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TALEN: A Tool for Genome Editing

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ABSTRACT

Genome editing by engineered nuclease enzymes, which, induce DNA double strand break (DSB) followed by the activation of repair mechanisms, is of great importance to researchers in the field of biology and medicine. TALEN (Transcription activator-like effector nuclease) as a tool for genome editing is widely used in a variety of organisms and cells to study the role of genes or mutations in biological or pathological pathways, create model organisms or a therapeutic approach to single genetic disorders such as sickle cell anemia. TALE consists of: 1) N-terminal T3S signal, 2) Central tandem repeat domain, 3) C-terminal nuclear localization signal (NLS) and 4). Acidic transcriptional activation domain (AD, fused to a non-specific domain for DNA double strand break). DSB can undergo changes such as insertion, deletion, inversion, translocation through two major repair pathways of NHEJ and HDR. In this review article, we will first discuss the TALEN technology, assembly of custom TALEN and its transmission techniques. This is followed by studies on genome editing, various repair mechanisms and donor design in the HDR pathway. Finally, the applications of this technology will be discussed.

Keywords: ZFN, CRISPR/CAS9, Gene therapy, DNA repair mechanisms, TALEN transmission

INTRODUCTION

Genome editing refers to a precise change in a target DNA sequence, obtained by controlling the accurate DNA double strand break and manipulating the cell repair system [1,2]. Genome editing is a broad approach that can produce various changes in different sequences of living cells or organisms [3]. There are a number of known approaches for genome editing including those, using engineered nuclease enzymes such as mega nuclease, ZFN (zinc finger nuclease), TALEN (Transcription activator-like effector nuclease), CRISPR/CAS9 [4,5] or viral vectors such as RAAV (Recombinant adeno associated virus) [6] and Transposons [7]. Engineered nuclease enzymes induce DNA double strand break (DSB) and stimulation of the cell repair machinery to make modifications [5]. There are two pathways for DSB repair [8]. Non-homologous end joining (NHEJ) is the dominant mechanism in multi-cellular organisms [9] in which the two break ends are directly ligated [5]. This pathway is error prone [4,9], because even if the break ends are related to a chromosome, the DNA repair mechanism leads to the deletion of several nucleotides [8]. In fact, NHEJ can cause changes such as insertion, deletion, or a combination of both and also large deletions, Inversions and translocations [2,10]. The second mechanism is homology directed repair (HDR), through which homologous chromosome sequence is used to repair and replace damaged nucleotides. Unlike NHEJ, genetic information of the break ends can be substituted by DNA sequence pattern [10,11]. Generally, genomic changes are classified into two categories: 1) loss of function and 2) gain of function [12]. Until recently, most of the loss of function and gain of function changes were performed, respectively, through RNAi [13,14] and transgenesis, two powerful techniques, however, with limitations [15].

Genome editing can be used to answer a variety of questions, such as the role of a gene or a specific mutation in a pathological or biological pathway, or can be applied as a therapeutic approach for genetic disorders, including single gene diseases, such as sickle cell anemia and cystic fibrosis. Due to its broad importance, genome editing with engineered nucleases was introduced as the 2011 method [3].

TALE STRUCTURE

TALE (Transcription activator-like effector) is the

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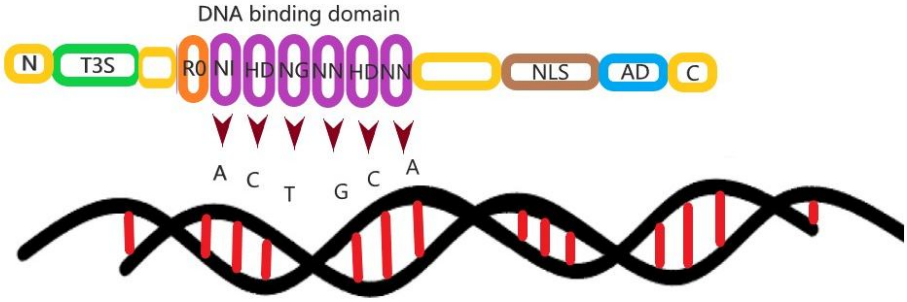

