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REVIEW ARTICLE

ZINC FINGER NUCLEASES: TAILOR-MADE FOR GENE THERAPY

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SUMMARY

Genome editing with the use of zinc finger nucleases (ZFNs) has been successfully applied to a variety of eukaryotic cells. Furthermore, the proof of concept for this approach has been extended to diverse animal models from Drosophila to mice. Engineered ZFNs are able to specifically target and manipulate disease-causing genes through site-specific double-strand DNA breaks followed by non-homologous end joining or homologous recombination mechanisms. Consequently, this technology has considerable flexibility, which can result in either a gain or loss of function of the targeted gene. In addition to this flexibility, gene therapy with ZFNs may enable persistent long-term gene modification without continuous transfection – a potential advantage over RNA interference or direct gene inhibitors. With systemic viral delivery systems, this gene-editing approach corrected mutant coagulation factor IX in mouse models of hemophilia. Moreover, phase I clinical trials have been initiated with ZFNs in patients with glioblastoma and HIV. Thus, this emerging field has significant promise as a therapeutic strategy for human genetic diseases, infectious diseases and oncology. In this article, we will review recent advances and potential risks in ZFN gene therapy.

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DISCOVERY OF ZINC FINGER NUCLEASES AND THEIR COMPONENTS

Homologous recombination (or homology-directed repair, HDR) is a powerful gene targeting technique in which DNA sequences are transferred from one sister chromosome to another (1). A particular gene may be corrected or inactivated in cells or living organisms by this type of genetic recombination. However, the frequency of HDR, particularly in higher eukaryotic cells, is as low as 1 per million treated cells. Nevertheless, double-strand breaks (DSBs) can stimulate recombination efficiency several thousand-fold, approaching gene targeting frequencies as high as 29% without selection. A method that induces a DSB at a specific site will promote the frequency of gene targeting and potentially have significant therapeutic utility (2-4). The need for such an approach spurred the development of zinc finger nucleases (ZFNs).

Each subunit of the ZFN is composed of three domains: 1) a non-sequence-specific cleavage domain at the *C*-terminal mediated by the restriction enzyme FokI; 2) a DNA-binding zinc finger domain, Cys_2-His_2 (C2H2), at the *N*-terminal, essential for its specificity; and 3) a peptide linker that connects the zinc finger domain with the nuclease. The restriction enzyme FokI (5, 6) induces a DNA double-strand break as a catalytic dimer. Thus, for DNA cleavage to occur, two zinc finger subunits must bind to the gene target sequence in the opposite orientation, leading to FokI dimerization (Fig. 1). In addition to its nuclease activity, effects of engineered zinc fingers on DNA extend to artificial transcription factors (7) and methylases (8).

The zinc finger motif was first discovered in transcription factor IIIA (9) and exhibited specific DNA binding in eukaryotic cells (10, 11). The binding domain of the zinc finger can insert its α-helix into the major groove of DNA in a sequence-specific manner (12, 13). Currently, the C2H2 zinc finger is the most common DNA binding domain in humans, with nearly 1,000 different zinc finger motifs identified in transcription factors. This predominance in nature to bind specifically to DNA sequences provides the framework for their therapeutic use. Moreover, linking different zinc fingers together in a subunit has enabled investigators to design targeted ZFNs specifically to almost

