

Targeted Modifications of the Human Genome using Zinc-Finger Nucleases

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Abstract

Methods to precisely and efficiently modify the human genome hold great promise to revolutionize the gene therapy arena. One promising technology is based on the zinc-finger nuclease (ZFN) technology. Recent advances in generating customized ZFNs to create a DNA double strand break at a pre-selected site in the human genome have paved the way for both knock-out and knock-in strategies in gene therapy. Gene modification frequencies in primary human cells of up to 50% in the absence of selection demonstrate the power of this approach. Here, recent advances of and upcoming challenges for this emerging technology are reviewed and future experimental work needed to bring ZFNs safely into the clinic discussed.

Introduction

The ability to modify with high fidelity a complex genome has revolutionized biomedical research in the late 1980s and early 1990s [1]. The underlying technology is known as gene targeting and is based on the cellular homologous recombination (HR) pathway, which has evolved to promote genetic recombination during meiosis and the repair of DNA double strand breaks (DSBs) before mitosis. Until recently, the low frequency of HR in mammalian cells (~ 1 HR event per 10^6 cells) and the resulting dependence on elaborate selection strategies to identify rare recombinants has prevented gene targeting from being applied in a therapeutic context. A recent technological breakthrough, however, has changed this dreary perspective. By fusing engineered zinc-finger (ZF) DNA-binding domains to a non-specific nuclease domain, so called zinc-finger nucleases (ZFNs) were generated. These ZFNs can be designed to introduce a DSB into a desired target locus and, as a consequence, stimulate gene targeting 100 to 10,000-fold by activating cellular DNA repair pathways [2]. Given recently published gene conversion frequencies of up to 50% [3], it can now be envisioned that this technology might be used to correct inborn mutations in adult stem cells derived from patients with genetic disorders. These corrected cells could subsequently be used to repopulate an affected organ and thereby reverse the disorder.

Targeted genome modifications: gene knockout

To exploit DSBs for therapeutic genome modifications customizable nucleases had to be developed. Such engineered endonucleases basically fall into two classes: re-designed homing endonucleases [4] and ZFNs [2]. ZFNs consist of an engineered Cys₂-His₂ zinc-finger domain fused to the nuclease domain of the type II restriction enzyme *FokI* [5]. In this configuration, the DNA-binding ZF domain directs the non-specific *FokI* cleavage domain to a specific DNA target site. Because the *FokI* cleavage domain is only enzymatically active as a dimer [6], introduction of a DSB is dependent upon dimerization of a ZFN pair. Accordingly, two ZFN subunits are typically designed to recognize the target sequence in a tail-to-tail conformation, with each monomer binding to “half-sites” that are separated by a “spacer” sequence [7] (**Fig. 1a**).

Repair of a DSB induced by a pair of ZFNs can occur by one of two potential pathways: non-homologous end-joining (NHEJ) or homologous recombination (HR). If repair is mediated by NHEJ, an

error-prone pathway that can lead to insertions or deletions [8], the target gene can be disrupted by frameshift mutations which in many cases will lead to expression of a truncated and/or non-functional protein (**Fig. 1b**). The feasibility of such an approach was initially demonstrated in *Drosophila* and more recently in zebrafish. Endogenous expression of ZFNs either targeting the *y*, *ry*, or *bw* loci in *Drosophila* was controlled by the *hsp70* heat-shock promoter, which allowed transient expression of the ZFNs in developing larvae [9, 10]. Between 0% and 78% of the resulting flies carried the targeted mutation in the germline, depending on the target locus and the temperature used to induce the *hsp70* promoter. For zebrafish, mRNAs encoding the ZFNs were co-injected into one-cell stage embryos. Expression of the ZFNs led to mutagenic DNA repair at the respective target sites in ~20% of the embryos and most of these animals transmitted the disrupted alleles to the progeny [11, 12]. In both animal models the ZFN expression levels and the chromosomal context of the target locus were crucial parameters for the level of NHEJ-mediated mutagenesis.