Fig. 1. TALE structure. 1) N-terminal T3S signal, 2) central tandem repeat domain, 3) C-terminal nuclear localization signal (NLS) and 4) Acidic transcriptional activation domain (AD). A repeat 0 (R0) which is located before the central domain plays crucial role in interaction with thymine.

family of protein effector which is secreted by *Xanthomonas spp* [5,8,10]. *Xanthomonas* is gram-negative bacterium [16] and pathogenesis of plants like rice [4,10], tomato and pepper. When these bacteria infect plants, TALE is delivered to the host plant cell nucleus through type lll secretion system [10,17] which is caused transcriptionally changes in gene expression of the host cells. Typically, this process facilitates the colonization of bacteria [16,18,19].

TALE structure includes: 1) N-terminal T3S signal (signal for secretion and transmission), 2) Central tandem repeat domain, 3) C-terminal nuclear localization signal (NLS) and 4) Acidic transcriptional activation domain (AD) [10,16,20] (Fig. 1). Each repetition of part 2 includes 33-35 conserved amino acids [8,21] differing only at positions 12 and 13 and are referred to as the RVDs (repeat variable di-residues) [5,8,22]. Each RVD binds specifically to one DNA nucleotide in a 1:1 ratio [5,18]. However, residue number 13 plays a key role in specific bond, while residue number 12 has a helper role for the stability of this structure [3,19]. There is also a repeat with 20 amino acids refered to as a half-repeat [23]. Repeat 0 in the structure of the TAL effector, located before the central domain [10], plays a crucial role in the interaction with thymine [16]. Repeat 0 has a helix-turn-helix motif which contains tryptophan 232 in a turn region that makes a van der Waals contact with the methyl group of thymine [24]. Presence of thymine in this location limits the number of DNA sequences targeted by TALE. Researchers have seen that through TALE like domain, which is related to plant pathogenesis, they can identify G instead of T. But, investigation for finding A and C bases will be continued [25].

Repeat Variable Diresidues (RVDs)

There are more than 25 types of RVDs, all capable of recognizing specific nucleotides [23]. The most common RVDs include NI, HD, NG and NN that are able to recognize A, C, T and G/A, respectively [8] (Fig. 1). Some RVDs such as NS and NN have codon degeneracy which bind to any nucleotide and A/G, respectively, therefore, they can bind to multiple DNA sequences [26]. Each repeat unit of RVD forms a left-handed helix-loop-helix, which enable the RVD to be exposed to the major groove of DNA [19]. Each RVD consists of two residues that the first residue (residue 12 is Histidine or Asparagine) does not directly bind to the DNA sequence. The side chain of residue 12 forms a hydrogen bond with the alanine (residue 8). The hydrogen bond is bonded to the oxygen in the carbonyl group (Alanine) which is caused a stable structure. The second residue directly bind to the DNA [3,10]. An RVD called NK, which is more specific than NN for G, has a lower catalytic activity [3]. HD and NN RVDs are able to make strong hydrogen bonds with DNA nucleotides, however, an efficient TALEN would require a minimum of 3-4 RVDs with strong hydrogen bonding capability [23]. *Xanthomonas* includes 1.5-33.5 repeated units (RVD) but whether all of them are effective in DNA sequence cleavage or not is unknown [23,27]. Types of RVD can affect the efficiency of TALE. For example, a TALE contains 10.5 repeated units which includes RVD with weak

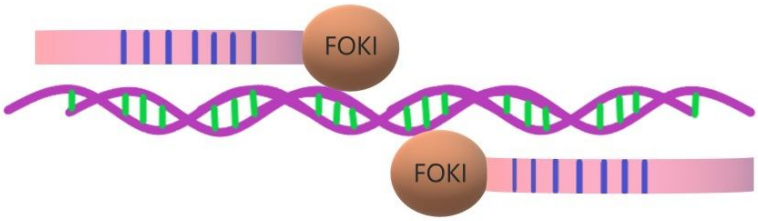

Fig. 2. FokI endonuclease is a bacterial restriction endonuclease type llS. Two TALEN subunits should be assembled at the cleavage site to activate catalytic domain of Fokl.

binding, does not induce target changes [23]. Natural TALEs have a level of mismatch tolerance [28]. Primary mismatches usually have serious complications since binding to the target sequence is in the N-terminal domain [10,23].