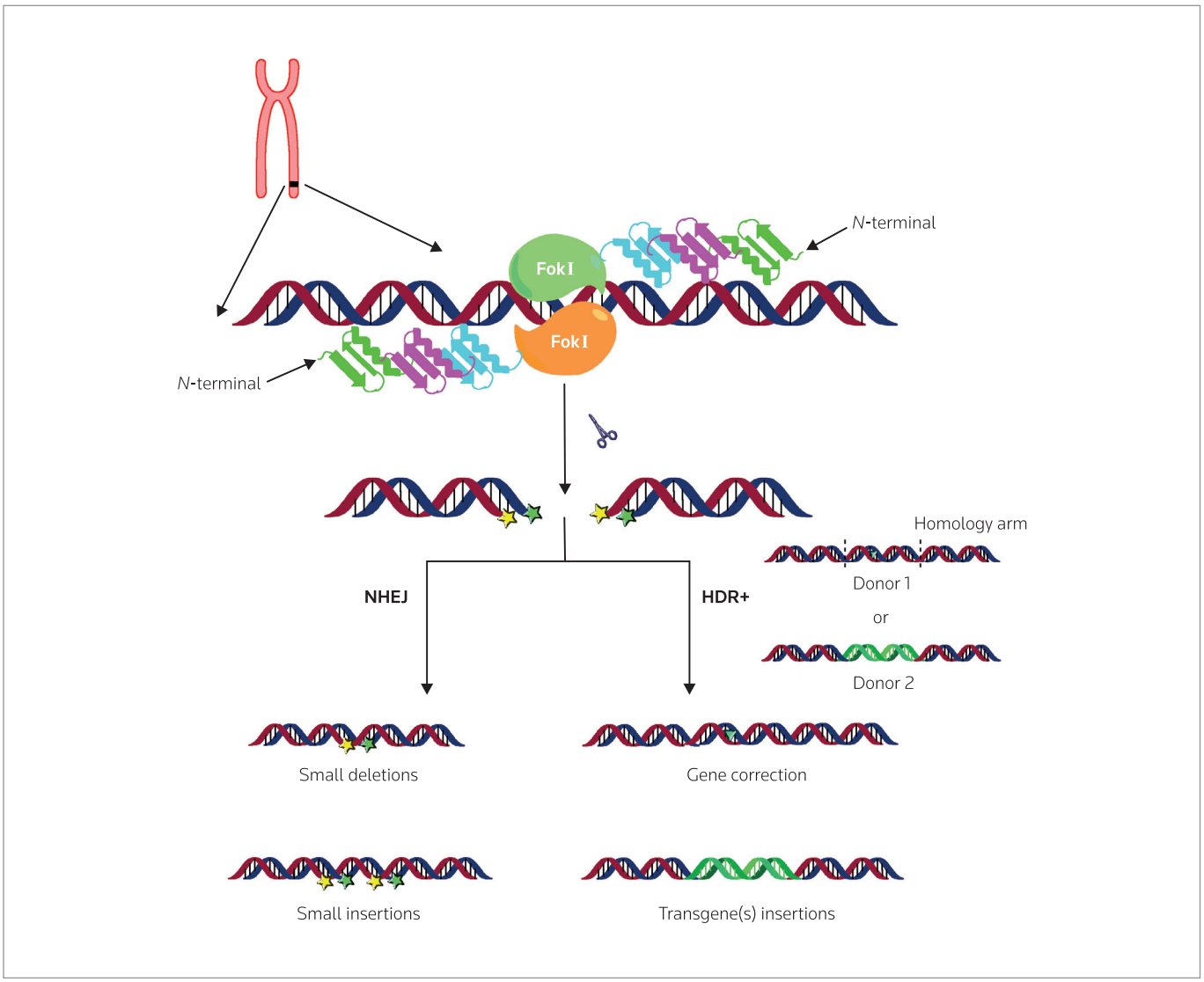

Figure 1. The components and mechanisms of zinc finger nuclease (ZFN). ZFN is composed of a three-finger binding domain and a FokI nuclease cleavage domain that are connected by a linker. Each finger binds to three base pairs. Site-specific double-strand break (DSB) can be induced by ZFN at endogenous loci. The repair process leads to small gene deletions or insertions in the non-homologous end joining (NHEJ) pathway. Corrected gene or transgene (one or multiple) can be integrated into the mutant gene by an alternative mechanism, homology-directed repair (HDR), while coexpressing ZFN and donor DNA.

any gene. Unfortunately, it is not usually possible to link different zinc fingers together based on their three-base pair recognition code, because of interfinger dependence, which may alter the base pair specificity of the zinc finger subunit.

With each finger recognizing about three base pairs, a three-finger subunit of ZFN binds to nine base pairs on the DNA. Typically, 2 ZFN subunits containing 6-12 zinc fingers (or 3-6 zinc finger pairs), respectively, bind to between 18 and 36 nucleotides. Several studies have indicated that ZFNs with a higher number of zinc fingers (four, five and six finger pairs) have increased specificity (14, 15). Comparison of a pair of 3-finger ZFNs and a pair of 4-finger ZFNs detected off-target cleavage in human cells at 31 loci for the 3-finger ZFNs and at 9 loci for the 4-finger ZFNs (15). In some cases, however, the activity of ZFNs may be reduced with five- and six-paired ZFNs compared to three- or four-paired ZFNs (16). Moreover, Guo et al. found that subunit affinity for the DNA sequence was critical in determining ZFN activity and may be more important than the number of fingers, that is, the subunits with lowest and highest binding affinity had reduced activity compared to the subunit with intermediate activity (17).

MECHANISM OF GENE MODIFICATION

The DNA DSB induced by ZFN at endogenous loci can be repaired primarily by two pathways in eukaryotic cells: error-prone non-homologous end joining (NHEJ) or HDR in the presence of a donor DNA (Fig. 1) (18-22). Because small base pair insertions or deletions

can be directly induced by the NHEJ-driven DNA repair process, knockout of specific genes within eukaryotic cells can be readily achieved by this ZFN-mediated approach. More complicated modifications of DNA may also be accomplished by ZFN with the NHEJ pathways. For example, with two pairs of ZFNs, large deletions of 15 megabases occurred efficiently (23). For HDR modifications, single or multiple transgenes can be introduced into the DSB site by co-expression of ZFN and donor DNA. With donor DNA homologous to the sequences flanking the DSB, HDR mediated by ZFN can be quite versatile, including insertion of marker genes, replacement of mutant with wild-type genes, or insertion of different transgenes at the same or different loci on chromosomes. Although the NHEJ pathway can be used in all eukaryotic cells without extensive knowledge of the sequence of the targeted gene, HDR can only be used in eukaryotic cells/organisms in which the gene sequence of the targeted locus is known. The option of utilizing a selection marker as part of donor DNA can be advantageous for HDR, particularly if target modification with zinc fingers is quite low. Moreover, NHEJ occurs primarily in G_1 of the cell cycle, whereas HDR occurs primarily in G_2. Consequently, HDR activity of ZFN was increased by sevenfold in cells treated with vinblastine, which increased the number of cells in G_1 (24). Whether the activity of NHEJ is altered by cell cycle agents that increase transit time or cause G_1 arrest has not been reported. Nevertheless, lower temperatures of 30 °C may increase ZFN activity, presumably by NHEJ, although the mechanism has not been established (25, 26). These post-DSB DNA repair mechanisms have been comprehensively reviewed (27).

SELECTION OF ZFN DOMAIN

Since the DNA binding domain of ZFN is essential for site-specific cleavage, advances in generating ZFN have continued, with six strategies developed thus far. These include modular assembly (12, 28), sequential context-sensitive selection (29-32), bipartite library strategy (33), oligomerized pool engineering (OPEN) (34, 35), context-dependent assembly (CoDA) (36) and the "2 + 2" approach (see Table I) (27, 37, 38). Because the development of ZFNs requires specific libraries and is labor-intensive, several academic laboratories formed the Zinc Finger Consortium and have as their objective the widespread use of ZFN among investigators [e.g., ZiFiTsoftware, screen DNA for potential ZFN (39); specific ZFN protocols (http://www.zincfingers.org/) and plasmids encoding ZFN are available through or linked to this website]. Although ZFN strategies have been extensively discussed, we briefly summarize here a few of these methodologies.

Modular assembly (12, 28) is a parallel selection method that uses a phage display system (40). This is the earliest ZFN approach and identifies a single finger triplet that targets base pair sequences individually from a large zinc finger archive and links them to form zinc finger proteins. Zinc finger domains have been revealed that bind to most sequences of 5′-GNN-3′, 5′-ANN-3′ and 5′-CNN-3′ (14, 41, 42). Although the modular assembly is relatively simple and reveals efficient DNA binding activity in lower eukaryotic cells, some studies showed reduced sequence specificity and binding affinity associated with lower efficacy and higher toxicity, compared to more recently developed approaches (see Table I) (43, 44).

