Zinc-Finger Nucleases for Somatic Gene Therapy: The Next Frontier

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Abstract

Zinc-finger nucleases (ZFNs) are a powerful tool that can be used to edit the human genome *ad libitum*. The technology has experienced remarkable development in the last few years with regard to both the target site specificity and the engineering platforms used to generate zinc-finger proteins. As a result, two phase I clinical trials aimed at knocking out the *CCR5* receptor in T cells isolated from HIV patients to protect these lymphocytes from infection with the virus have been initiated. Moreover, ZFNs have been successfully employed to knockout or correct disease-related genes in human stem cells, including hematopoietic precursor cells and induced pluripotent stem cells. Targeted genome engineering approaches in multipotent and pluripotent stem cells hold great promise for future strategies geared toward correcting inborn mutations for personalized cell replacement therapies. This review describes how ZFNs have been applied to models of gene therapy, discusses the opportunities and the risks associated with this novel technology, and suggests future directions for their safe application in therapeutic genome engineering.

Introduction

SINCE ROGERS'S PROPOSAL in the 1970s to use "exogenous 'good' DNA to replace the defective DNA in those who suffer from genetic defects" (Friedmann and Roblin, 1972), a wide variety of technologies enabling gene transfer have emerged from different fields of science, including chemistry, physics, biology, and virology. The first successful gene therapy clinical trial aimed at the phenotypic correction of X-linked severe combined immunodeficiency (SCID-X1) was published in 2000 (Cavazzana-Calvo *et al.*, 2000). This study was based on retroviral transfer of a "healthy" copy of the defective *IL2RG* gene into autologous hematopoietic stem cells (HSCs). Although overall successful, this gene addition strategy revealed unwanted genotoxic side effects, such as the activation of a cellular proto-oncogene by strong *cis*-regulatory elements present in the viral vector genome (Hacein-Bey-Abina *et al.*, 2008). This kind of insertional mutagenesis could be avoided by using precise genome surgery with the goal of directly correcting the defective endogenous gene *in situ*.

One potential approach for performing precise gene correction is to utilize the homologous recombination (HR) pathway. However, the low frequency of HR in mammalian cells ($\sim 10^{-6}$) has for many years prevented the exploitation

of this pathway in a therapeutic context. Fifteen years ago, two pioneering studies demonstrated that targeted DNA double-strand breaks (DSBs) could be used to stimulate the HR frequency several 1000-fold in a variety of mammalian cell types (Rouet *et al.*, 1994; Choulika *et al.*, 1995)—the dawn of the concept that a highly specific endonuclease might be harnessed to correct an inborn mutation. The next challenge has been the development of artificial nucleases that can be designed to specifically cleave DNA at a chosen target gene. Zinc-finger nucleases (ZFNs) represent a successful class of such artificial nucleases (Kim *et al.*, 1996; Bibikova *et al.*, 2001) and have been shown to promote HR at several loci associated with human disease (Urnov *et al.*, 2005; Maeder *et al.*, 2008; Perez *et al.*, 2008; Hockemeyer *et al.*, 2009; Zou *et al.*, 2009). This review summarizes how ZFNs have been employed in relevant gene therapy settings, describes the opportunities and the risks associated with the technology, and discusses future strategies for "safer" ZFN-based gene therapy protocols.

ZFNs: A Rapid Coming of Age

The key parameters for successful design of custom-made nucleases include the following: (i) the nuclease should be

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made up of customizable DNA-binding domains; (ii) the DNA-binding domain should recognize at least 18bp in order to specify a unique site in the human genome; and (iii) cleavage activity should only be triggered upon binding to the target site, thus adding an extra layer of specificity.