TALEN

TALE is a versatile protein and can be fused to different functional [29] proteins such as activators, repressors, histone modifiers, DNA demethylases, recombinases [13] and the FokI endonuclease. TALEN is generated by fusion of TALE to FokI endonuclease domain like ZFN (Zinc finger nuclease) [4,16]. This enzyme is found in *Flavobacterium okeanakoites* that is a bacterial restriction endonuclease type llS which consists of N-terminal DNA binding domain and C-terminal nonspecific DNA cleavage domain [19,30]. FokI endonuclease must dimerize to become active [8]. Two TALEN subunits should be assembled at the cleavage site to activate catalytic domain of Fokl [16] (Fig. 2). Wild type nuclease is homodimer and high risk for off-target cleavages, so reduce genome editing efficiency [8]. To overcome this problem, mutations were introduced in the catalytic domain that interfere with electrostatic and hydrophobic interactions and prevent the formation of the homodimer. These mutations in Fokl that create heterodimer, successfully reduce off-target cleavage [13,25].

DSB (double strand break) efficiency can be affected by the length of N-terminal and C-terminal domains. If the N-terminal is +136 and the C-terminal is +63, they will be more active and useful [8]. The +136/+63 scaffold can cleave 13-33 bps spacer nucleotides. It is interesting that non-RVD variations of each repetition unit can also affect genome editing activity. In the natural TALEs, some amino acids, especially those located in 4 and 32 positions, are non-RVD variations. The application of such changes in TALEN increases its efficiency than the conventional ones [31,32].

Cleavage function of TALEN is affected by epigenetic changes in the target site. There are strategies to overcome this limitation such as chemical inhibition of DNA methyl transferase and /the use of a TALEN in which asparagine is in position 12, but residue number 13 should be deleted [16].

Off-target cleavage causes cytotoxicity and disruption in the organism. There are two main ways to decrease off-target cleavage: 1) using a TALEN with higher repetitious units [28]; 2) using a heterodimer FokI to prevent random cleavage [33].

Assembly of Custom TALEN

Generation of plasmids encoding similar repeat sequences and long arrays can be difficult and challenging so to overcome this problem, different techniques have been developed for assembling the central repeat domain [25]. The most common of these platforms that are fast and cost effective are: 1) Golden gate cloning-based assembly, 2) Golden gate PCR-based assembly, 3) Restriction enzyme and ligation assembly (REAL) and 4) Fast ligation-based automatable solid-phase high throughput system (FLASH)(4). Each of these methods are discussed briefly below.

1) Golden gate cloning-based assembly: In this method a set of elements including the repetitive units, ligase and restriction enzyme type IIS [8] are used just in one reaction. The enzyme eventually creates an overhang that is a non-palindromic 4-nucleotide sequence [8,17]. Construction of

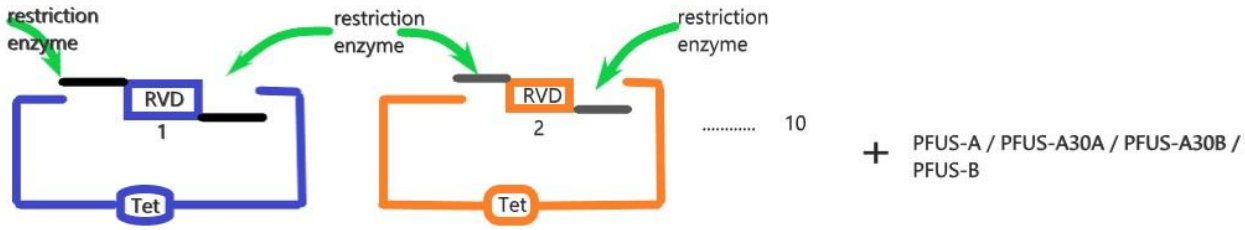

Fig. 3. Golden gate cloning-based assembly. Restriction enzymes are used to cut plasmid units encoding primary 10 target RVDs. This array must be cloned in PFUS-A and for RVDs 11-15 cloned in PFUS-B plasmid. In the arrays containing 22-31 repetitive units, the first 10 units are cloned in PFUS-A30A, the second 10 units are cloned in PFUS-A30B and the rest in PFUS-B.