OPEN is a robust and sensitive ZFN design strategy (34). This approach, supported by the Zinc Finger Consortium, involves screening previously characterized ZFN for each target DNA triplet, followed by random polymerase chain reaction (PCR) assembly and selection with the bacterial two-hybrid system. Since three zinc fingers are selected simultaneously, the optimized ZFN binds efficiently to the target gene (34, 35). The ZFNs developed by the OPEN method are highly specific for the target DNA, but this approach is still labor-intensive and requires significant expertise.

Most recently, CoDA has been developed, also by investigators (36) associated with the Zinc Finger Consortium. The group assembled 181 3-zinc finger arrays with known 9-base pair target sites. To obtain the desired ZFN subunit with new DNA specificity, two different zinc finger arrays with a common middle finger have their first fingers and the third fingers exchanged. Nearly 75% of these ZFNs activated the bacterial two-hybrid system more than threefold, indicating a high likelihood for site-specific cleavage. Indeed, when tested in vivo, the success rate per target for obtaining NHEJ-driven mutations with CoDA-generated ZFNs was 50% in zebrafish and plants, which was similar to the success rate of the OPEN-generated ZFNs (36). Compared to the OPEN approach, the CoDA may be slightly less specific, but is significantly easier to develop. Of the methods discussed, this provides the investigator the best opportunity to develop a functional ZFN in a cost-effective manner.

In addition to these strategies, Sangamo Biosciences has developed a proprietary method ("2 + 2") whereby investigators can obtain a tailor-made ZFN either through collaboration or purchasing from Sigma-Aldrich (CompoZr® Custom ZFN Service). Sigma-Aldrich/Sangamo Biosciences has constructed ZFNs that target a large number of different genes. The "2 + 2" method involves two steps in which promising lead four ZFN subunits assembled from an archive of preexisting two-finger units with known DNA specificities are used, with further optimization done with a proprietary algorithm (24) (for review of different approaches see 37). Sigma-Aldrich, through the CompoZr Custom Service, offers an extensive list of ZFN-targeting genes, but the disadvantage of these ZFNs is their cost (www.sigmaaldrich.com/life-science/zinc-finger-nuclease-technology/knockout.html).

CELLS AND MODEL ORGANISMS

We have divided the applications of ZFN into utilitarian- and disease-specific applications. We are aware that such divisions are somewhat arbitrary, since the ZFN methods relevant for non-disease subjects have been applied to human diseases. Moreover, this review primarily examines the importance of ZFN and its application to animal models, but importantly, this technology has also been established in plants, including *Zea mays* (45), tobacco (46) and *Arabidopsis* (47). As the global food crisis has become a serious issue, there is expectation that genetic modification of crop plants with this technology will improve agricultural productivity by enhancing stability of crop yield and tolerance to herbicides.

UTILITARIAN APPLICATIONS

In vitro and in vivo models

CHO cells

NHEJ-mediated target gene knockout is the most common approach in non-primate mammalian cells, by transfection of plasmids containing ZFNs (see Table I). Several genes have been modified by this approach with a frequency up to 30% in CHO cells,

Table I. *Zinc finger nuclease-mediated gene modification.*

Cell line	Target gene	Selection	Mechanism	Transfection	Ref.
In vitro					
CHO cells	*DHFR*	2 + 2	NHEJ	Electroporation	48
	BAK/BAX	2 + 2	NHEJ	Electroporation	50
	DHFR/GLUL/FUT8	2 + 2	NHEJ	Electroporation	49
	FUT8	2 + 2	NHEJ	Electroporation	51
	GLUL/BAK	2 + 2	HDR/NHEJ	Electroporation	57
HEK-293 cells	*IL2RG*	2 + 2	HDR	Lipofectamine/electroporation	24
	HBB/IL2RG/CD8	Modular assembly	HDR	Electroporation	52
	VEGF/HOXB13/CFTR	OPEN	NHEJ	Electroporation	34
	CCR5	2 + 2	NHEJ	Electroporation	54
	CCR5	Modular assembly	NHEJ	Lipofectamine	55
	ERBB2/ BCR-ABL/HIV[§]	Context	HDR	Calcium phosphate precipitation	117
K-562 cells	*IL2RG*	2 + 2	HDR	Lipofectamin/electroporation	24, 53
	VEGF/IL2RG	OPEN	NHEJ	Electroporation	34
	IL2RG	2 + 2	HDR	IDLV	58
Human T cells	*IL2RG*	2 + 2	HDR	Electroporation	24
	CCR5	2 + 2	NHEJ	Electroporation	56
	CXCR4	2 + 2	NHEJ	Ad5/F35	83
Human lymphoblastoid cells	*IL2RG*	2 + 2	HDR	IDLV	58
Mouse ESCs	*H3f3b*	2 + 2	HDR	Electroporation	123
Human ESCs	*IL2RG/CCR5*	2 + 2	HDR	IDLV	58
	OCT4/AAVS1	2 + 2	HDR	Electroporation	59
	PIGA	OPEN	HDR	Electroporation	60
	CCR5	2 + 2	NHEJ	Electroporation	61
Human iPSCs	*PITX3*	2 + 2	HDR	Electroporation	59
	PIG-A	OPEN	HDR	Electroporation	60
	AAVS1	2 + 2	HDR	Electroporation	86
	HBB	OPEN	HDR	Electroporation	84
	HBB	2 + 2	HDR	Electroporation	85
In vivo					
Drosophila	*y*	Modular assembly	NHEJ	Embryonic microinjection	65
	y	Modular assembly	HDR	Embryonic microinjection	66
	ry/bw	Modular assembly	NHEJ/HDR	Embryonic microinjection	67
	Coil/Pask	Modular assembly	NHEJ/HDR	Embryonic microinjection	68
Zebrafish	*kdr*	Context	NHEJ	Embryonic microinjection	32
	ntl	2 + 2	NHEJ	Embryonic microinjection	25
	tfr2/slc6a3/tert/hif1aa/hey2	OPEN	NHEJ	Embryonic microinjection	35
	actn1[¶]	CoDA	NHEJ	Embryonic microinjection	36
Rats	*IgM/Rab38*	2 + 2	NHEJ	Embryonic microinjection	70
	IL2RG	2 + 2	NHEJ	Embryonic microinjection	71
	Mdr1a/Pxr	2 + 2	HDR	Embryonic microinjection	74
Mice	*Mdr1a/Jag1/Notch3*	2 + 2	NHEJ	Embryonic microinjection	72
	Rosa26	2 + 2	HDR	Embryonic microinjection	73
	Ccr5	2 + 2	NHEJ	Electroporation (ex vivo treated human T cells)	56
	Ccr5	2 + 2	NHEJ	Electroporation (ex vivo treated human stem cells)	61
	Cxcr4	2 + 2	NHEJ	Ad5/F35 (ex vivo treated human T cells)	83
	F9	2 + 2	HDR	AAV	87