ZFNs are modular proteins with two functional domains, one to recognize the DNA and one to cleave it. The concept of fusing a tailor-made zinc-finger array to the nonspecific endonuclease domain of the well-characterized restriction enzyme *FokI* (Fig. 1A) to create an artificial nuclease was developed by Chandrasegaran's team only some 15 years ago (Kim *et al.*, 1996). Specific binding to the target site is mediated by an array of three to six zinc-finger tandem repeats, and each of the zinc-fingers recognizes primarily three bases of the DNA target site (Pavletich and Pabo, 1991). Each zinc-finger array confers binding to a respective target half-site and directs one *FokI* domain to the target site (Fig. 1B). Upon binding of a ZFN dimer to the DNA, a DSB is introduced within the spacer sequence that separates the two target half-sites (Fig. 1C). ZFNs have been shown to introduce DSBs specifically and efficiently in a variety of cell types and organisms, including nematodes (Morton *et al.*, 2006), fruit flies (Beumer *et al.*, 2008), zebrafish (Doyon *et al.*,

2008; Meng *et al.*, 2008; Foley *et al.*, 2009), plants (Townsend *et al.*, 2009), rats (Geurts *et al.*, 2009; Mashimo *et al.*, 2010), *Xenopus* (Young *et al.*, 2011), pigs (Yang *et al.*, 2011), and primary human cells, such as embryonic stem cells (ESCs) (Lombardo *et al.*, 2007), T cells (Perez *et al.*, 2008), induced pluripotent stem cells (iPSCs) (Hockemeyer *et al.*, 2009; Zou *et al.*, 2009, 2011), mesenchymal stromal cells (Benabdallah *et al.*, 2010), and HSCs (Holt *et al.*, 2010). The broad range of organisms and cell types in which ZFN-based genome editing has been applied suggests that it utilizes mechanisms of cellular DNA recognition and DNA repair that developed early in evolution.

Since their development, ZFNs have undergone several refinements, and today's third generation ZFNs are more specific and less toxic than their predecessors (Carroll, 2008; Cathomen and Joung, 2008). In total, the activities of ZFNs have been improved in three different ways. First, affinity and specificity of the zinc-finger array in each subunit are key determinants of ZFN specificity, and it was shown that DNA-binding specificity correlates with ZFN activity at the target locus and inversely correlates with ZFN-associated toxicity (Fig. 1D) (Cornu *et al.*, 2008). Different platforms are available to engineer target-specific zinc-finger arrays

A Zinc-finger nuclease

B ZFN target site

C On-target site cleavage

D Off-target site cleavage

FIG. 1. Zinc-finger nuclease-mediated DNA cleavage. **(A)** Architecture of a zinc-finger nuclease (ZFN) subunit. A ZFN subunit encompasses three to six zinc-fingers arranged in a tandem array (depicted here is a three-finger array) and the catalytic domain of the *FokI* endonuclease. A short linker connects the two domains. **(B)** ZFN target site. The target site is composed of the two target half-sites, which are separated by a short DNA spacer. **(C)** After dimerization of the two ZFN subunits, the nuclease is activated and cuts the DNA within the spacer sequence, leaving a 5'-overhang. **(D)** ZFN off-target activity. Insufficient specificity of DNA-binding permits ZFN binding to and cleaving at unintended DNA sites. Color images available online at www.liebertonline.com/hum

(Carroll *et al.*, 2006; Mandell and Barbas, 2006; Maeder *et al.*, 2008; Meng *et al.*, 2008; Sander *et al.*, 2011), and the strengths and weaknesses of some of these platforms have been discussed elsewhere (Cathomen and Joung, 2008; Ramirez *et al.*, 2008; Joung *et al.*, 2010; Kim *et al.*, 2010). Second, *FokI* cleavage domains with altered dimerization specificities have been created. By exchanging key residues in the dimer interface, obligate heterodimeric *FokI* domains that do not homodimerize have been created (Miller *et al.*, 2007; Szczeppek *et al.*, 2007; Söllü *et al.*, 2010). The resulting obligate heterodimeric ZFN revealed a significant reduction in the numbers of off-target cleavage events, which can be mutagenic or induce cell death (Alwin *et al.*, 2005). Third, the sequence and length of the linker that connects the DNA-binding domain with the nuclease domain can influence ZFN activity and target-site selectivity. Linker variants for defined spacer lengths of 5 to 18 bp have been described, thereby expanding the repertoire of potential ZFN target sites (Bibikova *et al.*, 2001; Händel *et al.*, 2009).