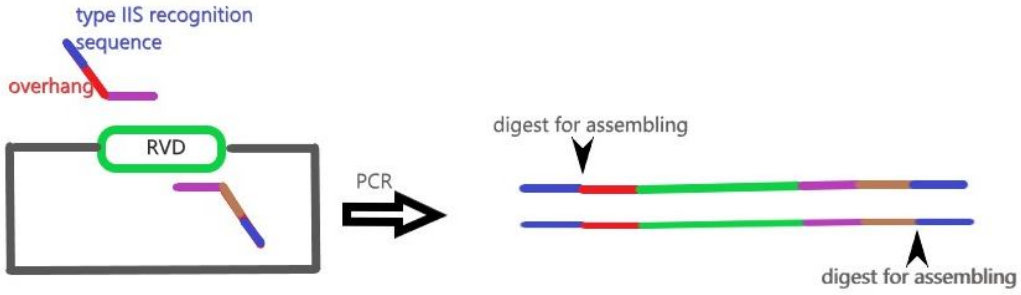

Fig. 4. Golden gate PCR based assembly. Repeat units are initially replicated through appropriate primers. The replicated fragments are assembled and duplicated through PCR for the second round of the assembling process.

TALEN includes two steps: a) Construction of an array containing 1-10 repeats through assembling repetitive units. b) Binding the arrays to a basic plasmid to create the final structure.

There is a library of plasmids for the synthesis of repetitive units. On the first day restriction enzymes are used to cut plasmid units encoding primary 10 target RVDs. This array must be cloned in a PFUS-A plasmid. Similar Previous step, is performed for RVDs 11-15 and cloned in PFUS-B. Note that the 16th and the last RVD is encoded by a different plasmid and added on the third day. In the arrays containing 22-31 repetitive units, the first 10 units are cloned in PFUS-A30A, the second 10 units are cloned in PFUS-A30B and the rest in PFUS-B (Fig. 3). Plasmids are introduced into *E. Coli* and grown in a culture medium containing x-gal and IPTG for white/blue screening. Some white colonies are grown overnight on the second day. On the third day, after extraction of plasmid DNA, identification of clones include target arrays through restriction enzymes and gel electrophoresis. At this step, arrays and the last repeat insert in to the plasmids. Finally, with the entry into the *E. coli*, the ampicillin is used to select the transformants. The procedure on the fourth day is similar to that of the second day. DNA extraction and detecting clones containing the final product with the appropriate length of repetitions are performed on the fifth day. The length of the repetition can be checked through sequencing or restriction enzyme analysis [17,29,34].

2) Golden gate PCR based assembly: In this approach, repeat units are initially replicated through appropriate primers using 4 plasmids encoding NN, NG, NI and HD. The replicated fragments are purified and assembled for the first stage. The ligated fragments are duplicated through PCR and used for the second round of the assembly process (Fig. 4). Compared to the cloning technique, a lower number of required plasmids and time are needed [30].

3) Restriction enzyme and ligation assembly: This method is based on the restriction enzyme type lls. Through

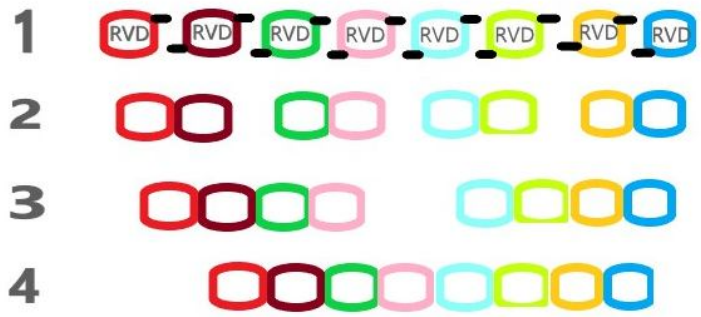

Fig. 5. Restriction enzyme and ligation assembly. Through compatible sequence overhangs, two TALE repeats are assembled. The product, which is a dimmer, is connected to another dimmer and so goes in the same way.

