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REVIEWS

Biological and Biomedical Applications of Engineered Nucleases

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Abstract The development of engineered nucleases is the fruit of a new technological approach developed in the last two decades which has led to significant benefits on genome engineering, particularly on gene therapy. These applications enable efficient and specific genetic modifications via the induction of a double-strand break (DSB) in a specific genomic target sequence, followed by the homology-directed repair (HDR) or non-homologous end joining (NHEJ) pathways. In addition to the application on gene modification in cells and intact organisms, a number of recent papers have reported that this gene editing technology can be applied effectively to human diseases. With the promising data obtained using engineered endonucleases in gene therapy, it appears reasonable to expect that more diseases could be treated and even be cured in this new era of individualized medicine. This paper first brief introduces the development of engineered nucleases with a special emphasis on zinc-finger nucleases (ZFNs) and transcription activator-like effector (TALE) nucleases (TALENs), and then takes CCR5-based gene therapy as an example to discuss the therapeutic applications of engineered nucleases.

Keywords Engineered nucleases · ZFNs · TALENs · Gene therapy · CCR5 · HIV

Introduction

Gene therapy is a genetic intervention used for the prevention or treatment of diseases by targeting selected genes with specific nucleotides. It includes both traditional forward gene therapy and the recently developed reversed gene therapy. The major purpose of traditional gene therapy is to normalize an abnormal gene, or in other words it is to restore the function a dysfunctional gene. As an opposite strategy, reversed gene therapy [1] aims at suppressing or abolishing the function of a gene to balance a complicated regulatory system or at depleting cellular molecules essential for a disease mechanism as in pathogen infections.

In the past three decades, few diseases and few cases have been cured solely by traditional forward gene therapy and two major obstacles contributed to hampering its clinical output: the selection of gene targets and the transgene delivery system.

The technological development of several engineered nucleases may help to overcome the aforementioned difficulties and pave the way to gene therapy. Among the engineered nucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and meganucleases have been successfully employed for both

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in vitro and *ex vivo* gene modifications. Based on these milestone events and their great potential, gene editing technology with ZFNs and TALENs was recognized as the Technology of the Year by the journal "Nature-Methods."

Zinc-finger nucleases (ZFNs) include a DNA binding domain of a serially repeated zinc fingers and a nuclease domain of the FokI restriction enzyme. Each zinc finger can precisely recognize and combine 3 nucleotides. ZFNs function as dimers, in which each monomer of ZFNs usually contains 3–4 or even more repeated elements to specifically digest one target per genome (Fig. 1). As the ZFNs, TALENs are also made of a number of DNA binding modules and the nuclease domain from FokI restriction enzyme. Each protein module typically contains 34 amino acids, and the 12th and 13th residues are the key site of the target identification, known as the repeated variable di-residues (RVDs) [2–5]. Currently, there are seven recognized units decoded and four commonly used identification units: NI identifies A, HD identifies C, NG identifies T, and NN identifies G or A (Fig. 2). According to the identity or similarity of the 12th amino acid among these units, one can easily know that the nucleotide identification is crucially performed by the 13th amino acid based on a common sense: the exclusive difference is located at the site of the 13th amino acid but it is almost the same as the one located at the 12th amino acid. Thus, the identification to different nucleotides must be attributed to the different composition of the amino acid rather than to the identical or largely overlapping amino acids. This logical inference has recently been confirmed by X-ray microfluorescence (XRF) data. When a ZFNs or TALENs have repeated DNA binding elements in its DNA binding domain, they can then efficiently and precisely cleave specific DNA sequences and induce a double-strand break (DSB), followed by the homology-directed repair (HDR) or non-homologous end joining (NHEJ) pathways [6, 7].

