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A highly effective TALEN-mediated approach for targeted gene disruption in *Xenopus tropicalis* and zebrafish

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ABSTRACT

Transcription activator like effector nucleases (TALENs) is a promising approach to disrupt intended genomic loci. The assembly of highly effective TALENs is critical for successful genome editing. Recently we reported a convenient and robust platform to construct customized TALENs. The TALENs generated by this platform have been proven to be highly effective for gene disruption in *Xenopus tropicalis* and zebrafish as well as large genomic deletions in zebrafish. The one-time success rate of targeted gene disruption is about 90% for more than 100 genomic loci tested, with the mutation frequencies often reaching above 50%. Here we describe the validated protocol for TALEN assembly, methods for generating gene knockout animals in *X. tropicalis* and zebrafish, as well as the protocol for engineering large genomic deletions in zebrafish.

1. Introduction

For many years, successful genome targeting by manipulation of embryonic stem cells was limited in most organisms. New breakthrough has been made in recent years using engineered nucleases [1]. In order to create such nucleases, the catalytic domain of FokI nuclease has been fused with the DNA binding domain from zinc finger proteins or transcription activator like effector (TALE) protein to generate zinc finger nucleases or transcription activator like effector nucleases (TALENs), respectively [2,3]. These engineered nucleases could induce specific double-stranded DNA break (DSB) in a genome. Repair of the DSB by error-prone non-homologous end-joining (NHEJ) can lead to the introduction of mutagenic insertions or deletions (indels) at the site of breakage. If an appropriate DNA donor is available, repair of the DSB by homologous recombination may also occur, which leads to knock-in of the exogenous DNA. More recently, specific genomic DSB has also been successfully generated by using RNA-guided Cas9 nuclease, providing an alternative approach for genome editing [4,5]. To date, genome editing mediated by these engineered nucleases has been successfully implemented in cells and in a number of organisms including *Xenopus tropicalis* and zebrafish [6–19].

TALENs provide a prominent genome-editing approach for their simplicity of design and their efficiency and specificity of on-target editing [20]. The engineered TALENs are a pair of artificial nucleases designed to bind onto two half-sites separated by a spacer (Fig. 1). Each TALEN monomer is a fusion construct composed of TALE repeats (DNA binding) and FokI nuclease (DNA cutting). The DNA binding specificity of TALEN is provided by the assembled TALE repeats. Each TALE repeat, which consists of nearly identical 34 amino acids (aa), could bind to one base. The specificity of binding is determined by two variable aa in the 12th and 13th positions called repeat-variable di-residues (RVDs), with RVDs NI, NG, HD and NN recognizing adenine (A), thymine (T), cytosine (C) and guanine (G), respectively [21,22]. Therefore, in order to bind to a specific DNA sequence, the TALE repeats were assembled and cloned into a vector containing a FokI nuclease domain. When translated, these engineered nucleases could introduce DSB in a pre-determined genomic locus.

Several platforms have been established to assemble the highly repetitive TALE repeats, including the PCR-based Golden Gate assembly [8,9,23], plasmid-based Golden Gate assembly [24–27], fast ligation-based automatable solid-phase high-throughput assembly [28] and ligation-independent cloning assembly [29]. Among these platforms, the plasmid-based Golden Gate assembly

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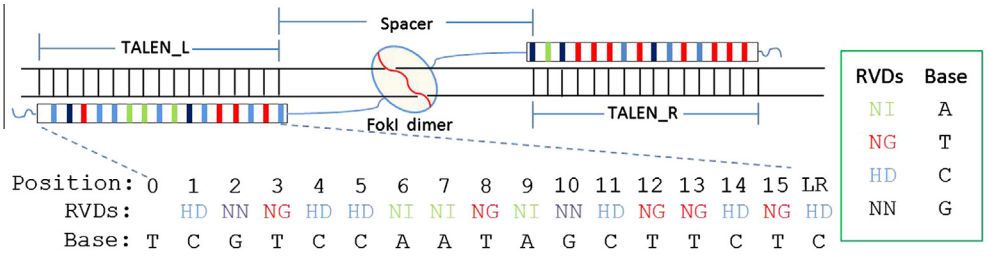

Fig. 1. A diagram of target recognition by engineered TALENs. A full TALENs target site is composed by two half-sites separated by a spacer. FokI dimer is formed in the spacer region where DSB is introduced. The binding specificity of TALENs is determined by RVDs in each TALE repeat. The codons for RVD-base interaction are shown on the right. LR, last repeat.