Fig. 6. Fast ligation based automatable solid-phase high throughput system. A repeating unit binds to the biotin from the N terminus, through which it can be attached to a magnetic beads coated with streptavidin and connected from the C terminus to the 4 pre-connected repetitive units.

compatible sequence overhangs, two TALE repeats are assembled. The product, a dimmer, is connected to another dimmer and so on (Fig. 5). TALENs with non RVD-variation can be synthesized *via* this method. REAL is the simplest method, but requires more time than other methods due to multiple cloning steps. The improved version is REAL-fast, in which 2-4 pre-connected units are used instead of single units. Although it is not time consuming, but more than 350 plasmids are needed in this technique [30].

4) Fast ligation based automatable solid-phase high throughput system: This method is similar to the REAL-fast method, however, the main difference is that repetitions are bound to magnetic beads. A repeating unit binds to the biotin from the N terminus, through which it can be attached to a magnetic bead coated with streptavidin and connected from the C terminus to the 4 pre-connected repetitive units (Fig. 6). Following purification, serial digestion and ligation are used for their assembly. A total of 96 different repeats are assembled in less than a day [25,35].

Transmission Techniques

To gain successful application of genome editing in a target cell, the transfer of nucleases into the cells is essential, which can be performed in vitro, in vivo and ex vivo. Nucleases can be transfered as DNA, RNA and protein *in vitro*, however, DNA transfection is the most commonly choice for primary research purposes. Three types of DNA transfection methods are used to transfer nucleases to mammalian cells [36]. 1) Biochemical methods: Several chemical compounds, such as lipofection (lipid-based transfection), are commonly used as early choices. But before use, the efficiency of these compounds should be compared with the target cell. 2) Physical methods: Electroporation and microinjection [37] are used for primary cells or stem cells where cell transformation is difficult. Electroporation through external electrical pulses generates transient pores in the cell membrane that delivers foreign DNA, which can be widely used in various types of prokaryotic and eukaryotic cells [36,38]. However, depending on the type of cell, electrical parameters such as

voltage should be determined. To increase the efficiency of this technique, the nucleofection was developed in 1998, which combines high voltage and high ionic strength. In 2006, the neon transfection system used an electronic pipette instead of the standard electroporation cuvettes. Nepagene, a Japanese biotechnology company created a new electroporator called NEPA21, which combines two types of poring and transfer electrical pulses. Poring pulse produces short high-voltage pulses to create pores in the membrane, while the transfer pulse produces a longer pulse with low voltage for DNA entry [36]. 3) Viral methods: Viral vectors are useful tools for the gene delivery into mammalian cells. For instance, adenoviral vector [5,37] and baculovirus [16] are used for the delivery of TALENs. Integrase-deficient lentiviral [16] vectors are used for DNA donor transport in a homology-directed repair (HDR) [39].

Bacterial transfection: Some pathogenic bacteria, such as *Pseudomonas aeruginosa*, have a needle-like appendage for transferring bacterial proteins to host cells. In this method, expression of the TALEN protein occurs in Pseudomonas, and then the Hela cells are infected by the bacteria. Therefore, TALEN proteins are injected into Hela cells directly through the Type III secretion system [36].

In *in vivo* conditions, there are several vectors that carry TALEN expression cassettes which include RNA POL II promoter, TALE encoding sequence, restriction endonuclease FokI, and a polyadenylation signal. Viral vectors include the Lentiviral and high-capacity Adenoviral (HC-Ad) vectors [37]. Since the HC-Ad vector does not contain viral encoding sequences, the vector does not stimulate the acquired and the innate immune systems [40]. It possesses inverted terminal repeats and a packaging signal at the 5′ end [37]. Another method uses the cationic polymer-based delivery vectors in which the plasmids encoding TALEN can easily inject into the tissue or target cells. Researchers utilized the Turbofect (cationic vector) for direct delivery of TALEN plasmids to the cervix of transgenic mouse model of human papillomavirus and cervical cancer which resulted in a decrease in tumor volume. Inflammation and off-target cleavage were also not detected [37].

TALEN proteins do not directly and alone penetrate the cell membrane. Therefore, cell-penetrating peptide (CPP) conjugation should be used. Covalent conjugation of TAT to TALEN [16] causes its entery into the target cells and damages the CCR5 encoding gene to prevent the HIV entry into the cells. As noted, due to the large size of TALENs and repeat arrays, gene transfer *in vivo* is difficult [41], while ex-vivo delivery can be more easily performed [42]. In fact, therapeutic genome editing can be done through two strategies: 1) direct *in vivo* delivery, 2) ex-vivo [5]: At first, a number of patient's body cells are taken and TALEN transfer in these cells occurs outside the body. Then, after cultivation and proliferation, these cells are eventually injected into the body of the same patient [5,42].