A potential therapeutic application of the ZFN-mediated knockout strategy includes the targeted disruption of the *CCR5* (chemokine receptor 5) locus. In a $CD4^+$ T cell line, the observed range of insertion and deletions due to mutagenic NHEJ repair upon expression of *CCR5*-specific ZFNs was from +8 to -43 bp [13]. Because *CCR5* serves as a co-receptor for HIV (human immunodeficiency virus), a *CCR5* knockout is expected to render cells resistant to infection with HIV. Indeed, HIV-1 infected mice engrafted with ZFN-modified human $CD4^+$ T cells revealed a lower virus load and higher $CD4^+$ T cell counts [13]. Hence, the adoptive transfer of *ex vivo* expanded *CCR5*-modified autologous $CD4^+$ T cells may be an attractive approach for the supplementary treatment of patients infected with HIV-1.

Targeted genome modifications: gene targeting

Gene targeting, on the other hand, relies on HR between the endogenous target locus and an exogenously introduced homologous DNA fragment, also referred to as the "donor DNA". In the absence of a DSB at the target locus, typically fewer than 1 in 10^5 of targeted cells will contain the desired genetic modification, a frequency too low to be useful for gene therapy [14]. However, proof-of-principle experiments involving the meganuclease *I-SceI*, which binds to an 18-bp recognition site, demonstrated that the insertion of a DSB in the target locus stimulates recombination with an exogenous donor DNA by several orders of magnitude [15, 16]. A concern has been that NHEJ will compete with HR to seal the broken chromosome. Recent experiments in two different human cell lines indicated that HR-mediated gene targeting accounted for 60% to 70% of all DSB repair events at an endogenous locus [17], suggesting that in the presence of large amounts of a donor DNA HR is the preferred pathway to repair DSBs.

The architecture of the donor DNA determines the outcome of the gene targeting event. For example, donor DNA can be designed to either create or correct a mutation in a specific gene locus (**Fig. 1c**). In the first study, in which an endogenous mammalian locus was targeted by ZFNs, gene editing at the human *IL2R γ* (interleukin-2 receptor γ -chain) locus was achieved in up to 18% of transiently transfected K562 cells, a human erythroleukemia cell line, and in ~5% of primary human T cells [18]. When integrase-deficient lentiviral vectors were used for ZFN gene transfer, the gene targeting frequency at the *IL2R γ* locus in K562 cells could be increased to up to 29% [17], suggesting that gene transfer efficiency and ZFN expression levels are crucial factors for the success of such an approach.

Alternatively, the donor DNA can contain either an entire expression cassette or a cDNA fragment of the gene to be corrected. In the latter case, the main benefit of gene addition is that a single pair of ZFNs combined with a single donor DNA can be used to "correct" all mutations located downstream of the DSB in a given gene, while still keeping gene expression under control of the endogenous promoter (**Fig. 1d**). Targeted insertions of entire expression cassettes, on the other hand, aim at restoring the cellular phenotype by integration of a therapeutic expression cassette into a yet to be defined "safe harbor". Such a "safe harbor" should include the support of high and sustained transgene expression levels without any signs of genotoxic side effects. Hence, at least in theory, a single pair of ZFNs combined with a customized donor DNA could be used to "correct" safely any given monogenetic inherited disorder amenable to combined gene/stem cell therapy protocols. Because individuals who harbor a homozygous deletion in the *CCR5* gene are healthy [19], this locus represents a candidate site for targeted transgene insertion. In proof-of-principle studies, targeted integration of an EGFP expression cassette into the human *CCR5* locus was achieved in 39% of Jurkat cells, 3.5% of embryonic stem cells, and 0.1% of hematopoietic stem cells [17]. Moreover, it has been shown that ZFNs can mediate targeted insertion of a 7.8-kb long DNA sequence into an endogenous locus in 6% of transfected K562 cells [20]. Surprisingly, two homology arms of 750 bp were sufficient for efficient gene targeting. These results demonstrate that the addition of large DNA fragments is feasible but that the HR frequency is highly cell-type specific, an observation made also in another study [21]. Although these experiments in immortalized cell lines and in embryonic stem cells appear to be promising, it remains to be determined in long-term follow-up studies whether the human *CCR5* locus will ultimately prove to be a "safe harbor".