ZFN-Mediated DNA Cleavage Activates the Cellular DNA Damage Response

After ZFN-mediated DNA cleavage at the target site, the resulting DSB leads to induction of the cellular DNA damage response. As a result, a human cell activates one of two major DSB repair pathways, which are each regulated in a cell cycle-dependent manner (Mao *et al.*, 2008; Shrivastav *et al.*, 2008). The nonhomologous end-joining pathway (NHEJ) is the predominant DSB repair pathway in mammalian cells and is most active during the G1 phase of the cell cycle. It is an error-prone repair mechanism, which simply re-ligates the two DNA ends. However, DNA ends can undergo processing prior to ligation in which nucleotides are removed or added. As a consequence, small insertions or deletions (indels) are created at the cleavage site, and if the DSB occurs within coding exons, indels can frequently result in frame-shift or nonsense mutations. The second important DSB repair pathway is homology-directed repair (HDR), which is based on HR and utilizes a homologous donor sequence as a repair template. This highly accurate pathway is mostly active during the S and G2 phases of the cell cycle during which homologous sister chromatids are available. Notably, the HDR pathway is the basis of both conventional gene targeting (e.g., in ESCs to generate knockout mice) and ZFN-induced gene targeting. Although NHEJ is the more prominent repair pathway in mammalian cells, it seems that the cell can be skewed to repair a DSB via HDR if high numbers of donor DNA are delivered to the nucleus (Gellhaus *et al.*, 2010; Lombardo *et al.*, 2007). It remains to be proven, however, whether these observations are cell-type or vector-type specific or a general phenomenon that can be harnessed to improve the gene-targeting frequency in a therapeutic setting.

ZFN-Mediated Gene Disruption

In the absence of donor DNA, a ZFN-induced DSB will be repaired by the NHEJ pathway and the resulting indels can be exploited to disrupt the coding sequence of a gene to generate a functional gene knockout (Fig. 2A). As elucidated in following text, a permanent gene knockout strategy can be envisioned for several applications in the context of somatic

gene therapy. The concept of a targeted, ZFN-induced knockout was introduced by Santiago *et al.* (2008). Transiently expressed ZFNs targeting a site within exon 1 of the gene encoding dihydrofolate reductase in Chinese hamster ovary cells led to disruption of up to 7% of alleles, with about 1% of cells harboring a bi-allelic knockout. This study revealed two basic findings. First, transient expression of ZFNs permits the generation of mammalian cell lines with bi-allelic gene disruption without any selection. Second, to improve the chances of creating a functional knockout, ZFNs should be designed to target an exon that encodes a critical region of the protein product. As later shown by Liu *et al.* (2010), even triple knockout cell lines can be generated by sequential bi-allelic disruption of three genomic loci.

In a related approach, chromosomal deletions can be created by concomitantly expressing two different ZFN pairs targeting adjacent sites on a chromosome (Fig. 2B). Lee *et al.* (2010) achieved forced chromosomal deletions of 15 kb between *CCR2* and *CCR5* in 10% of cells. In this context, it is important to mention that the use of autonomous ZFN pairs—that is, pairs in which the individual subunits of one pair cannot cross-react with the subunits of the second ZFN pair—significantly reduce ZFN-associated toxicity (Söllü *et al.*, 2010).

