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# Recent advances in the use of ZFN-mediated gene editing for human gene therapy

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## Abstract

Targeted genome editing with programmable nucleases has revolutionized biomedical research. The ability to make site-specific modifications to the human genome, has invoked a paradigm shift in gene therapy. Using gene editing technologies, the sequence in the human genome can now be precisely engineered to achieve a therapeutic effect. Zinc finger nucleases (ZFNs) were the first programmable nucleases designed to target and cleave custom sites. This article summarizes the advances in the use of ZFN-mediated gene editing for human gene therapy and discusses the challenges associated with translating this gene editing technology into clinical use.

## ZINC FINGER NUCLEASES: FIRST OF THE PROGRAMMABLE NUCLEASES

In the late seventies, scientists observed that when DNA is transfected into yeast cells, it integrates at homologous sites by homologous recombination (HR). In stark contrast, when DNA was transfected into mammalian cells, it was found to integrate randomly at non-homologous sites by non-homologous end joining (NHEJ). HR events were so rare that it required laborious positive and negative selection techniques to detect them in mammalian cells [1]. Later work performed by Maria Jasin's lab using I-SceI endonuclease (a meganuclease) and a homologous DNA fragment with sequences fanking the cleavage site, revealed that a targeted chromosomal double-strand break (DSB) at homologous sites can stimulate gene targeting by several orders of magnitude in mammalian cells that are refractory to spontaneous HR [2]. However, for this experiment to be successful, the recognition site for I-SceI endonuclease had to be incorporated at the desired chromosomal locus of the mammalian genome by classical HR techniques. Thus, generation of a unique, site-specific genomic DSB had remained the rate limiting step in using homology-directed repair (HDR) for robust and precise genome modifications of human cells, that is, until the creation of zinc finger nucleases (ZFNs) – the first of the programmable nucleases that could be designed to target and cleave custom sites [3,4].

FINANCIAL & COMPETING INTERESTS DISCLOSURE

Dr Chandrasegaran is the inventor of the ZFN technology. Johns Hopkins University (JHU) licensed the technology exclusively to Sangamo Biosciences, Inc. (concomitant to its formation in 1995) to develop ZFNs for various biological and biomedical applications. As part of the JHU licensing agreement, Dr Chanrasegaran served on the Sangamo scientific advisory board from 1995 to 2000 and received royalties and stock as per JHU guidelines. The JHU ZFN patents expired in 2012 and became part of the public domain. No writing assistance was utilized in the production of this manuscript.

Because HR events are very rare in human cells, classical gene therapy – use of genes to achieve a therapeutic effect – had focused on random integration of normal genes into the human genome to reverse the adverse effects of disease-causing mutations. The development of programmable nucleases – ZFNs, TALENs and CRISPR-Cas9 – to deliver a targeted DSB at a pre-determined chromosomal locus to induce genome editing, has revolutionized the biological and biomedical sciences. The ability to make site-specific modifications to the human genome has invoked a paradigm shift in gene therapy. Using gene editing technologies, the sequence in the human genome can now be precisely engineered to achieve a therapeutic effect. Several strategies are available for therapeutic gene editing which include: 1) knocking-out genes by NHEJ; 2) targeted addition of therapeutic genes to a safe harbor locus of the human genome for *in vivo* protein replacement therapy (IVPRT); and 3) correction of disease-causing mutations in genes.

The first truly targetable reagents were the ZFNs that showed that arbitrary DNA sequences in the human genome could be cleaved by protein engineering, ushering in the era of human genome editing [4]. We reported the creation of ZFNs by fusing modular zinc finger proteins (ZFPs) to the non-specific cleavage domain of FokI restriction enzyme in 1996 [3]. ZFPs are comprised of ZF motifs, each of which is composed of approximately 30 amino acid residues containing two invariant pairs of cysteines and histidines that bind a zinc atom. ZF motifs are highly prevalent in eukaryotes. The $Cys_2His_2$ ZF fold is a unique ββα structure that is stabilized by a zinc ion [5]. Each ZF usually recognizes a 3–4-bp sequence and binds to DNA by inserting the α-helix into the major groove of the double helix. Three to six such ZFs are linked together in tandem to generate a ZFP that binds to a 9–18-bp target site within the genome. Because the recognition specificities can be manipulated experimentally, ZFNs offered a general means of delivering a unique, site-specific DSB to the human genome. Furthermore, studies on the mechanism of cleavage by 3-finger ZFNs established that the cleavage domains must dimerize to affect an efficient DSB and that their preferred substrates were paired binding sites (inverted repeats) [6]. This realization immediately doubled the size of the target sequence recognition of 3-finger ZFNs from 9- to 18-bp, which is long enough to specify a unique genomic address within cells. Moreover, two ZFNs with different sequence specificities could cut at heterologous binding sites (other than inverted repeats), when they are appropriately positioned and oriented within a genome.