Donor Design

Donor template is used to make precise changes in HR repair system [2], which is usually carried *via* a plasmid [19]. For the homologous recombination, the length of DNA molecule, modification sites and the sequence heterology are important. Commonly on both sides of donor sequence, there are homology arms [13] with 800 bp [19]. Linear template [13] increases the rate of the homologous recombination compared to the circular, but also increases the degradation rate [43].

Genome Editing

By inducing DNA double-strand break (DSB) following binding of TALEN to the target site, two DNA repair pathways are induces: 1) HDR and non-homologous end joining (NHEJ) [4,44]. NHEJ is divided into classical and alternative (microhomology mediated end joining (MMEJ) [4]) classes. There is a competition between proteins from both repair pathways for the free end of DNA strand. How cell decides to use which repair mechanism is still unknown, but the most important factor is the cell cycle stage and the presence of the DNA template [4].

NHEJ. This method does not require any homologous sequences and is responsible for the rapid repair of DSB. NHEJ is error prone and causes INDEL in the DNA sequence [9]. These indels induce frame shifts leading to premature stop codons in DNA sequence resulting in nonsense mediated decay and gene knock-out [9,19]. During the Human Genome Project, about 20000-25000 protein-coding genes were detected [45], but in many cases the functions are unclear. Gene knock out is an important technique to study gene function. This technique can be

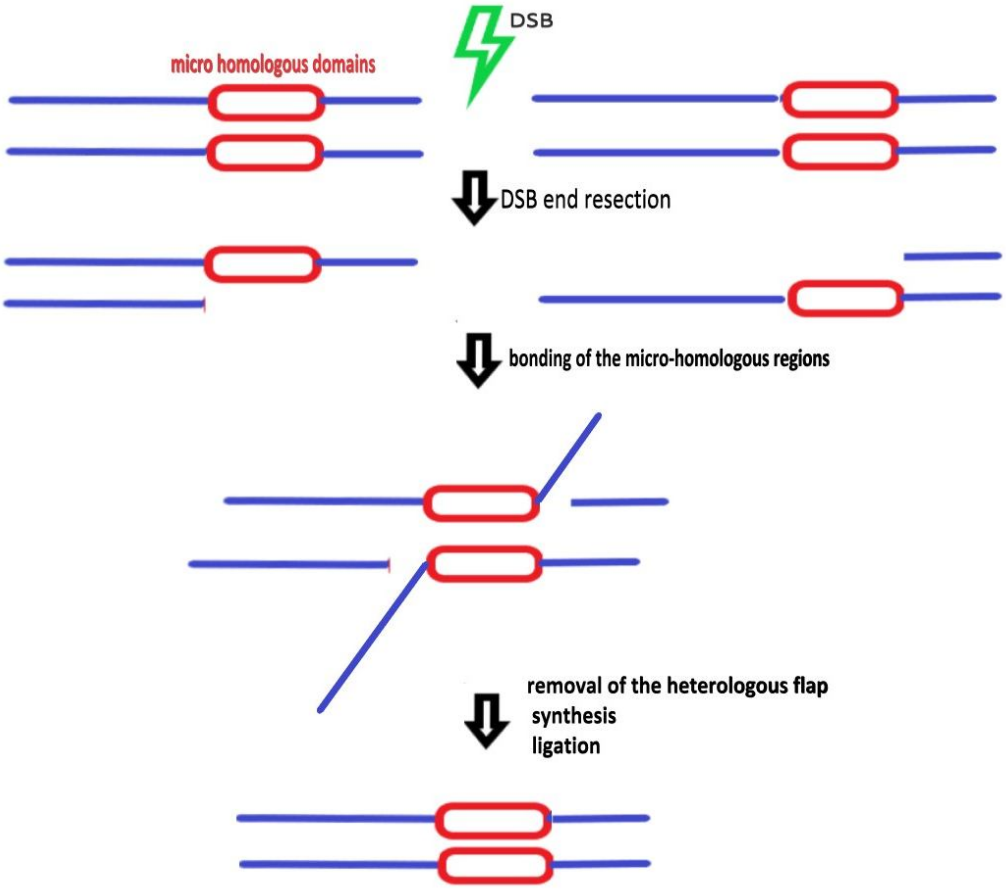

Fig. 7. MMEJ repair mechanism.

applied to make changes to the various types of human cells and animal models [46].