ZFN-associated toxicity

ZFN-induced cytotoxicity is a major potential issue, which has been reported in several studies [9, 10, 22-26]. Cell death and apoptosis associated with ZFN expression is most likely the result of excessive cleavage at off-target sites, which in turn suggests imperfect target site recognition by the ZF DNA-binding domains. Since therapeutic gene targeting strongly depends on creation of a DSB at a specific target site, the implementation of quantitative assays to assess immediate and long-term genotoxicity of artificial nucleases is paramount [27]. In some studies the extent of cytotoxicity upon ZFN expression was quantified by measuring cell survival [23, 28, 29] or apoptosis [22]; however, these assays are very coarse measures of assessing toxicity and provide little information about the contributing mechanisms. To address this problem, assays that directly document the number of off-target cleavage events have been developed. Using antibodies specific for phosphorylated histone H2AX (γ -H2AX) or p53 tumor suppressor-binding protein 1 (53BP1), we and others have quantified the relative number of ZFN-induced repair foci formed after formation of a DSB [25, 26]. Although these quantitative assays can be used to characterize the specificity and immediate genotoxicity of any artificial nuclease of interest, they are not very sensitive and they do not provide information about the sites at which off-target DSBs occur.

Assessing the *in vivo* cleavage specificity of ZFNs, i.e. the ratio of on-target vs. off-target cleavage events in a complex genome, remains a significant and thus far unsolved challenge. Although recently published *in vivo* and *in vitro* specificity profiling systems for ZF DNA-binding domains can provide information about the DNA-binding profile of monomeric ZF domains [13, 30, 31], they cannot predict actual cleavage sites of ZFN dimers in the human genome. A possible approach to directly identify ZFN cleavage sites might be to exploit the fact that DSBs in a cellular

genome serve as efficient integration sites for episomal DNA, such as vectors based on adeno-associated virus (AAV) [32]. Sequencing of the AAV vector integration sites upon ectopic ZFN expression could offer direct information about the locations of off-target DSBs in a cell.

The long-term effects of ZFN-induced mutagenesis to induce unpredictable oncogenicity can be assessed by soft agar transformation studies [33] or *in vitro* transformation assays using purified lineage-negative cells from murine bone marrow [34]. Moreover, cytogenetic analyses, like spectral karyotyping [35], can provide information about whether ZFN activity induces chromosomal abnormalities and/or translocations. It is emphasized here however that the long-term consequences of ZFN-induced DSBs can only be studied *in vivo*. Assays previously developed to evaluate the genotoxicity of retroviral vectors in gene therapy protocols [36, 37] should prove useful to study the malignant potential of cells upon overexpression of ZFNs.

Improved engineering platforms for ZFNs

Cys₂-His₂ ZF domains are the most abundant DNA-binding motif in eukaryotes and consist of ~30 residues that fold into a $\beta\beta\alpha$ -structure coordinated by a zinc ion [38]. A number of groups have demonstrated that changing one or more of the six critical residues located within or adjacent to the structurally conserved α -helix can alter the DNA-binding specificity of a single ZF (recently reviewed in [2]). ZFNs described in the literature to date contain three or four ZF domains arranged into tandem arrays. Because a single ZF recognizes ~3 bp of DNA sequence [39], a ZFN subunit binds to 9 or 12-bp long target sites, respectively. Assuming perfect specificity by each ZFN monomer, dimers of three-finger ZFNs would therefore be expected to bind to an 18-bp target DNA site while dimers of four-finger proteins recognize a 24-bp target site. Recognition sites of 18 or 24 bp are long enough to define a statistically unique sequence in a human genome.