## ZFNS PAVED THE WAY FOR HUMAN GENOME EDITING

In collaboration with Dana Carroll's lab, we then showed that a ZFN-induced DSB stimulates HR in frog oocytes in 2001 [7]. The groundbreaking experiments on ZFNs established the potential for inducing targeted recombination in a variety of organisms that are refractory to spontaneous HR, and ushered in the era of site-specific genome engineering, also commonly known as genome editing. A number of studies using ZFNs for genome editing in different organisms and cells, soon followed [4,8–10]. The modularity of DNA recognition by ZFs, made it possible to design ZFNs for a multitude of genomic targets for various biological and biomedical applications [4]. Thus, the ZFN platform laid the foundation for genome editing and helped to define the parameters and approaches for nuclease-based genome engineering.

Despite the remarkable successes of ZFNs, the modularity of ZF recognition did not readily translate into a simple code that enabled easy assembly of highly specific ZFPs from ZF modules. Generation of ZFNs with high sequence specificity was difficult to generate for routine use by at large scientists. This is because the ZF motifs do not always act as completely independent modules in their DNA sequence recognition; they are influenced more often than not by their neighbors. ZF motifs that recognize each of the 64 possible DNA triplets with high specificity, never materialized. Simple modular assembly of ZFs did not always yield highly specific ZFPs, hence ZFNs. Thus, DNA recognition by ZF motifs turned out to be more complex than originally perceived. With this realization came the understanding that the ZFPs have to be selected in a context-dependent manner that required several cycles of laborious selection techniques and further optimization. This is not to say that it can't be done, but just that it requires substantial cost and time-consuming effort. This is evidenced by the successful ZFN-in-duced genome editing applications to treat a variety of human diseases that are underway. For example, ZFN-induced mutagenesis of HIV co-receptor CCR5 as a form of gene therapy has the potential to provide a functional cure for HIV/AIDS.

Successor technologies – TALENs and CRISPR/Cas9 – have made the delivery of a site-specific DSB to the mammalian genome much easier and simpler. Custom nuclease design was facilitated further by the discovery of TAL effector proteins from plant pathogens, in which two amino acids (repeat variable di-residues, also known as RVDs) within a TAL module, recognize a single base pair, independent of the neighboring modules [11,12]. In a similar fashion to ZFNs, TAL effector modules were fused to FokI cleavage domain to form TAL effector nucleases, known as TALENs [13]. The development of TALENs simplified our ability to make custom nucleases by straightforward modular design for the purposes of genome editing. However, the discovery of CRISPR/Cas9 – an RNA-guided nuclease in bacterial adoptive immunity – has made it even easier and cheaper, given that no protein engineering is required [14–17]. A constant single nuclease (Cas9) is used for cleavage together with a RNA that directs the target site specificity based on Watson-Crick base pairing. CRISPR/Cas9 system has democratized the use of genome editing, by making it readily accessible and affordable by small labs around the world.

## ZFN SPECIFICITY & SAFETY

The efficacy of ZFNs to a large extent depends on the specificity of the ZFPs that are fused to the FokI nuclease domain. The higher the specificity of the ZFPs, the lower the ZFNs off-target cleavage, and hence toxicity. The early ZFNs designed for genomic targets displayed significant of-target activity and toxicity due to promiscuous binding and cleavage, particularly when encoded in plasmids and expressed in high levels in human cells. One way to increase the specificity of the ZFNs is to increase the number of ZF motifs within each ZFN of the pair. This helps to improve specificity, but it is not always sufficient. Many different mechanisms could account for the of-target activity. They include ZFNs binding to single or unintended target sites as well as to homodimer sites (the inverted repeat sites for each of the ZFN pair). Binding of a ZFN monomer to single or unintended target sites could be followed by dimerization of the cleavage domain to another monomer in solution. Therefore, one approach to reduce ZFNs toxicity is to re-design the dimer interface of the