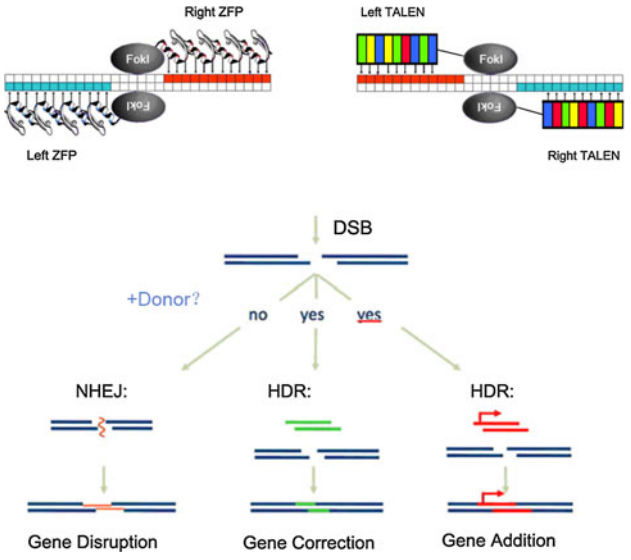

Fig. 1 Structures and applications of ZFNs and TALENs. ZFNs and TALENs recognize DNA targets through their DNA binding domains. While each zinc-finger protein module identifies three nucleotides, a TALE module only recognizes one nucleotide through its 13th amino acid. The cleavage domains of homodimers or heterodimers form an endonuclease that digests the target sequence and creates a double-strand break. Following the creation of double-strand break, gene editing efficiencies increase by two log scales as compared to those formed through spontaneous gene recombination, which greatly facilitates gene disruption, gene correction, or gene insertion

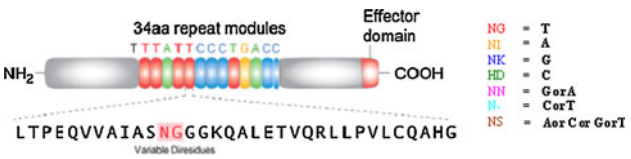

Fig. 2 TALE identification of specific nucleotide. Each TALE module typically contains 34 amino acids and the 12th and 13th residues are the key sites of the target identification known as the repeated variable di-residues (RVDs). There are seven recognized units decoded and four commonly used identification units: NI identifies A, HD identifies C, NG identifies T, and NN identifies G or A

Biomedical Applications of ZFNs and TALENs

ZFNs and TALENs have been successfully employed in genetic manipulations both in vitro and in vivo. Several endogenous genes modified by ZFNs and TALENs are listed in Table 1.

Gene Disruption

Gene disruption is the simplest means of gene editing. By causing a frameshift mutation, the disruption or abolishment of selected gene functions can easily be obtained. Engineered nucleases-driven gene disruption has already been used for model organisms and a variety of mammalian cells as listed in Table 1.

Injection of mRNA encoding ZFNs into the early Drosophila embryo has been used to disrupt a gene in *D. Melanogaster* [8]. As for ZFNs-mediated gene disruption in zebrafish [9–12], four separate studies illustrated that up to 50 % germline disruptions at the targeted genes were achieved. The first published example of the application of engineered ZFNs to disrupt an endogenous locus in a mammalian cell is a knockout of the dihydrofolate reductase (DHFR) gene in Chinese hamster ovary (CHO) cells, in which the disruption frequencies are up to 15 % of alleles in the cell population [13]. In addition, ZFNs have been used to make double [14] and triple [15] locus gene disruptions in CHO and K562 cells.

Table 1 Target genes modified by ZFNs and TALENs

Nucleases	Cells or Organisms	Gene	Refs
Zinc-finger nucleases	Fruit flies	Yellow	[42]
		Rosy, brown	[47]
		Coilin, pask	[8]
	CHO cells	Dhfr	[13]
		Dhfr, Glul	[15]
		Fut8	[48]
		Bax, bak1	[14]
	Zebrafish	kdr	[10]
		Golden, no tail	[9]
		tfr2, dat, telomerase, hif1aa, gridlock (also known as hey2)	[11]
		cxcr4a	[12]
	Human T cells	CCR5	[39]
	Hek293 cells	CCR5	[49] [61]
		TP53	[62]
		CDKN2A	[63]
	Rats	Rab38, IgM	[16]
		F9	[31]
		Il2rg	[50]
	SupT1 cells	CXCR4	[51]
	K562 cells, HeLa cells	PPP1R12C (the AAVS1 locus), TP73, MAP3K14, EP300, BTK, CARM1,	[52]
		GNAI2, TSC2, RIPK1, KDR, NR3C1	
	K562 cells, human T cells	IL2RG	[29]
	K562 cells	IL2RG, VEGF, HOXB13, CFTR	[30]
	Tobacco	SuRA, SuRB (acetolactate	[32]
		synthase genes)	
		Chitinase	[21]
	Arabidopsis thaliana	ABI4, KU80	[33]
		ADH1, TT4	[53]
	Mouse ES cells	H3f3b	[34]
	Mouse NIH3T3 cells	Thumpd3	[63]
	Human ES cells	IL2RG, CCR5	[25]
		PIGA	[54]
		OCT4 (also known as POU5F1), PPP1R12C (AAVS1 locus), PITX3	[27]
	Maize	lpk1, Zein protein 15	[22]
TAL effector nucleases	HEK293 cells	NTF3, CCR5	[18]
		TP53	[62]
	Human ES cells	SOX2, KLF4	[55]
	Nematode	Endogenous genes and cis-acting regulatory elements	[56]
	Rat S16 cells	IgM locus	[57]
	Zebra fish	tnikb gene	[19]
	Human pluripotent cells	PPP1R12C (the AAVS1 locus), OCT4 (also known as POU5F1), PITX3 genes	[28]
	Fish	pCSKAGFPS-I, paact-GFPI2	[58]
	Medaka	Sleeping Beauty transposon	[59]
	Fibroblast (COS-7) and BSR cells	HSV1 sequences	[60]