Single pair of nuclease (FOKI) induces small deletions (about 100 bp) while two pairs of TALEN can induce large deletions over 230 kbp, duplications about 15 kbp and inversions up to 140 kbp in HEL293T cells. In this way, scientists created a factor 8 gene inversion in human Induced Pluripotent Stem Cells (iPS) to use it as a cell line model [47]. To enhance NHEJ, coupling TALEN to an exonuclease is recommended for deletion of nucleotides from the DNA end at the cleavage site before a precise repair. Coupling TALEN to exonuclease 1 increases the efficiency to over 30% in rat cells, while coupling to Trex2 increases its efficiency to over 144% in human cells without any toxicity. Typically, NHEJ can occur in all stages of the cell cycle [44].

MMEJ. MMEJ is the alternative to the C-NHEJ, which is still not clear in some details. Unlike the NHEJ, DSB ends are linked to the micro homologous domains with 100% homology, on each side (5-25 nucleotides) of the cleavage region, which can lead to deletion in this site. The MMEJ repair usually occurs in the S phase of the cell cycle [4,44] and compete with the HDR. In fact, the MMEJ consists of five stages: DSB end resection, bonding of the micro-homologous regions, removal of the heterologous flap, synthesis and ligation [44] (Fig. 7).

HDR. Contrary to NHEJ, the HDR repair often due to a special template for accurate repair is not error prone [5]. Therefore, it uses sister chromatid as a pattern or an external template [10,30]. The DNA donor needs to have 800 bp homologous sequence on each side of the cleavage site. Applicable changes can range from a base pair to multi-

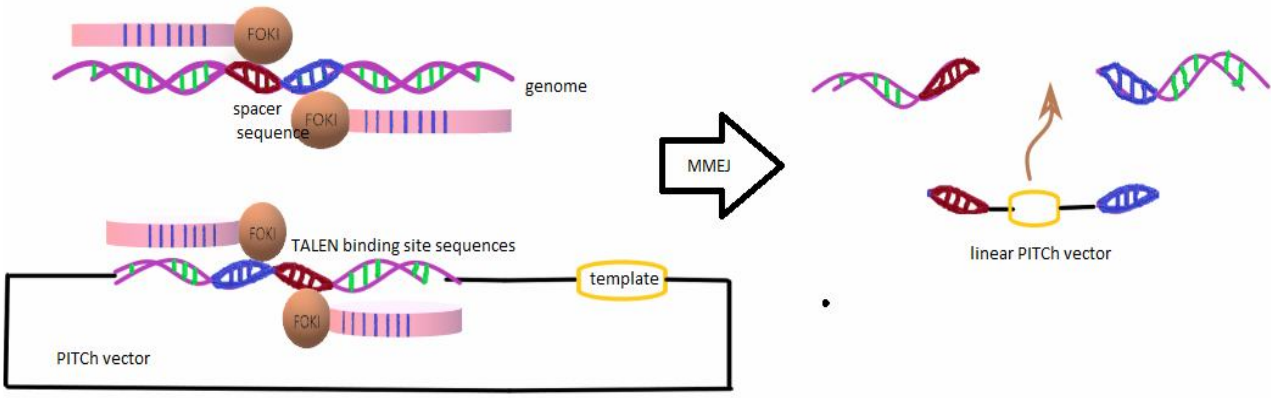

Fig. 8. TAL-PITCH for Gene knock-in. Vector contains template and TALEN binding site sequences. To construct microhomologous sequences, the spacer sequence of TALEN is used, except that the left half of the spacer sequence should be designed for right half of the microhomologous genomic sequence and the same way for the other side. After introducing TALEN into the genome, the vector become linear and entered into the genome *via* MMEJ repair mechanism.

kilobase pairs. Recently, linear single strand templates have been reported, but are used for smaller variations such as point mutation. Each of the DSB repair mechanisms is in competition with each other.