cleavage domains to weaken the interaction and generate a heterodimer variant pair that will actively cleave only at heterodimer binding sites and not at the homodimer or single or unintended binding sites. We had previously shown that the activity of the ZFNs could be abolished by mutating the amino acid residues that form the salt bridges at the FokI dimer interface [6]. Two groups achieved reduction in ZFNs of-target cleavage activity and toxicity by introducing amino acid substitutions at the dimer interface of the cleavage domain that inhibited homodimer formation, but promoted the obligate heterodimer formation and cleavage [18,19]. We showed further improvements to the obligate heterodimer ZFN pairs by combining the amino acid substitutions reported by the two groups [20].

Another approach to reduce ZFN toxicity is to use ZF nickases that cleave at only one predetermined DNA strand of a targeted site. ZFN nickases are produced by inactivating the catalytic domain of one monomer within the ZFN pair [4]. ZFN nickases induce greatly reduced levels of mutagenic NHEJ, since nicks are not efficient substrates for NHEJ. However, this comes at a cost, in terms of lowered efficiency of cleavage. A standard approach that has been widely used to increase the sequence specificity of ZFPs (and the DNA binding proteins in general) is to abolish non-specific protein contacts to the DNA backbone by amino acid substitutions. Again, this comes at the price of ZFPs' lowered binding affinity for their targets, resulting in lower efficiency of on-target cleavage.

## METHODS FOR ZFN DELIVERY INTO CELLS

The first experiments to show that ZFNs were able to cleave a chromatin substrate and stimulate HR in intact cells were performed by microinjection of ZFNs (proteins) and synthetic substrates into xenopus oocytes [7]. Plasmid-encoded ZFNs and donors have also been co-transfected into human cells by using electroporation, nucleofection or commercially available chemical reagents. This potentially has two drawbacks: 1) the plasmids continue to express the ZFNs that accumulate in high levels in cells, promoting promiscuous DNA binding and of-target cleavage; and 2) there is also the possibility that the plasmid could integrate into the genome of the cells. To circumvent these problems, one could transfect mRNAs coding for the ZFNs along with donor DNA into cells. Adeno-associated virus (AAV) and lentivirus (LV) are the common vehicles used for the delivery of ZFNs and the donor into human cells.

## FIRST-IN-HUMAN STUDY

ZFN-mediated CCR5 disruption was the first-in-human application of genome editing, which was aimed at blocking HIV entry into cells [21]. Most HIV strains use CCR5 co-receptor to enter into cells. The CCR5Δ32 allele contains a 32-bp deletion that results in a truncated protein; it is not expressed on the cell surface. The allele confers protection against HIV-1 infection without any adverse health effects in homozygotes. Heterozygotes show reduced levels of CCR5; their disease progression to AIDs is delayed by 1 to 2 years. The potential benefit of a *CCR5* targeted gene therapy was highlighted in the only reported case of an HIV cure. The so called "Berlin patient" received allogeneic bone marrow transplants from a CCR5Δ32 donor during treatment of acute myeloid leukemia and ever since has remained HIV-1 free without antiviral treatment (ART). This report gave impetus to gene

therapy efforts to create CCR5-negative autologous T cells or hematopoietic stem/progenitor cells (HSPCs) in HIV-infected patients. The expectation was that the edited cells will provide the same anti-HIV effects as in the Berlin patient, but without the risks associated with the allogeneic transplantation. CCR5 knockout via NHEJ was used in this strategy, since gene modification efficiency by HDR is relatively low. ZFN-in-duced genome editing of CCR5 is the most clinically advanced platform, with several ongoing clinical trials in T cells and HSPCs [22].