ZFN-based gene disruption has also been applied in animal models. In the fruit fly and zebrafish, direct embryo injection of ZFN-encoding mRNA has been used to generate heritable knockout mutations at specific loci (Beumer *et al.*, 2008; Doyon *et al.*, 2008; Meng *et al.*, 2008; Foley *et al.*, 2009). A major difference in these studies, from a technological point of view, was how the ZFNs were designed. Beumer *et al.* (2008) used modular assembly (Carroll *et al.*, 2006), Doyon *et al.* (2008) used ZFNs derived from a commercial source, Meng *et al.* (2008) used zinc-finger arrays selected from a bacterial-one-hybrid system, and Foley *et al.* (2009) used the OPEN protocol (Maeder *et al.*, 2009). All of these platforms seem to be useful to generate knockout animals, yielding high frequencies of mutated alleles in about 10%–30% of raised “healthy” animals. In a similar approach, knockout rats were created by microinjection of ZFN-encoding mRNA into one-cell embryos (Geurts *et al.*, 2009; Mashimo *et al.*, 2010). This technology has been used to create rat models of various diseases, such as SCID, which was generated by targeting exon 2 in the *IL2RG* locus (Mashimo *et al.*, 2010).

Cell surface proteins that serve as receptors for viral cell entry provide therapeutic targets of high clinical relevance. One of these proteins is the C-C chemokine receptor type 5 (*CCR5*), the co-receptor for infection of CD4⁺ cells with human immunodeficiency virus (HIV). Populations in Northern Europe that carry the naturally occurring genetic deletion *CCR5delta32* are largely protected from HIV infection (Oh *et al.*, 2008). With the rationale to phenocopy the natural *CCR5delta32* null genotype, Sangamo BioSciences, Inc., has led the development of a gene mutation approach that aimed at permanently disrupting the endogenous *CCR5* locus. Primary CD4⁺ T cells of healthy donors were transduced with an adenoviral ZFN expression vector to knock-out the *CCR5* locus on chromosome 3 (Perez *et al.*, 2008). Under nonselective conditions, 23% of T cell clones carried a disrupted *CCR5* allele, and about a third of these cells harbored a bi-allelic knockout. As an extension to this study,

FIG. 2. Zinc-finger nuclease mediated genome editing. **(A)** Targeted gene disruption. A DNA double-strand break (DSB) introduced by a ZFN is repaired by the error-prone nonhomologous end-joining (NHEJ) pathway. The resulting insertions and deletions at the cleavage site can disrupt the coding sequence by inserting frameshift or nonsense mutations (m), leading to a functional knockout. **(B)** Targeted deletion. A targeted chromosomal deletion can be achieved if two DSBs are created simultaneously by two pairs of ZFNs at two adjacent sites on a chromosome. **(C)** Gene correction. A genetic defect in the genome is restored by co-introducing ZFN with a donor DNA that encompasses wild-type sequences homologous to the mutated gene (X^m). The ZFN-induced DSB stimulates homology-directed repair (HDR) between the donor DNA and the defective gene. **(D)** Gene complementation by targeted integration into a safe harbor. In order to restore the phenotype of a cell harboring a genetic defect, a therapeutic expression cassette (V) flanked by sequences homologous to the safe harbor locus is embedded into a donor DNA. The ZFN-induced DSB stimulates HDR and results in targeted integration of the therapeutic gene. Color images available online at www.liebertonline.com/hum

Holt *et al.* (2010) knocked out the *CCR5* locus in human CD34⁺ cells using nucleofection to deliver the ZFN expression plasmids. When either ZFN-treated CD4⁺ or CD34⁺ cells were co-transplanted with HIV-infected peripheral blood mononuclear cells into immunodeficient mice, the CD4⁺ T-cell population with disrupted *CCR5* was enriched and the animals had significantly lower virus counts. These results show impressively that ZFN-induced *CCR5* disruption in stem or differentiated effector cells can at least partly protect the CD4⁺ T cell population from HIV infection *in vivo*. Currently, the Sangamo *CCR5*-specific ZFNs are being assessed in two phase I clinical trials to treat HIV/AIDS patients (NCT00842634; NCT01044654).

ZFN-Induced Gene Targeting

Precise genetic manipulation of the human genome by accurate HR-based replacement of one sequence with another in pluripotent or multipotent stem cells represents the gold standard for treatment of many monogenetic disorders. Depending on the design of the donor, HR-mediated sequence exchange can be used to correct an inborn mutation—here referred to as “gene correction”—or used to insert a cDNA or a whole expression cassette into a defined location—here referred to as “gene complementation.” For efficient gene correction (Fig. 2C), it is believed that a ZFN pair typically has to cut the target locus within 50 bp of the mutation. The reason for this is the relatively short conversion tracts in mammalian cells; i.e., the sequence information that is transferred from the donor to the genomic locus within the homologous regions (Elliott *et al.*, 1998).

