleavage of DNA by Zinc Finger Mutants", *Inorg. Chem.*, **43**, 1708 (2004).
16) S. Negi, M. Itazu, M. Imanishi, A. Nomura, Y. Sugiura, "Creation and Characteristics of Unnatural $CysHis_3$-type Zinc Finger Protein", *Biochem. Biophys. Res. Commun.*, **325**, 421 (2004).
17) A. Nomura, Y. Sugiura, "Hydrolytic Reaction by Zinc Finger Mutant Peptides: Successful Redesign of Structural Zinc Sites into Catalytic Zinc Sites", *J. Am. Chem. Soc.*, **126**, 15374 (2004).
18) D. E. Uehling, P. A. Harris, "Recent Progress on MAP Kinase Pathway Inhibitors", *Bioorg. Med. Chem. Lett.*, **25**, 4047 (2015).
19) M. Imanishi, K. Matsumura, S. Tsuji, T. Nakaya, S. Negi, S. Futaki, Y. Sugiura, "Zn(II) Binding and DNA Binding Properties of Ligand-Substituted CXHH-Type Zinc Finger Proteins", *Biochemistry*, **51**, 3342 (2012).
20) W. Nomura, "Development of Toolboxes for Precision Genome/Epigenome Editing and Imaging of Epigenetics", *Chem. Rec.*, **18**, 1717 (2018).
21) F. D. Urnov, J. C. Miller, Y. L. Lee, C. M. Beausejour, J. M. Rock, S. Augustus, A. C. Jamieson, M. H. Porteus, P. D. Gregory, M. C. Holmes, "Highly Efficient Endogenous Human Gene Correction Using Designed Zinc-Finger Nucleases", *Nature*, **435**, 646 (2005).
22) H. R. Jabalameli, H. Zahednasab, A. Karimi-Moghaddam, M. R. Jabalameli, "Zinc Finger Nuclease Technology: Advances and Obstacles in Modelling and Treating Genetic Disorders", *Gene*, **558**, 1 (2015).
23) F. D. Urnov, E. J. Rebar, M. C. Holmes, H. S. Zhang, P. D. Gregory, "Genome Editing with Engineered Zinc Finger Nucleases", *Nat. Rev. Genet*., **11**, 636 (2010).
24) P. D. Hsu, E. S. Lander, F. Zhang, "Development and Applications of CRISPR-Cas9 for Genome Engineering", *Cell*, **157**, 1262 (2014).