The Phase I clinical trial (#NCT00842634), of knocking-out CCR5 receptor to treat HIV, was conducted by Carl June's lab in collaboration with Sangamo Biosciences (California) scientists. The goal was to assess the safety of modifying autologous $CD4^+$ T cells in HIV-1–infected individuals [21]. Twelve patients on ART were infused with autologous $CD4^+$ T cells, in which the *CCR5* gene was inactivated by ZFN treatment. The study reported: 1) a significant increase in $CD4^+$ T cells post-infusion; and 2) long-term persistence of CCR5-modified $CD4^+$ T cells in peripheral blood and mucosal tissue. The therapeutic effects of the ZFN treatment in five patients were monitored by a 12-week interruption of ART. The study established that the rate of decline of the CCR5-modified $CD4^+$ T cells was slower than that of the unmodified cells, indicating a protective effect of CCR5 disruption [22]. One patient showed both delayed viral rebound and a peak viral count that was lower than the patient's historical levels. This patient was later identified as being heterozygous for CCR5Δ32, which suggested that the beneficial effects of the ZFN treatment was magnified in this patient, probably due to increased levels of bi-allelic modification [22]. Thus, heterozygous individuals may have a greater potential for a functional HIV cure. The obvious next step is to apply the ZFN treatment to earlier precursors or stem cells. Editing HSPCs instead of $CD4^+$ T cells has the potential to provide a long-lasting source of modified cells. Success of this strategy has been established in preclinical studies [23] and a recent clinical trial (#NCT02500849) has been initiated using this approach. Programs to disrupt CCR5 in T cells and HSPCs, using the other nuclease platforms that include TALENs, CRISPR/Cas9 and megaTALs (a meganuclease fused to TAL effector modules), are also underway; these are at the pre-clinical stage.

## ZFN PRECLINICAL TRALS AIMED AT TREATING HUMAN MONOGENIC DISEASES

Sangamo Biosciences, Inc. has leveraged its proprietary database of proven ZFNs (that includes an extensive library of functional ZF modules and 2-finger units for the assembly of highly specific ZFNs) and its ZFN patent portfolio to enter into research collaborations with academic scientists for the application of ZFN-mediated gene editing strategies to treat a number of human diseases. Many of these programs are at the preclinical stage.

An interesting gene editing approach is gene replacement therapy. ZFN-mediated gene editing has shown promise for *in vivo* correction of the *hFIX* gene in hepatocytes of hemophilia B mice. Katherine High's lab in collaboration with Sangamo scientists, is developing a general strategy for liver-directed protein replacement therapies using ZFN-mediated site-specific integration of therapeutic transgenes within the albumin gene locus

[24]. Using *in vivo* AAV delivery, they have achieved long-term expression of hFVIII and hFIX in mouse models of hemophilia A and B at therapeutic levels. Because albumin is very highly expressed, modifying less than 1% of liver cells can produce therapeutic levels of relevant proteins, essentially correcting the disorders. Several pre-clinical studies are now underway to develop liver-directed protein replacement therapies for lysosomal storage disorders that include Hurler, Hunter, Gaucher, Fabry and many others.

We have previously shown that the *CCR5* gene could serve as a safe harbor locus for protein replacement therapies [25]. We reported that by targeted addition of the large CFTR transcription unit at the CCR5 chromosomal locus of human-induced pluripotent stem cells (hiPSCs), one could achieve efficient CFTR expression. Thus, therapeutic genes could be expressed from the CCR5 chromosomal locus for autologous cell-based transgene-correction therapy to treat various recessive monogenic human disorders. Other safe harbor loci such as AAVS1 in the human genome are also available for gene replacement therapy.

Many labs around the world are also working to develop gene editing strategies to treat several other diseases such as sickle cell anemia, SCID, cancer (CAR T cells for immunotherapy) and many others, which are not discussed here. A list of clinical and pre-clinical studies using genome editing technologies for gene and cell therapy of various diseases is outlined elsewhere [26].

## CHALLENGES FACING ZFN-BASED GENE EDITING BEFORE ROUTINE TRANSLATION TO CLINIC

Several challenges still remain that need to be addressed before we see routine translation of ZFN-based gene editing to clinic. They include: 1) potential harmful human genome perturbations due to of-target DSBs, which may be genotoxic or oncogenic; 2) current gene editing efficiencies may not be sufficient for certain diseases, particularly where gene edited cells have no survival advantage; 3) safe and efficient delivery of ZFNs into target cells and tissues, when using the *in vivo* approach; and 4) the treatment costs, if and when ZFN-based gene editing is translated to clinic for routine use.