In a study related to a rat model of severe combined immune deficiency (SCID), ZFNs was used to target an integrated reporter and two endogenous rat genes of immunoglobulin M (IgM) and Rab38 [16]. From 25 to 100 % of the rats developed from these ZFNs-treated one-cell embryos showed genotypes carrying disrupted target loci. These mutations are faithfully and efficiently transmitted through the germlines. In human and other eukaryotic cells, specific ZFNs deleted predetermined genomic DNA segments in the range of 15 to several-hundred base pairs (bp) at frequencies of 10^{-3}–10^{-1}[17]. TALENs can also efficiently disrupt the zebrafish genes as well as the endogenous human genes NTF3 and CCR5 at efficiencies of up to 25 % [18, 19].

Gene addition

The great potential of genetically modified endonucleases in genetic medicine, biotechnology, and molecular biology made this genomic approach a hot topic in biomedical research. Currently, more and more cases in gene therapy seem to be focusing on targeted gene additions. This approach consists of introducing DNA sequences into a predetermined genomic location. In order to make this strategy work, two essential properties should be considered [20]. First, the inserted DNA sequences must functionally integrate within the modified cells. Second, the normal function and gene regulation in cells harboring the predetermined genomic location should not be blocked, or in other words, no off-target effect is required following the gene modification or gene addition.

ZFNs-driven gene addition was achieved in plants in two different ways. A Ti plasmid harboring both the ZFNs and a donor DNA construct including a disease resistance gene cassette was delivered in tobacco cells. This strategy achieved targeted, homology-directed transgene integration in approximately 10 % of the ZFN cleavage site [21]. Alternatively, simultaneous expression of ZFNs and delivery of a simple heterologous donor molecule also led to precise targeted addition of an herbicide-tolerance gene at the intended locus in plants of the crop species Zea mays [22].

In addition to plant cells, this approach has been applied to gene addition in cells from various species including human cells. In mouse embryo stem (ES) cells, ZFNs were used to generate an isogenic panel carrying a defined series of alleles for an endogenous gene [23]. Using ZFNs directed against IL2RG and an extrachromosomal DNA donor carrying homology arms of 750 bp, transgenes positioned precisely in the ZFNs recognition site. In this study, ZFNs could drive the addition of an as long as 8 kb sequence carrying three distinct promoter-transcription units into an endogenous locus at a frequency of 6 % [24].

Gene addition has also been achieved in human ES and induced pluripotent stem (iPS) cells. The first demonstration of ZFN-mediated gene addition was done with a ZFN expression cassette and a donor construct delivered by an integrase-defective lentiviral vectors (IDLV) in human ES cells [25]. While a high ratio of gene addition (up to 50 %) was obtained in a panel of human cell lines, a relatively low efficiency was observed in human embryonic stem cells (5 % with gene addition). In human mesenchymal stromal cells (MSC), zinc-finger nuclease (ZFN)-mediated targeted addition of the erythropoietin (Epo) gene into