There are two strategies for HDR to overcome NHEJ. 1) NHEJ blocking, which is the most important repair mechanism in mammals, is done through inhibition of factors such as KU70/80 and ligase IV. 2) The target nuclease transfers in the late S and G2 phases of the cell cycle(30).

TAL-PITCH

Gene knock-in is usually done through HDR, which is a precise repaire system. But a better repair mechanism can be applied for this purpose, which is done by MMEJ and is known as PITCh (Precise Integration into Target Chromosome) system. TAL-PITCH can be used for a variety of cells and organisms, especially those with low HDR rate. Another advantage is that it requires short (10-40bp) homologous sequences, which can easily be added to the vector through PCR or cloning. In this method, one requires a single TALEN pair and a PITCH vector. The vector contains template and TALEN binding site sequences. To construct microhomologous sequences, the spacer sequence of TALEN is used, except that the left half of the spacer sequence should be designed for right half of the microhomologous genomic sequence and the same way for the other side. After introducing TALEN into the genome, the vector becomes linear and enteres into the genome *via* MMEJ repair mechanism [48,49] (Fig. 8).

Genome Editing Applications

Model organisms: TALENs provide targeted changes in a number of model organisms such as Drosophila, Zebrafish [3,50], frogs, rats and pigs [3] that were previously difficult or inaccessible to genetic manipulation. In most of these cases, single TALEN pair was used to induce knockout mutations through NHEJ. The ability to introduce mutations in different organisms has led to the development of new animal models of human disease. For example, TALEN has been applied to inactivate the LDL receptor gene in pigs to create a model for Familial hypercholesterolemia.

Plants and livestock: Unlike model organisms, several plants have long reproductive cycles. Genome editing through nucleases in plants and livestock can significantly reduce the time needed to create new species compared to traditional strategies. This technique has been used to make resistance against Xanthomonas bacteria in rice by disrupting the TALE target sites [3,51,52].

Therapeutic applications: Since there is no successful treatment for HIV infection to date, it is a candidate for gene therapy through engineering of nucleases. This virus

requires a co-reporter chemokine receptor type 5 (CCR5) on the white blood cells. Individuals with ahomozygote CCR5-delta32 mutation are resistant to CCR5-tropic HIV-1 infection [53]. CCR5 knockout through TALEN(5, 42) compared to ZFN significantly reduces cytotoxicity(37, 54) and off-target cleavage. During this method, DSB is induced in human primary CD^{+}_{4} T Cells and CD^{+}_{34} hematopoietic stem cells in an in vitro condition, thereby activating the NHEJ pathway and causing CCR5 knockout [13,42,54].

Beta-thalassemia [41] is a type of hereditary disease that results from a point mutation or a deletion in the beta-globin gene. One way to cure the disease is through hematopoietic stem cell transplantation, however with limitations like human leukocyte antigen-matched (HLA match) [55]. Among the various types of point mutations, the IVS2-654 (C > T) mutation is the most common. This mutation is targeted by all three types of ZFN, TALEN and Crispr/cas9 in beta-thalassemia-derived ipsc. The DSB is repaired by HDR. Efficiency of TALEN [21,56] and Crispr is higher than ZFN [56].

CONCLUSIONS

Engineered nuclease enzymes such as ZFN, TALEN and CRISPER/CAS9 are powerful and efficient tools for genome editing. The choice of these techniques requires a knowledge on their methodology and, most importantly, the purpose and application of the genome editing. TALEN has advantages such as fewer cytotoxicity versus ZFN, and is much easier to engineer. Also, the advantages of this method compared with CRISPR, are the reduction of off-target cleavage and the lack of need to protospacer adjacent motif (PAM). However, TALEN has disadvantages including a difficult protein engineering step and cannot cleavage methylated DNA. Today, CRISPR is used as a dominant method in various fields, and is a newer technology than TALEN. But TALEN is also very valuable due to its high specificity, being capable of targeting different sites and application in different organisms. However, there are significant challenges in each of these methods from design to the induction of changes. The genome editing has helped to understand complex diseases, the role of each gene, and effort to improve diseases.

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