Continued

Table I. *(Cont.) Zinc finger nuclease-mediated gene modification.*

Organism	Target gene	Selection	Mechanism	ZFN delivery (treatment)	Ref.
In vivo					
Rabbits	*IgM*	2 + 2	HDR	Embryonic microinjection	75
Pigs	*PPARG*	2 + 2	NHEJ	Embryonic microinjection (SCNT)	77
	GGTA1	2 + 2	NHEJ	Electroporation (SCNT)	76
Cattle	*LGB*	2 + 2	NHEJ	Electroporation (SCNT)	78

AAV, adeno-associated virus; Ad5/F35, chimeric adenoviral vector; *BAK*, apoptosis regulator BAK; *BAX*, apoptosis regulator BAX; *CCR5*, chemokine receptor CCR5; *CFTR*, cystic fibrosis transmission conductance regulator; *CXCR4*, chemokine receptor CXCR4; *DHFR*, dihydrofolate reductase; *F9*, coagulation factor IX; *FUT8*, α-(1,6)-fucosyltransferase; *GGTA1*, inactive *N*-acetyllactosaminide alpha-1,3-galactosyltransferase; *GLUL*, glutamine synthetase; *HBB,* beta-globin; HDR, homology-directed repair; *H3f3b*, histone H3.3; *HOXB13*, homeobox protein Hox-B13; IDLV, integrase-defective lentiviral vector; *IL2RG*, IL-2 receptor subunit gamma; *kdr*, kinase insert domain receptor-like; *LGB*, beta-lactoglobulin; NHEJ, error-prone non-homologous end joining; *PIGA*, phosphatidylinositol *N*-acetylglucosaminyltransferase subunit A; *PITX3*, pituitary homeobox 3; *PPARG*, peroxisome proliferator-activated receptor-γ; SCNT, somatic cell nuclear transfer; *VEGF*, vascular endothelial growth factor. §Retroviruses or plasmids containing zinc finger nuclease (ZFN) target sequences for erbB-2, BCR-ABL translocation sequence, or the HIV-1 promoter were introduced into HEK-293 cells. ¶, *actn1* was one of the eight genes targeted in zebrafish that had a success rate of > 1%.

including dihydrofolate reductase (*DHFR*) (48, 49), apoptosis regulator BAK/BAX (*BAK/BAX)* (50), glutamine synthetase (*GLUL*) (49) and α-(1,6)-fucosyltransferase (*FUT8*) (49, 51). In contrast to small interfering RNA (siRNA), transient intraellular increases of ZFN are sufficient to induce permanent knockouts in both alleles.

The NHEJ pathway is independent of the homologous recombination donors, thereby making it the preferred approach for unsequenced genomes, such as those in CHO cells. The simplicity of this mechanism allows the ZFN to generate multiple gene knockouts simultaneously in the same cell. For example, knockout of dual proapoptotic genes (*BAK* and *BAX*) in CHO cells has been shown to increase the viability of the cells while grown in a large-scale bioreactor. Such customized cell lines may be used by the pharmaceutical industry to increase therapeutic peptides/proteins. Table I lists ZFN-mediated gene modification in various eukaryotic cells and organisms.

Non-stem human cells

Although NHEJ or HDR may be used in human cells, only the HDR method has been used to insert donor homologous DNA into specific loci of the chromosome. In addition to different disease targets, NHEJ and HDR methods have been applied to several human somatic cells, including HEK-293 cells (24, 34, 52-55), human chronic myelogenous leukemia K-562 cells (24, 34, 53) and T cells (24, 56).

Enhancement of the efficacy of ZFN technology in vitro and in vivo requires increasing the efficiency of gene targeting. Nonetheless, there have been significant advances in this field. Until recently, insertion of donor DNA was restricted to large arm lengths (~1.5 kb, 0.75 kb/arm), which delayed the experimental output until entire loss of donor plasmid expression. More recently, Orlando et al. (57) demonstrated that ZFN-driven transgene integration with a donor as small as 100 base pairs of chromosomal homology could be generated at a frequency of 10% using a synthetic oligonucleotide. By improving the specificity of the "2 + 2" (24) and engineered OPEN ZFN approaches (34), gene targeting frequency has been enhanced to 20% and 50%, respectively. The success of gene addition in vitro suggests that ZFN approaches may be effective in vivo.

ZFN also has an important role in targeting dynamic biological processes that occur in a cell. Using a ZFN-mediated approach, Doyon et al. fused fluorescent markers in-frame with genes of interest in mammalian cells to study the function and location of proteins essential in clathrin-mediated endocytosis (25). Compared to the randomly inserted overexpressing transgenes, ZFN-mediated genome editing in which the stoichiometry of clathrin light chain A and dynamin 2 is preserved proved important for understanding the dynamics of endocytosis. Indicative of a highly efficient endocytic process, the use of ZFN-modified cells showed that clathrin was downregulated with recruitment of dynamin to vesicles; in contrast, cells in which the gene constructs were randomly inserted showed the likely artifact of co-localization of the two proteins in vesicles. The ability to add a maker and/or to mediate gain or loss of function of genes makes ZFN a very powerful tool for understanding the role of proteins in biological processes (see p53 gain of function in Cancer section below).

Human stem cells

Aberrant human stem cells obtained from genetic heritable disorders are potential candidates for gene editing, as they can differentiate, propagate and restore normal tissue function. Whereas the application of stem cells for gene and cell therapies draws much attention, the lack of precise gene targeting to a disease-linked allele, as well as efficient exogenous transgene integration, have been major challenges to stem cell-based therapies.