First, these gene-editing tools need further refinement before they can be safely and effectively used in the clinic. The of-target effects of gene editing technologies are discussed in detail elsewhere [4]. The efficacy of ZFNs is largely governed by the specificity of the ZFPs that are fused to the FokI cleavage domain. Higher the specificity of the ZFPs, the lower the ZFNs' of-target cleavage is and hence toxicity. As seen with the CCR5 clinical trial, some highly evolved ZFNs are very specific. In the clinic, engineered highly specific ZFNs will be used repeatedly to treat many different individuals [4]. Therefore, design and construction of highly evolved ZFNs for a particular disease target, will likely be a small part of the overall effort.

Second, further improvements to gene editing efficiencies are needed for successful therapeutic genome editing. HSPCs gene editing may not yield sufficient number of edited cells for autologous transplantation due to the difficulties associated with the *ex vivo* culture and expansion. An alternative approach is to modify patient-specific iPSCs, which then

could be reprogrammed into HSPCs. Since clonal selection, expansion and differentiation of gene edited iPSCs is performed *ex vivo*, this may enable very high editing efficiencies, particularly when coupled with HDR-mediated insertion of a selection cassette. This would also allow for complete genome-wide analysis of gene edited cells for off-target effects. The patient-specific *ex vivo* approach has the potential to become a viable clinical alternative to modifying autologous HSPCs [25,27]. In the case of autosomal recessive disorders that require two copies of the gene to be mutated, correction of mono-allele in sufficient number of cells may be enough to confer a therapeutic effect in patients. However, in the case of autosomal dominant disorders that require only one mutated copy of the gene, bi-allelic modification in sufficient number of cells, will be essential to achieve a therapeutic effect in patients. Therefore, methods need to be developed to increase the levels of bi-allelic modification in human cells.

Third, another potential issue pertains to the safe and efficient delivery of ZFNs into the appropriate target cells and tissues [4]. ZFNs are much smaller than TALENs or Cas9. Therefore, ZFNs can be readily delivered using AAV or LV constructs. The method of ZFN delivery could also vary depending on the human cell types. For example, Ad5/F35-mediated delivery of ZFNs was very efficient in $CD4^+$ T cells while it was less efficient in HSPCs [23]. The nontoxic mRNA electroporation has been efficient for the introduction of ZFNs into HSPCs. This approach has been adapted in a recent clinical trial (#NCT02500849). Recently, Kohn's lab compared the efficiency, specificity, and mutational signatures during the reactivation of fetal hemoglobin expression by BC-L11A knock-out in human $CD34^+$ progenitor cells, using ZFNs, TALENs and CRISPR/Cas9 [28]. ZFNs showed more allelic disruption in the BCL11A locus when compared to the TALENs or CRIS-PR/Cas9. This was consistent with increased levels of fetal hemoglobin in erythroid cells generated *in vitro* from gene edited $CD34^+$ cells. Genome-wide analysis revealed high specific BCL11A cleavage by ZFNs, while evaluated TALENs and CRISPR/Cas9 showed off-target cleavage activity. This study highlights the high variability in cleavage efficiencies at different loci and in different cell types by the different technology platforms. Therefore, there is a critical need to investigate ways to further optimize the delivery of these nucleases into human cells.

Fourth, if and when therapeutic gene editing is translated to clinic for routine use, a major challenge will relate to the treatment costs associated with these technologies. In the age of $1000 per pill and $100,000 – $300,000 per year treatment costs for certain chronic disease conditions, it is critical to simplify these $21^{st}$ century cures, if they are to be become accessible and affordable for the average citizen and the poor populations of the third world. Many labs are working towards simultaneous gene correction and generation of patient-specific iPSCs to simplify treatment [4]. CRISPR/Cas9 may be best suited for this strategy [29].

Finally, since all these gene editing platforms have been shown to cleave at off-target sites with mutagenic consequences, a word of caution is warranted: careful, systematic and thorough investigation of off-target effects at the genome-wide scale, for each and every reagent that will be used to treat human diseases, is absolutely essential to ensure patient

safety. For these reasons, therapeutic gene editing by these technology platforms, will ultimately depend on risk versus benefit analysis and informed consent.

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