Mutations in the gene coding for the IL-2 receptor γ chain (*IL2RG*) lead to SCID-X1, which is characterized by the lack of T lymphocytes and natural killer cells. The impairment of the adaptive immune response is lethal and the predicted life span of the patients is only about 9 months without bone marrow transplantation. In a gene therapy approach, an *IL2RG* expression cassette was added via *ex vivo* retroviral transduction and subsequent autologous transplantation of CD34⁺ HSCs (Cavazzana-Calvo *et al.*, 2000; Gaspar *et al.*, 2004). Although the adaptive immune system recovered in most treated patients, some developed leukemia due to retroviral activation of cellular proto-oncogenes (Hacein-Bey-Abina *et al.*, 2008). A ZFN-mediated strategy may provide a safer approach without the risk of insertional mutagenesis. ZFNs targeting a mutation hot spot within *IL2RG* exon 5 and homologous donor molecules were designed and subsequently shown to promote gene targeting in 10% of a transfected human cell line (Urnov *et al.*, 2005). The frequency was increased to 18% by transiently arresting the cells in the G2 phase using vinblastine, which prolongs the time window for HR-based gene targeting. Remarkably, gene modification at the *IL2RG* locus was bi-allelic in up to 6.6% of cells, and the technology was robust enough to achieve gene conversion in 5% of human primary CD4⁺ T cells. This study indicated, for the first time, that ZFNs could be used to specifically modify an endogenous human disease locus without the need for selection.

For monogenic diseases in which different patient mutations may be scattered along the length of a gene, gene complementation may be a more suitable approach. In such a scenario, a cDNA encompassing the sequence of all exons

downstream of the most 5' mutation could be inserted into an early exon of the affected gene. In a proof-of-concept approach, Lombardo *et al.* (2007) integrated an 800-bp long partial cDNA into exon 5 of the *IL2RG* gene to restore wild-type function. For transfer of the ZFN and the donor DNA, they used a delivery system based on integrase-deficient lentivirus (IDLV) (Wanisch and Yanez-Munoz, 2009) and observed gene targeting in up to 6% of the *IL2RG* alleles in a human lymphoblastoid cell line. An alternative approach is the targeted insertion of an entire therapeutic expression cassette—including an autonomous promoter, transgene and polyadenylation signal—into a “safe harbor” locus (Fig. 2D). Ideally, a true safe harbor should satisfy the following criteria. First, the endogenous gene function should not be disrupted or disruption should not have any effect on the cell physiology. Second, native insulator regions should (i) protect transgene expression from position-effect variegation and silencing and (ii) prevent the transgene promoter from affecting endogenous promoters in the neighborhood. Although *CCR5* does not meet all of these requirements, the locus has been advanced by some as a potential safe harbor because naturally occurring null mutations seem to be well tolerated. Employing the Sangamo *CCR5*-specific ZFNs, Lombardo *et al.* (2007) achieved targeted addition of an *EGFP* marker cassette in 39% of Jurkat cells, 35% of K562 cells, 3.5% of human ESCs, and 0.11% of CD34⁺ hematopoietic progenitor cells, confirming that the efficiency of DSB-based gene targeting is highly cell-type specific. In another study, *CCR5*-specific ZFNs were used to target integration of a mouse erythropoietin expression cassette in human mesenchymal stromal cells (MSCs) (Benabdallah *et al.*, 2010). Transduction of these cells with adenoviral vectors for transient ZFN expression and an IDLV-based donor resulted in targeted transgene addition in 30% of cells. Immunodeficient NOD/SCID/ γ C mice injected intraperitoneally with these MSCs exhibited a significantly increased hematocrit, demonstrating that genetically modified MSCs can serve as a cellular production factory for secreted therapeutic proteins.