Table 2 Major types of mutations causing human diseases

Data type	Number of entries (public release for academic/non-profits only) Total (public release) 85840	No. of entries (HGMD professional release 2011.4) Total (HGMD professional 2011.4) 120004
Mutation data		
Missense/nonsense	48,633	66,902
Splicing	8,131	11,220
Regulatory	1,395	2,245
Small deletions	13,680	18,799
Small insertions	5,599	7,719
Small indels	1,243	1,783
Repeat variations	260	377
Gross insertions/duplications	1,001	1,757
Complex rearrangements	737	1,170
Gross deletions	5,161	8,032
Gene/sequence data		
Genes	3,253	4,411
cDNA reference sequences	3,195	4,320

The top six types of mutations are the major targets editable by TALENs because these mutations are relatively short and can be efficiently edited. Indels listed in the table indicate mutations with both inserted and deleted nucleotides simultaneously

CCR5 gene locus [26] would result in 30–40 % targeted gene addition and stable transgene expression in vivo. With a similar approach for the addition of genes into hESCs [27] using ZFNs, TALEN expression constructs and the corresponding donor plasmids were introduced into human embryonic stem cell (ESC); the TALEN-mediated targeting of PPP1R12C [28] resulted in a robust transgene expression in pluripotent as well as in differentiated cells.

Gene correction

According to the Human Genome Mutation Database (Table 2), in more than a hundred thousand mutations causing human diseases, 80–90 % is caused by the substitution, deletion, and insertion of one nucleotide. Therefore, the application of engineered nucleases for gene correction at a specific locus has been considered as a crucial target for gene therapy. As evidenced by its widespread use in experimental model organisms, plant, and mammalian cells, engineered nucleases-mediated gene corrections appear to have high therapeutic potential, particularly to point mutations and several other types of mutations with short sequences involved.

The gene correction based on zinc-finger nucleases in human cells was first reported in a X-linked SCID mutation in the IL2Rγ gene in which more than 18 % of gene-modified human cells were observed [29]. It is noteworthy that about 7 % of the cells acquired the desired genetic modification on both X chromosomes. High frequencies were also observed at this locus in human $CD4^+$ T cell. The same approach was applied with relatively high efficiencies (1–50 %) to three additional human genes (VEGF-A, HoxB13, and CFTR) in transformed cells [30].

In a mouse model of hemophilia B [31], ZFNs co-delivered with an appropriately designed gene-targeting vector stimulated gene correction in vivo. As a consequence, the gene therapy treatment was sufficient to correct the prolonged clotting times in this mouse model of hemophilia B, and remained a persistent effect after the induced liver regeneration. In addition, gene corrections using ZFNs and TALENs have also been achieved in tobacco, fruit flies, *Arabidopsis thaliana*, and mouse ES cells for a variety of gene abnormalities [32–34].

Comparison Between the Enzymatic Properties and Potential Application Values of ZFNs and TALENs

TALENs have shown some advantages over ZFNs in genetic modifications, gene therapy, and selected other uses [35]. The base identification mechanisms of these enzymes are different. Each zinc finger can recognize three nucleotides whereas each TALE only recognizes one nucleotide. Compared with ZFNs, the cloning process of TALENs is easier, the specificity of recognized target sequences is higher, and their off-target effects are lower. Additionally, there will be less possible intellectual property disputes in the application of TALENs as compared to ZFNs. In Table 3, the characteristic features of ZFNs and TALENs are summarized.

The proper targets of TALENs used in gene therapy are single base mutations, small deletions, or small insertions. As reported in the Human Genome Mutation Database, these three types of mutations cover over 80 % of known monogenic diseases. Additionally, TALENs can be used in certain polygenic diseases such as essential hypertension or infectious diseases such as HIV. Interestingly, a recent study by Dr. JS Kim demonstrated that ZFNs are able to edit DNA over a range of kilobase-pairs and cause mutations [36]. In addition to the possible application in the making of selected disease models, Dr. Kim's study may suggest the therapeutic potential of engineered nucleases of both ZFNs and TALENs in complicated genetic diseases [17].

As mentioned previously, reversed gene therapy involves functional or structural normal gene knockout. Functional gene knockout usually refers to simple gene code frameshifting. However, structural gene knockout requires exogenous gene fragment. Specific DNA sequences are precisely cleaved to form a DSB, followed by the HDR or NHEJ pathways. Therefore, the engineered nucleases such as ZFNs and TALENs could achieve efficient and designed homologous recombinations with their specific cleavage of gene targets in the presence of exogenous homologous genes.