Various methods for engineering multi-ZF domains have been described in the literature, including rational design and selection from randomized libraries using phage display or bacterial selection systems (recently reviewed in [2]). These ZF engineering platforms can basically be grouped into three categories: "modular assembly", "context-sensitive selection", and the "2+2 method" of the company *Sangamo BioSciences*. The most promising "open-source" approach to date has been developed by the Zinc Finger Consortium (<http://www.zincfingers.org>) and is termed OPEN for "oligomerized pool engineering" [3]. The platform is based on the bacterial-two-hybrid (B2H) system and has proven to be robust and to yield multi-finger domains with high DNA-binding affinities and specificities [3]. Upon transient transfection of OPEN-derived ZFNs, the gene targeting frequency at the *VEGF-A* (vascular epithelial growth factor A) locus in the human K562 cell line reached up to 50%.

As mentioned before, cleavage of a target locus requires that two different ZFN subunits bind as a heterodimer at the desired cleavage site. However, symmetry at the *FokI* dimerization interface also permits homodimers to form, thus enabling cleavage at additional sites. By altering interacting residues in the protein-protein interface of the *FokI* dimerization domain, we and others have engineered asymmetric ZFN variants that prevent the undesirable homodimerization of ZFN subunits, resulting in significantly reduced toxicity [25, 26]. An ideal ZFN architecture therefore includes not only a highly specific DNA-binding domain but also an asymmetric dimerization interface in the *FokI* cleavage domain.

Towards the clinic

It has long been envisaged that by applying the ZFN technology to stem cells, inherited mutations could be repaired *ex vivo* and functionally corrected stem cells transplanted back into patients to repopulate the affected tissues and cure the disease. Importantly, gene correction would restore the functionality of the affected gene product and, at the same time, retain its normal endogenous expression pattern, thereby overcoming a major limitation of conventional gene therapy approaches. Gene correction might work even more efficiently if the repaired gene provides the modified stem cell with a growth advantage. For example, in the case of X-SCID, which is caused by mutations in the *IL2R γ* locus, correction of only a small number of genetically corrected HSCs will be sufficient to restore proper function of the immune system [40].

Several obstacles continue to limit the exploitation of ZFNs in a therapeutic setting. The following criteria should be met in order for ZFNs to be successfully applied in a clinical setting: *(i) ZFN architecture.* As outlined above, ZFNs must consist of a DNA-binding domain with high specificity to the target site and a "regulated" nuclease cleavage domain. *(ii) Choice of delivery system.* To date four different systems have been reported to be suitable to mediate DSB-stimulated targeted genome editing in human cells: plasmid-DNA introduced by transfection [20, 22, 23, 25, 26, 28, 29], AAV vectors [24, 41], integrase-deficient lentiviral vectors (IDLVs) [17, 29] and modified adenoviral vectors [13]. Plasmid-DNA has been the most commonly used ZFN expression vector thus far. Owing to their superior transduction record, AAV vectors, IDLVs and adenovirus type 5 vectors substituted with a type 35 fiber structure (Ad5/35) are promising tools to deliver high numbers of ZFN expression cassettes into stem cells, such as hematopoietic [17] and mesenchymal human stem cells [42]. However, independent of the nature of the expression vector, the delivery of DNA expression cassettes containing strong promoters – and this can include donor DNAs for targeted gene addition – is associated with the potential risk of insertional mutagenesis, as reported for both integrating and "episomal" vectors [43-45]. Moreover, transient expression of ZFNs is strongly preferred over permanent expression of the nucleases. The efficient *ex vivo* delivery of ZFNs in the form of mRNA has therefore been an important breakthrough to achieve this goal and to reduce the risk of vector integration [11, 12]. *(iii) Genotoxic side effects.* As mentioned above, a comprehensive evaluation of treated cells for potential ZFN-induced side-effects both short-term and long-term is paramount. *(iv) Immune response.* Assessment of the potential immune reactivity against ZFNs, especially against the bacterial *FokI* domain should be included, especially when applying ZFNs *in vivo*.

Conclusions

The main advantage of using ZFN-stimulated gene targeting over conventional gene-addition-type gene therapy is the potential to preserve temporal and tissue-specific gene expression. Gene knockout via NHEJ-mediated repair of ZFN-induced DSBs is another promising application of this technology. While the overall efficiency of ZFN-induced genome editing depends on the activity and specificity of custom-made ZFNs, additional parameters, such as the apoptotic threshold of a cell and the proficiency to activate the appropriate DNA repair pathways, both of which may vary significantly among different cell types, will be important too.