Non-dividing human stem cells are difficult to transfect compared to somatic cells. To co-deliver ZFN and donor DNA efficiently into human stem cells, lentivirus has been applied for cell transduction. Based on low-background integration of integrase defective lentiviral vectors (IDLVs), transient expression of ZFN was able to induce site-specific integration. Gene addition at the chemokine receptor CCR5 (*CCR5*) loci had a 5% success rate in $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) (58). In another study with lentivirus, investigators were able to target the safe harbor *AAVS1* locus efficiently with ZFN in both human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) (59). Although IDLVs appear relatively safe, non-specific random integration remains a potential risk.

Electroporation has also been utilized to deliver ZFN to hESCs and hiPSCs. With electroporation, successful gene insertion at the *OCT4* (*POU5F1*) and *PITX3* loci (59) and gene correction of glucosylphosphatidyl inositol-anchored protein (GPI-AP) (60) was achieved in human stem cells. Neither karyotypic abnormalities nor effects on pluripotency were observed in HDR-driven gene addition. Holt et al. have also used electroporation in CD34$^+$ HSPCs to disrupt the *CCR5* gene, with a frequency of 17% (61). Moreover, the use of ZFN to insert a site-specific reporter into stem cells has recently been suggested as a means to study their lineage in disease progression (62). Although ZFN provides an alternative for gene targeting, technical challenges in stem cell biology are significant. For example, cell culture conditions for these cells have an important role in determining the precision and stability of DSB repair (63).

Drosophila melanogaster and Caenorhabditis elegans

Germline and somatic cells of *D. melanogaster* (5) and *C. elegans* (64) were among the earliest models used to study gene disruption with ZFN (see Table I). A group led by Carroll used ZFN to target efficiently several loci of genes in flies by embryonic injection (65-68). The results indicated that ZFNs were capable of inducing mutant offspring from more than half of the parents, and that mutant offspring represented approximately 10% of all offspring. These studies with *Drosophila* also provided greater insight into mechanisms of DNA repair. When different forms of donor DNA (linear vs. circular plasmids) were compared, HDR occurred most efficiently with co-injection of the circular donor plasmid and ZFN mRNA. Notably, Carroll's lab found that in the absence of ligase IV, the frequency of HDR compared to NHEJ increased to nearly 100% (68). A more recent study determined that the synthesis-dependent strand annealing mechanism has a significantly greater role in HDR repair compared to the single-strand annealing mechanism (69).

Embryo injection and cloning: from zebrafish to pigs

The zebrafish is a non-mammalian vertebrate model that has been studied for decades. Because of its transparent embryo and forward genetics, the zebrafish is an ideal candidate for gene modification with engineered ZFN in vivo. Gene disruption in zebrafish has been investigated with mRNA injection of one-cell embryos. Four-finger ZFNs that targeted the golden loci (eye color) generated at least 30% mutant phenotypes in the injected embryo, while ZFNs targeting the no tail (*ntl*) loci generated mutations in the germline with an average frequency of 20%. Another context-dependent bacterial one-hybrid ZFN that targeted vascular endothelial growth factor receptor 2 (VEGFR-2; *KDR*) (32) resulted in insertions or deletions with 20% frequency. Recently, Foley et al. validated the OPEN-engineered ZFN approach in zebrafish by efficiently modifying five genes (*tfr2*, *slc6a3*, *tert*, *hif1aa* and *hey2*) (35).

Injection of embryos with ZFN encoding mRNA can also be applied to mammals such as rats and mice that are genetically closer to humans. Numerous gene knockout rats (*IgM, Rab38* and *Il2rg*) (70, 71) and mice (*Mdr1a*, *Jag1, Notch3*) (72) have been generated through an NHEJ mechanism. By circumventing embryonic stem cells, germline transmission of modified alleles can be accomplished in offspring more efficiently. Before using the ZFN approach, knockout rats were very difficult to generate. More recently, HDR-mediated target integration in rat and mouse embryos has been reported (73, 74). These studies showed that mutant or wild-type animals can be developed by inserting the gene modification into an endogenous locus. Similar ZFN methods targeting the embryonic loci of immunoglobulin M (*IgM*) generated knockout rabbits with deficient serum *IgM* (75); approximately 30% of the founder rabbits had a mutation at the *IgM* locus. In addition, homologous recombination with a neomycin gene was successful in 20% of the fetuses at the *IgM* locus.

Despite the success of injecting ZFN mRNA directly into the embryos of many species, this approach has not been successful with larger domestic animals. Nevertheless, somatic cell nuclear transfer (SCNT) with ZFN has been an effective approach to knockout several genes (*PPARG*, *LGB* and *GGTA1*) in pigs and cattle (76-78). Such knockouts may prove important for human health, as well as for agriculture. For example, *LGB*, which is a major allergen in the milk of cattle, was knocked out by this method with apparently healthy cattle produced. Although inactivation of genes in larger domestic animals has been done with homologous recombination strategies, the approach with SCNT and zinc fingers makes such gene knockouts far more feasible and easy to perform.

SPECIFIC DISEASE-BASED THERAPIES

HIV

One of the major co-receptors for HIV-1 entry, the chemokine CCR5 receptor, has been targeted by several gene-inhibitory approaches, including RNAi (79, 80), anti-CCR ribozymes (81, 82), and most recently by ZFN. The specificity and efficiency of *CCR5*-targeted ZFN has been studied in mammalian cells (55) and human stem cells (58). ZFN-mediated *CCR5* disruption in human CD4$^+$ T cells, which resulted in HIV-1 resistance, was the first demonstration of ZFN as an anti-HIV agent (56). By transplanting ZFN-modified human CD4$^+$ T cells in which more than 50% of the *CCR5* gene was disrupted, the therapeutic potential of these modified cells was validated by marked reduction of viral titers in the plasma 50 days post-HIV infection in a mouse model. These findings have resulted in testing the efficacy of ZFN-targeting the *CCR5* gene ex vivo in three phase I clinical trials (ClinicalTrials.gov Identifiers NCT00842634, NCT01044654 and NCT01252641).