The *AAVS1* site may represent a better candidate for a safe harbor. *AAVS1* is a natural integration hotspot of adeno-associated virus type 2 within the *PPP1R12C* locus on chromosome 19 and has been reported to contain insulator regions (Ogata *et al.*, 2003) and to support stable and long-term transgene expression (Smith *et al.*, 2008). ZFNs designed to target intron 1 of the *PPP1R12C* locus mediated targeted integration in up to 50% of alleles in human ESCs and iPSCs after drug selection (Hockemeyer *et al.*, 2009). More recently, ZFNs targeted to *AAVS1* were also used to insert a minigene encoding gp91 in iPS cells derived from patients with X-linked chronic granulomatous disease who harbored disease-causing mutations in their endogenous gp91 locus. Introduction of the gp91 expression cassette led to partial restoration of gp91 function and restoration of reactive oxygen species production (Zou *et al.*, 2011).

In another human iPSC study, ZFN-mediated gene targeting was used to create a knockout mutation by inserting a drug resistance gene into the human *PIG-A* with the goal of creating a disease model for paroxysmal nocturnal hemoglobinuria (Zou *et al.*, 2009). Of note, in this and all but one study published to date, the use of drug selection has been required to identify iPS cells that have undergone successful gene-targeting events. This selection is required because the

frequency of gene targeting, although higher than in the absence of a DSB, is still low, with rates in the range of 1 in 10⁴ transfected cells or less (Cathomen and Schambach, 2010). In a therapeutic setting it will be critical to remove the antibiotic marker after selection for the desired gene targeting event, for example by flanking the selection cassette with *loxP* sites, which will then allow excision by transient expression of Cre recombinase (Maetzig *et al.*, 2010). The relatively high targeting efficiency in human iPSCs after selection demonstrates that ZFN-mediated gene targeting has the potential to develop into an efficient tool for safe genome engineering in patient-derived iPSCs and therefore holds great promise for personalized cell replacement therapy in the future.

Risk Assessment of ZFN-Mediated Genome Engineering

An ideal ZFN pair would introduce only two specific DSBs in the genome—one in each target allele (assuming the gene is not present on a monoploid chromosome). Such a specificity will probably never be achieved because even highly specific natural homing endonucleases, such as *I-SceI*, which cleaves an 18-bp target site that is not present in the human genome, revealed off-target DNA-cleavage activity when expressed in human cells (Petek *et al.*, 2010). Therefore, it is crucial to have tools at hand that allow researchers to assess ZFN specificity. One approach is to estimate off-target activity by visualizing the number of DSBs in the cell nucleus upon expression of the ZFN (Miller *et al.*, 2007; Szczepek *et al.*, 2007). While somewhat useful with some ZFNs, this assay is not sensitive enough to pick up ZFN-associated genotoxicity of third generation ZFNs (Perez *et al.*, 2008). Another simple experiment involves measuring cell survival upon ectopic ZFN expression (Cornu *et al.*, 2008). Although more sensitive because the recorded time period can be extended to several days, this assay gives no direct insight into the locations of DSBs that drive ZFN-induced cytotoxicity. A more informative approach is to determine the *in vitro* DNA-binding profile of each of the two ZFN monomers using SELEX (systematic evolution of ligands by exponential enrichment) or a bacterial one-hybrid system (Meng *et al.*, 2008; Gupta *et al.*, 2011). Based on the combined DNA-binding profiles of both ZFN subunits, putative off-target sites in the human genome can be predicted using bioinformatics. For example, Perez *et al.* (2008) performed such an analysis for the *CCR5*-specific ZFNs and analyzed the top 15 alternative target sites containing up to two mismatches per target half-site by pyrosequencing and a PCR-based assay to screen for ZFN-induced mutations at predicted genomic loci (also referred to as the “surveyor assay”). These assays revealed ZFN off-target activity in two additional loci, *ABLIM2* and *CCR2*. The frequency of ZFN-associated mutagenesis at *CCR2* was rather high, with 4% of alleles revealing indels. Although mutations in the *CCR2* locus in CD4⁺ cells are not likely to be deleterious (Smith *et al.*, 1997), these results underscore the importance of performing off-target analyses.