Despite tremendous recent progress, the development of methods to better understand and quantify ZFN-associated genotoxicity remains a major priority and challenge for future research. Cleavage at unintended, off-target DNA sites is likely to be the decisive factor for ZFN-associated toxicity. Although the development of a malignant phenotype is hypothesized to require multiple

genetic insults, every genetic manipulation poses a risk, especially in stem and progenitor cells with their high proliferative potential. In view of recent adverse events in gene therapy trials to treat X-SCID, development of both "safer" ZFNs and "safer" delivery methods to minimize genotoxic side effects will be important in order to bring the next generation of gene therapy tools into the clinic.

Acknowledgments

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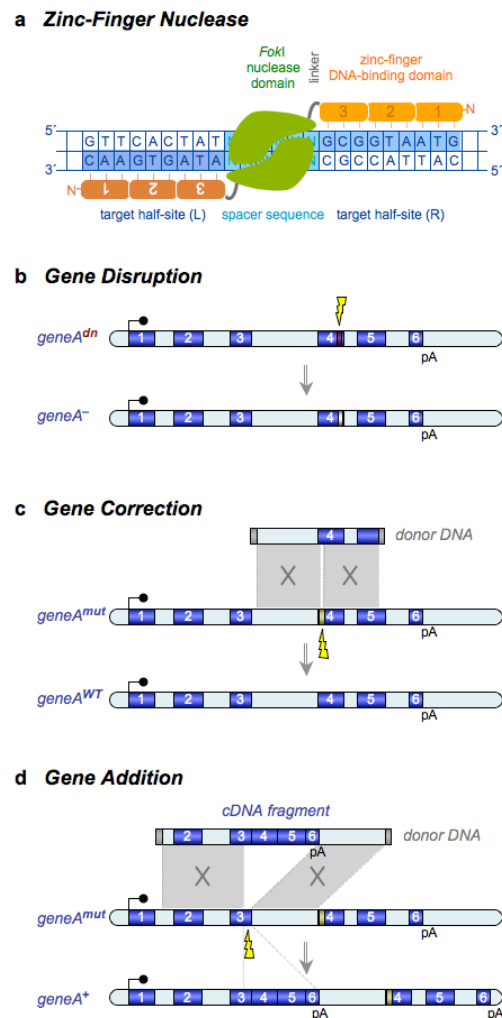


Figure 1 Zinc-finger nuclease mediated genome editing. **(a)** Architecture and application of zinc-finger nucleases (ZFNs). A ZFN designed to create a DNA double strand break (DSB) in the target locus is composed of two monomer subunits. Each subunit encompasses three zinc-fingers (yellow, 1-2-3), which recognize 9-bp within the full target site, and the *FokI* endonuclease domain (green). A short linker (grey) connects the two domains. After dimerization, the nuclease is activated and cuts the DNA in the spacer sequence, which separates the two target half-sites (L) and (R). **(b)** Gene disruption. A DSB (yellow flash) introduced by the ZFN into a dominant mutant allele ($geneA^{dn}$) is repaired by the error-prone non-homologous end-joining (NHEJ) pathway. Deletions and insertions that can occur disrupt the coding sequence ($geneA^-$) and render the expressed protein non-functional. **(c)** Gene correction. In order to restore a genetic defect directly in the genome ($geneA^{mut}$), a targeting vector (donor DNA) encompassing wild-type sequences homologous to the mutant gene (grey areas) is transduced into the target cell. A ZFN-induced DSB stimulates homologous recombination (HR) between the donor DNA and the defective gene ($geneA^{mut}$) to generate a corrected locus ($geneA^{WT}$). **(d)** Gene addition. In order to restore the phenotype of a cell harboring a genetic defect ($geneA^{mut}$), a partial cDNA flanked by sequences homologous to the mutant gene is embedded in a targeting vector. A ZFN-induced DSB stimulates HR between the donor DNA and the mutant gene. Expression of the gene is reconstituted ($geneA^+$) and remains under control of the endogenous promoter.