Two recent studies advanced the use of ZFN to enhance the augmentation and duration of natural immunity to HIV-1 infection. The multipotency of human CD34$^+$ HSPCs, which give rise to myeloid and lymphoid cell lines, may create a greater opportunity than the use of T cells for reducing HIV infection. Transplantation of CCR5/ZFN-treated HSPCs showed a persistent and stable HIV-resistant phenotype for more than 10 weeks, indicating long-term control of virus replication and reconstitution of the immune system (61). Nevertheless, CCR5-suppressed T cells are still vulnerable to infection by other strains of the HIV virus. As a result, ZFN targeting the HIV-associated surface receptor chemokine CXCR4 receptor was investigated. CD4$^+$ T cells treated with ZFN targeting CXCR4 were observed to have similar therapeutic effects against CXCR4-tropic HIV without significant loss of cell viability (83). Therefore, the goal to optimize HIV resistance by disruption of both CCR5 and CXCR4 in human T cells appears to be promising and attainable.

Heritable diseases – ex vivo approach

The development of ZFN optimization and selection in human stem cells has resulted in a number of studies targeting aberrant genes of inherited monogenetic disorders with wild-type donor DNA, leading to reversal of disease. Among these studies, ZFN targeting the *IL2RG* locus, important in X-linked severe combined immune deficiency (X-SCID), has been extensively studied. By using ZFN and wild-type donor DNA, Urnov et al. were able to correct 7% of an exon 5 mutation in the *IL2RG* locus of both X chromosomes in K-562 cells; this resulted in normal mRNA and protein levels of *IL2RG* (24). In $CD4^+$ cells, they determined that ZFN-mediated correction of a green fluorescent protein (GFP) mutant located in the *IL2RG* locus occurred at a frequency of 5.3%. These ZFN models targeting the *IL2RG* locus indicate that this strategy may be effective for the treatment of X-SCID patients. By modifying the $CD4^+$ cells before expansion and reinjection of these modified cells, the ZFN approach has advantages over random viral insertion into chromosomes that may result in insertional oncogenesis.

hiPSCs derived from sickle cell anemia (84, 85) and X-linked chronic granulomatous disease (X-CGD) (86) patients have been treated with ZFN to correct disease-causing alleles. Targeted integration of the beta-globin (*HBB*) gene to correct the sickle cell mutation in hiPSCs had success rates of up to 40%, without significant off-target effects. Pluripotency was retained in gene-corrected, transgene-removed hiPSCs. Furthermore, ZFN targeting the safe harbor *AASV1* locus in X-CGD hiPSCs successfully inserted the *gp91* transgene (86). Neutrophils differentiated from these treated X-CGD hiPSCs had functional phenotypes similar to normal neutrophils. Nevertheless, scale-up and transplant of hiPSC-derived cells present challenges before this therapy can be used clinically.

Hemophilia – in vivo approach

Hemophilia B is an X-linked genetic disorder in which the liver produces inadequate levels of coagulation factor IX for clotting. Numerous strategies for preclinical and clinical gene therapy have been tried, most of which have failed because of inadequate, long-lasting production of coagulation factor IX. The ZFN approach has considerable promise for this disease in that the mutant coagulation factor IX loci can be targeted and replaced with the wild-type sequence (87). By using a hepatotropic adeno-associated virus (AAV), the coagulation factor IX-specific ZFN was systemically delivered to a humanized mouse model with coagulation factor IX deficiency. ZFN-driven gene correction specifically replaced the mutated coagulation factor IX gene (*F9*) with a wild-type gene that resulted in persistently restored coagulation function for more than 30 weeks, whereas the control mice in which AAV carrying coagulation factor IX was used had elevated levels for only 6 weeks. In contrast to the ex vivo cell manipulation, which cannot be easily applied to this disease, this study indicates that systemic delivery with ZFN by HDR can effectively target the hepatocyte in vivo. Although this approach was highly specific, the ZFN did have at least one off-target site and AAV was integrated at several sites in the genome. The mice showed no effects on growth or weight over an 8-month period, however. In addition to coagulation factor IX deficiency, this study has important implications for diseases of the liver, from heritable disorders to infectious diseases.

Cancer

Cancer genes and the pathways they regulate have been identified and characterized over the past several decades. Oncogenes and mutant tumor suppressor genes clearly provide an opportunity for the use of a ZFN-mediated approach. Indeed, ZFNs have been used to downregulate specific growth factors or replace *TP53* mutations. In targeting the tumor angiogenic factor vascular endothelial growth factor A (VEGF-A; *VEGFA*), an OPEN-driven ZFN strategy induced gene alterations with 7.7 % and 54% efficiency, respectively, in K-562 cells treated with and without vinblastine (34). Although vinblastine was associated with significant toxicity in these cells, other agents that arrest G_2 may increase ZFN efficacy with less cellular toxicity.

Furthermore, a yeast one-hybrid four-finger ZFN was designed to replace mutant *TP53* with wild-type *TP53* in two cancer cell lines (26). With a liposomal delivery system, the apparent success rate was about 0.1%. Although the homologous recombination events were not particularly effective in this case, modifications at the *TP53* and *VEGFA* loci provide the framework for further investigation. A more efficient delivery system may be more effective than the liposome carriers for these in vitro systems. Alternatively, a more active *TP53*-targeting ZFN would also be attractive, particularly for less efficient but safer liposomal or peptide delivery systems.

In addition to modifying cancer cell genes, investigators have utilized ZFN to augment T-cell-mediated antitumor therapy. The therapy is based on two results: 1) apoptosis is induced by glucocorticoids on cytolytic T lymphocytes (CTLs) of glioblastoma patients (88, 89); and 2) IL-13γ zetakine-expressing CTLs induced significant cell death of glioblastomas in an animal model (90). To prevent apoptosis of "zetakine"-expressing CTLs in patients administered steroids, Reik et al. knocked down the glucocorticoid receptor in the modified CTLs with ZFN (91). Consequently, the cytolytic activity of the "zetakine"-expressing CTLs toward glioblastomas was retained in the presence or absence of treatment with glucocorticoids. This achievement in an animal model was a pioneering study that has now been translated into the clinic (phase I; ClinicalTrials.gov Identifier NCT01082926).