Although computational prediction of putative off-target sites based on *in vitro* DNA-binding profiles and subsequent analysis of the top sites by pyrosequencing is arguably the best current approach available to look at ZFN specificity, this strategy still has at least two significant limitations. First, the method only defines putative off-target sites based on the

DNA-binding specificities of ZFN monomers and not on the cleavage specificity of the dimeric ZFN complex. This distinction is potentially very important because mutations in the ZFN target site may have a greater impact on the binding of a monomeric zinc finger array than on the cleavage by a dimeric ZFN. Stated another way, the cooperativity between the two zinc-finger arrays may allow binding to and cleavage of sequences with a greater number of differences from the target site than might be predicted from the DNA-binding specificity profiles of a single monomer. Thus, the current computational approach may be failing to identify *bona fide* off-target sequences for analysis by next-generation sequencing. Second, the current approach will only detect ZFN-induced mutations that fall within the short fragment of the investigated DNA, meaning that large indels or translocations will be missed. As previously mentioned, Lee *et al.* (2010) reported that expression of CCR5-specific ZFNs induced a 15-kb chromosomal deletion in 10% of a transfected human cell line by simultaneously cleaving at the CCR5 and CCR2 locus. It is not yet known whether chromosomal deletions occurred in the ZFN-treated human CD4⁺ cells in the clinical trial. A concomitant loss of CCR2 and CCR5 may not necessarily be problematic (Smith *et al.*, 1997) but a potential CCR2-CCR5 fusion epitope may lead to unwanted immune reactions and elimination of the engineered T cells by the host immune system. A thorough cytogenetic analysis to exclude the occurrence of gross chromosomal aberrations would partially help to address this limitation.

In summary, an unbiased, genome-wide approach to detect ZFN-induced mutations is urgently needed and several laboratories are working towards this goal. A safe approach to assess the accuracy of therapeutic genome engineering will comprise a thorough genetic analysis, including whole genome sequencing on clonal cell populations, such as genetically corrected iPSCs (Cathomen and Schambach, 2010). The combination of “safe” genome engineering with clonal selection will permit stringent quality control to characterize and select for the “perfect” cell clone for further downstream applications in regenerative medicine, gene therapy, or cancer therapy.

Other Customized Nuclease Platforms

Two alternative platforms to ZFNs for engineering customized nucleases have been described: meganucleases and engineered TALE nucleases (TALENs).

Meganuclease technology involved redesign of the DNA-interacting residues of naturally occurring LAGLIDADG homing endonucleases, such as I-CreI, that cleave extended DNA sequences (Stoddard, 2011). Meganucleases have been engineered to target sequences in two human genes, *RAG1* and *XPC* (Redondo *et al.*, 2008; Grizot *et al.*, 2009), as well as the herpes simplex virus type 1 genome (Grosse *et al.*, 2011). Reasons for the slow coming of age of engineered meganucleases may be the complex structure of homing endonucleases. While a proprietary selection-based platform to design customized meganucleases has been developed by the company Collectis (Smith *et al.*, 2006), such high-throughput design processes are not readily convertible to academic laboratories. However, recent progress in structure-based computational design of meganucleases may facilitate the design process in the future (Ashworth *et al.*, 2010; Ulge *et al.*, 2011).