Therapeutic Application of ZFNs and TALENs in HIV/AIDS Treatment

Conventional strategies including chemotherapy and vaccination have limited efficacy in fighting HIV infection. Chemotherapy treatment is restricted due to drug resistant virus and debilitating side effects. HIV vaccine development has never succeeded to pass the clinical trial phase. As listed in Table 4, HIV infection is a candidate for nuclease-mediated gene therapy and a few projects are actually in phase II clinical trials.

HIV infection requires the presence of the co-receptors chemokine (C–C motif) receptor type 5 (CCR5) [37] or chemokine (C-X-C motif) receptor type 4 (CXCR4) [38]. Epidemiological data showed that individuals with homozygous Δ32 deletion are absolutely resistant to the infection by CCR5-tropic HIV-1. Individuals with the heterozygous CCR5-Δ32 genotype have a lower possibility of being infected with HIV and take a longer time to

Table 3 Characteristic features of ZFNs and TALENs

Items	ZFNs	TALENs
First reported	ZFP: Miller et al. [66]	TALE: Bonas et al. [65]
First developed	ZFN: Kim et al. [67]	TALEN: Christian et al. [6]
Composition	A DNA binding domain of a serially repeated zinc fingers and a nuclease domain of the FokI restriction enzyme	A DNA binding domain of a serially repeated TALE and a nuclease domain of the FokI restriction enzyme
The code of DNA binding specificity	Each zinc figure can recognize three nucleotides	Each TALE can recognize one nucleotide
Off-target cleavage specificity	4/12	2/18
Cloning difficulty	Difficult to clone a ZFN with high activity; more off-target effects reported	Golden gate and its derivatives are available for cloning; less tolerant to mismatched targets

Table 4 Therapeutic applications of ZFNs and TALENs in gene therapy

Type of nucleases	Disease treated	Research stage	References or websites
Zinc-finger nucleases	HIV/AIDS	The phase I clinical trial (NCT00842634 and NCT01044654) The phase II clinical trial	http://www.sangamo.com/pipeline/sb-728.html
	Glioblastoma	The phase I clinical trial (NCT01082926)	http://www.sangamo.com/pipeline/sb-313.html
	Hemophilia B	Preclinical research	http://www.sangamo.com/pipeline/index.html
	Parkinson's disease		
	Neuropathic pain		
	Huntington's disease		
	Spinal cord injury		
	Traumatic brain injury		
	Stroke		
	Hemoglobinopathies	Research stage	http://www.sangamo.com/pipeline/rare-diseases.html
	Lysosomal storage disorders		
TAL effector nucleases	Sickle cell disease	Research stage	[64]
	HIV/AIDS	Research stage	[42]
	Thalassemia	Research stage	http://en.wikipedia.org/wiki/Transcription_Activator-Like_Effector_Nuclease
	HCM		

progress to AIDS after the infection. On the other hand, the protective effect of anti-CCR5 IgA against HIV infection has been confirmed [39]. The aforementioned pieces of evidence prompted us and other groups to target CCR5 as a novel gene therapy strategy. In a paper published in April 2005, we described these HIV gene therapy strategies as follows: "In a therapeutic point of view AIDS can be treated as aplastic anemia or leukemia and the patients transplanted with bone marrow carrying CCR5-Δ32. … However, allogenic bone marrow transplantation has two drawbacks: immune rejection and the limited number of available donors. … Thus, in order to be more beneficial to more HIV-infected people, it is technically easier to introduce the CCR5-Δ32 genotype through genetic engineering of hemopoietic stem cells isolated from the patients themselves."

Due to its efficacy and specificity, ZFN-mediated gene therapy has been used for the clinical treatment of HIV. Delivery of ZFNs targeting CCR5 using recombinant adenoviral vector was shown to disrupt >50 % of CCR5 alleles both in model cell lines and the primary human $CD4^+$ T cells [40]. In the HIV-1-infected mice that received ZFN-modified $CD4^+$ T cells, there was a substantial reduction in viral load and an increase in $CD4^+$ T cell counts in peripheral tissues. Another study showed that ZFNs can disrupt CCR5 in human $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) at a mean frequency of 17 % of the total alleles in a population [41]. The results of this study suggested that ZFN-modified autologous hematopoietic stem cells could be considered a clinical approach to treating HIV-1.