Infectious diseases

Direct inactivation of episomal DNA of infectious viruses is another feasible target. The formation of covalently closed, circular double-stranded DNA (cccDNA) occurs in hepatocytes infected with hepatitis B virus (HBV). Currently, there are no therapies that have focused on targeting cccDNA, which may be one reason why more than 50% of treated hepatitis patients fail therapy. Cradick et al. developed an HBV-specific ZFN that inactivated at least 36% of the episomal DNA and reduced 30% of pregenomic RNA in hepatoma cells transfected with plasmid containing the HBV genome (92). This study provides proof of principle that ZFN induced DSBs in episomal viral DNA, and further reduction is anticipated when viral vectors replace the liposomal carrier that was used in the above study.

The ZFN approach has been suggested as a treatment for malaria. Carried by a cell penetration peptide (CPP), the ZFN has been hypothesized to be delivered to infected red blood cells to target the *Plasmodium* genome (93). This approach may result in functional

gene knockout in the parasite and lead to the death of *Plasmodium* if the delivery system is effective.

ZFN DELIVERY

To manipulate gene expression, an efficient transient transfection agent is required. In many cell culture experiments, electroporation has been widely used for ZFN transfection (Table I). Several less frequently used alternatives have also been used, including adenoviruses, AAVs, IDLVs and Lipofectamine™ 2000. Most of the carriers for ZFN have been used without any obvious unwanted phenotypic changes attributed to them.

Nevertheless, one has to be cautious regardless of the delivery system. For example, although IDLV integrates at rates in chromosomes similar to naked DNA (94), the virus in combination with zinc finger integrates at a discernibly higher rate (95). In addition, enhanced incorporation of exogenous DNA at off-target sites with "naked DNA" has also been reported with agents that promote double-stranded DNA breaks (96, 97). Increased insertion of the donor or vector DNA has not always been observed and this variability may be attributed to the specificity of ZFN and the repair system of the cell (87, 98). If the goal of the investigator is to knock out a gene, then delivery of mRNA encoding ZFN would prevent the off-target integration from the vector DNA. Although ZFN mRNA has only been delivered by microinjection of embryos (25, 68, 99, 100), several non-viral delivery systems (liposomal, electroporation, peptide, hydrodynamic) could easily be adapted to deliver the ZFN mRNA to other cells (101-103).

Three approaches have been developed to exploit ZFN technology in animal models (Fig. 2). First, embryonic microinjection followed by implantation or SCNT has demonstrated effective gene addition in insects, zebrafish and mammals (25, 65, 70, 75, 77). Although these techniques will continue to be utilized to develop important animal models, they are unlikely to be used in humans based on technical and ethical grounds. Second, whereas HIV resistance is observed by autologous ZFN-modified $CD4^+$ T cells and $CD34^+$ HSPCs (56, 61), use of these ZFN-treated cells ex vivo is limited to specific infectious diseases and heritable disorders. Nevertheless, the ex vivo approach has tremendous promise for the treatment or correction of these diseases. Several vectors have been used for ex vivo therapy, including non-integrating lentiviruses and electroporation. Third, for in vivo delivery, AAV is particularly attractive for gene therapy, as demonstrated by the preclinical trial in hemophilia. Nevertheless, integration of AAV in the genome is of concern, although the preclinical study did not reveal an increased insertion of AAV compared to the control AAV without the ZFN construct (87).

While the hydrodynamic delivery method has not been applied to ZFN-directed therapy, the computer-assisted hydrodynamic method is highly efficient and safe for delivery to the liver, and this approach may have significant utility with ZFNs in treating various diseases of the liver (104, 105). Despite the potential utility of hydrodynamic and possibly electroporation approaches, most non-viral methods, such as liposomal or peptide delivery technologies, may not currently be successful for in vivo application of the ZFN technology due to lower transfection levels (106-108). Compared to RNA interference (RNAi) therapies, this is a potential disadvantage of plasmid-based ZFNs since many non-viral delivery carriers of siRNA have been effective in vivo, at least in preclinical trials. That said, there have been significant advances in packaging, targeting and/or extending the circulatory half-life of non-viral nucleic acid carriers (109-114). For instance, dual-targeting nanoparticles have recently been found to be significantly more effective carriers of plasmids compared to single-targeting or the pegylated control formulations (113). These advancements offer the expectation that non-viral carriers, if not now, will in the future be effective carriers of ZFNs for in vivo applications.

SPECIFICITY AND OFF-TARGET EFFECT

As the title of the review article (115) states, "It's all about the specificity." The importance of ZFN as a therapeutic agent is dependent on its specificity, particularly for ex vivo and in vivo applications. Since untoward events occurred in SCID-diseased individuals who developed leukemia as a result of retroviral therapy (116), reduction of off-target effects and increased specificity of ZFN have assumed greater importance. Although increased ZFN specificity is expected to enhance the frequency of gene modification and minimize off-target effects (117), consideration about specificity depends on the method of delivery, the particular target and organism, and the off-target generated. Selection strategies for ZFN that we discussed in the previous section have markedly increased the affinity and specificity of the DNA binding domain to the target sequence.

Several additional studies have exploited approaches aimed at greater specificity. Self-dimerization of wild-type FokI increased off-target effects by potentially increasing the number of DNA binding sites. In order to overcome this problem, creation of FokI variants that function as obligate heterodimers and do not self-dimerize markedly reduced nonspecific, genome-wide cleavage and genotoxicity (118, 119). In addition to the DNA binding and cleavage domain, the composition and length of inter-domain linkers are essential factors of target site selectivity (120). These specificity-related factors should be characterized and screened to select the optimal ZFN.

Besides the intrinsic ZFN binding affinity, the random integration induced by AAV or lentiviral vectors may be problematic, particularly for delivery in vivo. Notably, delivery systems that result in sustained high levels of the ZFN are correlated with nonspecific effects (32, 35). Thus, integrating viruses may not be desirable, and more ideal vectors that provide transient high levels of ZFN are preferable. What defines sufficient levels of ZFN in the nucleus is not totally clear, because the optimal levels of ZFN are likely based on the design of the ZFN, the target chromosomal locus and the cell.