An alternative DNA-binding domain that has recently been used to generate engineered nucleases is that of tran-

scription activator-like effectors (TALEs) from the plant pathogen *Xanthomonas* (Christian *et al.*, 2010; Cermak *et al.*, 2011; Li *et al.*, 2011; Miller *et al.*, 2011). The central TALE repeat units are highly conserved ~34 amino acid motifs that mediate binding to DNA. Recent work has shown that an individual TALE repeat unit binds to a single basepair of DNA and a simple code has been described that relates the binding specificity of a TALE repeat to the identity of two variable residues within the repeat unit. Hence, this “one-repeat-to-one-base” code enables the prediction of the DNA binding sites of natural TALEs and, vice versa, the engineering of customized TALE repeat arrays that recognize a user-defined target sequence. Joining together multiple TALE domain repeats into an extended array can generate proteins capable of binding to DNA sequences of 12 bp or more in length. To date, TALENs have been used to target the human *CCR5*, *HPRT1*, and *NTF3* genes in transformed cell lines (Cermak *et al.*, 2011; Miller *et al.*, 2011). Although the present reports suggest that TALENs can be generated by simple “modular assembly” of individual TALE repeats, maximizing the activities and specificities of these nucleases may require additional protein engineering efforts. Moreover, similar to ZFNs, additional studies to determine the range of off-target effects induced by TALENs and meganucleases are needed.

Conclusions

ZFN technology allows for efficient and precise modification of the human genome. An important aspect of DSB-based genome engineering is the method and the efficiency of ZFN delivery. Ideally, ZFNs are present at a high concentration for a limited period of time, just long enough to create the DSB. Short-lived ZFN expression from episomal DNA-based expression vectors—such as plasmid DNA (Hockemeyer *et al.*, 2009; Holt *et al.*, 2010; Zou *et al.*, 2009), integrase-deficient lentiviral vectors (Cornu and Cathomen, 2007; Lombardo *et al.*, 2007), adenoviral vectors (Perez *et al.*, 2008), and vectors based on adeno-associated virus (Porteus *et al.*, 2003; Gellhaus *et al.*, 2010; Metzger *et al.*, 2011)—can only be achieved in mitotic cells, which ensures rapid dilution of the vectors during cell divisions. Because DNA-based vector systems have a tendency to integrate into the host genome (Cornu and Cathomen, 2007; Lombardo *et al.*, 2007; Gellhaus *et al.*, 2010; Olsen *et al.*, 2010), it will be important to closely follow the fate of the ZFN expression vectors in the target cells. An alternative way of delivering ZFNs is the transfer of ZFN-encoding mRNA, which ensures rapid but transient ZFN expression and avoids the issue of illegitimate integration (Doyon *et al.*, 2008; Meng *et al.*, 2008; Foley *et al.*, 2009; Geurts *et al.*, 2009; Mashimo *et al.*, 2010; Zou *et al.*, 2011). Microinjection of ZFN-encoding mRNA has been performed in zebrafish and rat single-cell embryos, and the ZFN-mediated gene disruption frequency was comparable to plasmid DNA delivery (Geurts *et al.*, 2009). Moreover, delivery of ZFNs by mRNA transfection has been used to target the integration of a transgene into the *AAVS1* locus in human iPSCs (Zou *et al.*, 2011).

If direct *in situ* correction of a disease locus is not an option, an important consideration will be to determine where to integrate a therapeutic transgene cassette into the human genome. The *AAVS1* site on chromosome 19 is thus far the

most promising candidate for such a safe harbor, as a native insulator region appears to both protect transgene expression from position-effect variegation and silencing and prevent the transgene promoter from affecting the host transcriptome.

The fact that ZFNs can be used to create knockout animals is especially encouraging and emphasizes the high specificity the technology has reached in the last 3 years. Moreover, the development of alternative designer nucleases, such as TALENs and meganucleases, has further spurred interest in targeted genome engineering approaches. Conversely, studies reporting ZFN off-target activities in zebrafish and human cells must not be overlooked and should serve as the basis for further improvement of the technology. The employment of highly specific designer nucleases is especially important when DSB-based genome engineering is applied to multipotent or pluripotent stem cells, such as HSCs or iPSCs, with their high proliferative potential. Even so, the remarkable progress achieved in the last few years demonstrates that ZFNs represent a tool that allows researchers and clinicians for the first time to rationally edit the genome of human cells and to take this technology from the bench to the bedside for therapeutic applications.

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