Currently, two phase I clinical trials (NCT00842634 and NCT01044654) have been completed and one phase II trial

investigating the clinical application of ZFNs-modified $CD4^+$ T cells targeting CCR5 in patients with HIV/AIDS is in progress. These trials should bring support to the use of CCR5 ZFN-modified autologous $CD4^+$ T cells in HIV patients as an attractive approach for the treatment of HIV-1 infection.

As one of the valuable engineered nucleases, TALENs were also designed to disrupt an EGFP marker gene and the human loci CCR5 [42]. Gene editing was achieved in up to 45 % of transfected cells. In comparison with ZFNs, TALENs showed similar gene disruption activities but significantly reduced nuclease-associated cytotoxicity and revealed only minimal off-target activity. Consequently, TALENs are considered as a key technology for targeted modifications of complex genomes.

Recently, the reported cure of an HIV-1 patient with acute myeloid leukemia appeared as an important milestone for the application of strategies targeting CCR5 [43, 44]. These results strongly supported the application of engineered nucleases in *ex vivo* and in vivo gene therapies to HIV infection.

Future Expectations

The fundamental barriers preventing the application of ZFNs and TALENs in patient treatment are their off-target effects and the immunological response of the organism to new genetically modified or corrected proteins. To some extent, reversed gene therapy may help to bypass these barriers [45, 46]. First, reversed gene therapy involves either local or temporal gene knock-out and may have a therapeutic effect or even cure selected diseases. It is less toxic compared to systematic or life time gene knock-out. Second, reversed gene therapy can be achieved simply by functional knock-out. Off-target effects can be better controlled in reversed gene therapy compared to gene correction as reversed gene therapy has a nearly whole-gene zone for the target of choice.

As far as the therapeutic outcomes of reversed gene therapy are concerned, the following points need to be understood. In genetic disorders such as enzyme deficiencies, a 5–10 % elevation of a missed enzyme may significantly relieve or even eliminate the clinical symptoms. Although a proportional disruption of an oncogene may have limited therapeutic effects on tumor, reversed gene therapy can be a component of a combined or synergistic cancer therapy. At this early age of reversed gene therapy, it would not be used to replace traditional chemotherapy. Instead, it will be mainly used for diseases lacking effective therapies, such as the aforementioned inherited genetic diseases and cancers. Infectious diseases are another type of candidates suitable for reversed gene therapy. Since most of the infections are strictly dependent on the pathogen–host interactions, genetic manipulations blocking these interactions either by targeting the pathogen or the host may successfully prevent or inhibit an infection. Just like chemotherapy treatments, an infection is not cured by direct pathogen killing by antibiotics, but by the immune system. For example, the transfusion of in vitro modified CD4 cells have no effect at all in wiping out existing HIV, but the increased level of CD4 cells helps to improve host's immune system and to slow down the replication process of HIV, which makes a cure of HIV infection possible.

The newly developed reversed gene therapy appears promising for the treatment of hemophilia, thalassemia, DMD, neuronal deafness, Stargardt's syndrome, essential hypertension, and TP53 gene mutation as well as lung, breast, cervix, prostate, colon, rectum cancers, and leukemia. This list will get longer with a better understanding of the pathophysiology of human diseases. TALENs are a new type of "drugable" macromolecules. As compared to traditional drugs, the effectiveness and toxicity of TALENs are easy to determine by screening their gene editing spectra and off-target effects. With the availability of the next generation sequencing technology, both gene editing spectra and off-target effects can be efficiently assayed using cultured human cells. Thus, engineered nucleases particularly the "drugable" TALENs are expected to be invaluable for future gene therapy. However, the gene editing technology is only one approach in gene therapy as the lack of gene delivery is still an obstacle to overcome. Fortunately, both lentiviral and adenoviral vectors are efficient enough to deliver ZFN and TALENs in vivo and *ex vivo*, particularly when used in reversed gene therapies that only requires a partial knockout of a selected gene to reach an observable clinical symptom improvement.

On the basis of the current state of knowledge, it is reasonable to suggest that the development of engineered nucleases described in this review will hold much promise for their use in safe and effective gene therapy approaches. We also have reasons to believe that gene therapy including reversed gene therapy with these nucleases and advances in stem cell technologies will provide effective treatments to lower the clinical severities or even cure a number of currently untreated or poorly treated diseases.

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