Cell toxicity and genomic assays are commonly done to evaluate the nonspecific targeting of ZFN. Cell toxicity assays have included the MTT reduction assay, cell viability and apoptosis, whereas antibodies to factors (αH2AX, p53BP) associated with DNA repair can identify DSB within a specific time window. As the specificity of ZFN has improved, these assays have become less effective and more sensitive assays have been sought. Several more recent assays have utilized bioinformatics to identify and rank similar DNA binding to the intended target sites in which *pyro*-sequencing or a CEL-1 assay was done to determine unintended DNA incorporation. Although these modalities are useful, they are subject to bias ascertainment. More recently, a genome-wide analysis identified on- and off-target activity by mapping the location of IDLV clustered integration sites (95). Importantly, this study also revealed that off-target site binding and

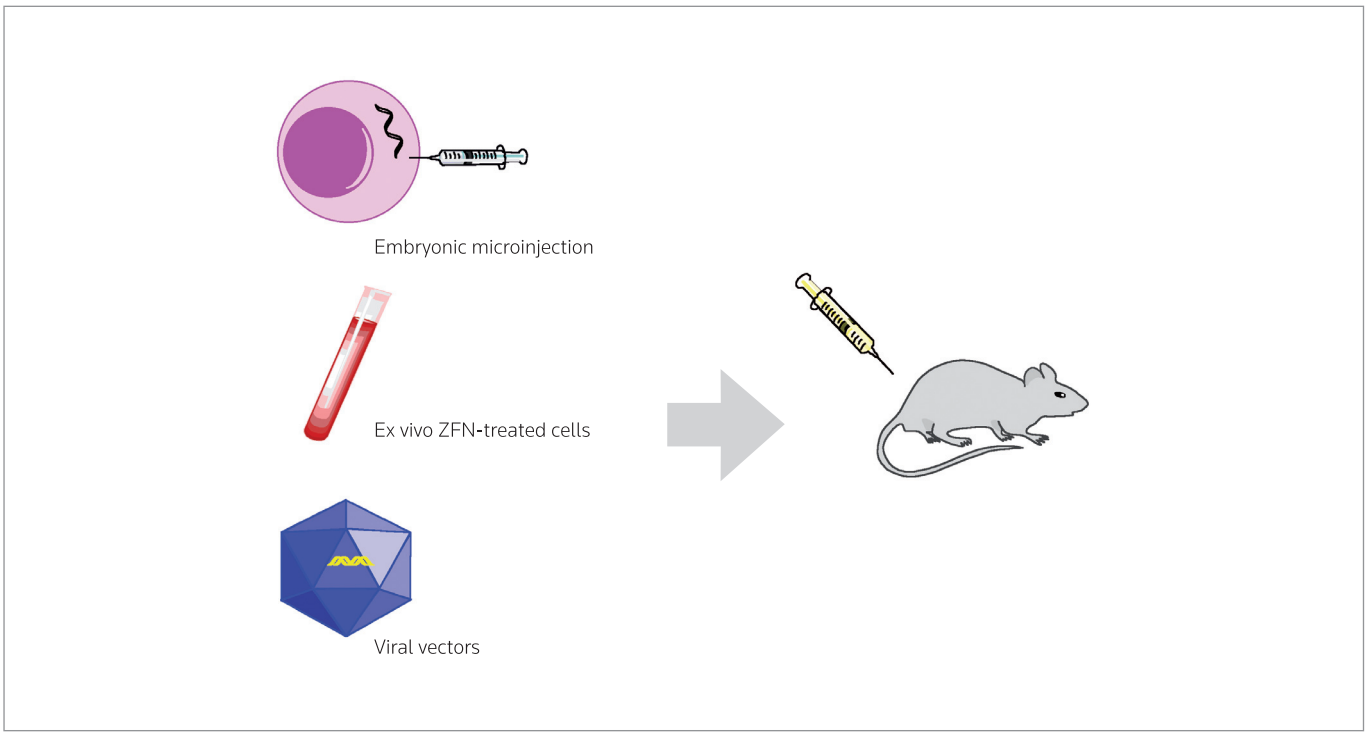

Figure 2. Therapeutic applications of zinc finger nuclease (ZFN). Three general strategies have been used to deliver ZFN in animal models. **Upper**, gene-modified offspring can be generated by directly injecting ZFN-encoded DNA or mRNA into the embryos, followed by implantation in foster mothers. **Middle**, human T cells or stem cells modified by ZFN ex vivo can then be transplanted into an animal model. **Lower**, ZFN can be delivered to the target cells to correct mutant genes by using adeno-associated virus (AAV) as a vector.

cleavage by ZFN predicted in silico did not correlate with the actual cleavage site in vivo.

Besides lack of specificity, toxicity can occur due to accumulation of ZFN protein in a concentration-dependent manner (121). By linking the *N*-terminus of ZFN to a destabilization ubiquitin moiety or a modified FKBP12 protein, respectively, the ZFN protein level can be regulated by a proteasome inhibitor or small blocking molecule. As a result, cytotoxicity was significantly reduced in several cell lines without loss of gene targeting efficacy (122). Another potential problem is an immune reaction to the bacteria-derived FokI that may occur with repeated injections in mammalian species.

CONCLUSION

The low frequency of homologous recombination in cells has presented a significant obstacle to modify until the emergence of ZFN. The proficiency of precise gene modification in plants, insects and mammals, as well as in human cells, has bolstered the use of ZFN in a variety of applications. The difficulty of site-specific ZFN selection and optimization has been at least partially overcome by the development and validation of ZFN engineering. Several challenges remain for further study to promote the use of this approach:

- In contrast to RNAi methods, the ZFN technology cannot be readily used by many laboratories to silence specific targets. As a result, continued expansion of a publicly available database of ZFNs and plasmid constructs, covering genes of particular interest to investigators, is required.
- Development of safe and robust viral and non-viral vectors of ZFNs is particularly desirable for in vivo use.
- Increased efficacy of ZFNs may enable their use on less accessible targets/cells. Implicit in this is a greater understanding of the factors that increase target efficiency and decrease off-target DNA modification. Factors involved with greater gene-specific cleavage can be incorporated into the selection process for optimal ZFN assembly.
- Development of improved screening for off-target effect and potential toxicity, particularly for ex vivo and in vivo applications.

With further improvement and addressing the problems associated with ZFN, this approach has considerable promise for biomedical research and gene therapy.

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DISCLOSURES

The authors state no conflicts of interest.

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