has been proven to be an easy, fast and effective method which has been adopted by most laboratories. The Golden Gate assembly relies on type IIS restriction enzymes which can cleave DNA out of their binding site, thus generating user-defined 4-bp overhangs [30]. We have adopted and modified the Golden Gate toolkit developed by Voytas' laboratory to assemble TALENs [24]. This toolkit was originally designed for gene targeting in yeast and plants. We have made two TALEN expression vectors, namely pCS2-TALEN-ELD and pCS2-TALEN-KKR, which are suitable for genome editing in animals. The optimized TALENs expression vectors contain SP6/CMV promoters, a nuclear localization signal, a truncated TALE architecture [31], an improved ELD/KKR obligate heterodimeric FokI [32], and two Esp3I sites allowing Golden Gate assembly. The optimized TALENs platform has several properties. First, the optimized TALENs expression vectors are compatible with the TALENs toolkit developed by Voytas' laboratory. Second, customized TALENs recognizing 12–21 bp half-sites can be routinely assembled by two digestion–ligation steps in five days. Third, the optimized TALE architecture and the enhanced ELD/KKR obligate heterodimeric FokI domain enable high cleavage efficiency and high specificity. Our optimized Golden Gate TALEN system has been proven to be highly effective in *X. tropicalis* and zebrafish embryos [12,33].

Although TALENs could induce indel-mutations with high frequency, such indel-mutations are often small (<30 bp) and are created in a random manner. This particular feature cannot satisfy requirements where larger and more predictable genomic alterations are desired. Several studies indicate that simultaneous targeting of two loci using two pairs of TALENs can induce large genomic deletions [34–37], inversions as well as chromosomal translocations [38,39]. Using this dual TALEN approach, we and other groups have demonstrated that large deletions of 1–80 kb can be efficiently created in zebrafish [33,40–42]. These large deletions have been used to knock out non-coding RNA genes, gene clusters and gene regulatory elements.

Herein we describe a validated protocol for assembling highly effective TALENs. The detailed methods for generating gene knockout animals in *X. tropicalis* and zebrafish, as well as the method for engineering large genomic deletions in zebrafish are provided.

2. Experimental procedures

2.1. Choosing the TALEN binding site

A full TALEN target site is composed of two half-sites separated by a spacer (Fig. 1). Most of the indel-mutations will occur in the spacer region. To disrupt a protein-coding gene, the targeted region should preferably be placed in the exons at the 5′ end or the exon encoding the functional domain. To disrupt non-coding RNA genes, gene clusters or gene regulatory elements, knockout of the entire DNA fragment is preferred. In this case, two target regions flanking the target fragment should be selected (Fig. 2).

We adopt the following rules when choosing the TALEN binding site. First, position 0 ought to be T, as this is always observed in the target sites of the natural TALEs. Second, the half-site length is recommended to be 16–18 bp. In our hands, longer half-site length decreases TALENs activity. Third, the ideal spacer length is around 16–18 bp. The spacer length is critical since an ideal spatial space is needed to form the FokI dimer. Fourth, less than five G in a half-site is recommended since the RVDs NN could also bind to A.

2.2. TALENs assembly

We have adopted the Golden Gate kit to assemble TALENs (Fig. 3). We have optimized the TALEN expression vectors as well as the buffer system and the reaction parameters for Golden Gate reactions (see protocol). Using our optimized TALEN platform, customized TALENs recognizing 12–21 bp half-sites can be routinely assembled by two digestion–ligation steps in five days with high

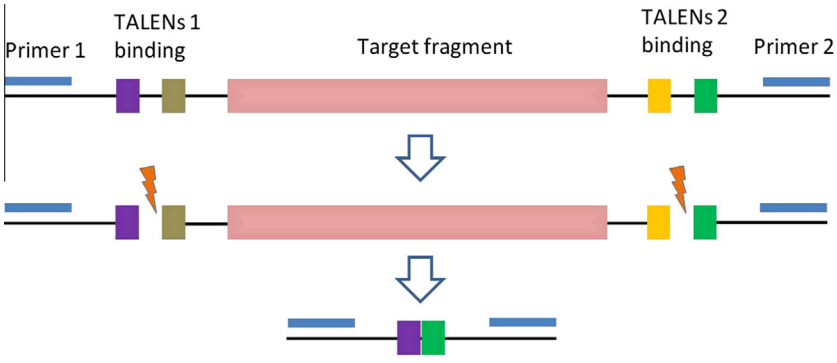

Fig. 2. Schematic diagram of large genomic deletion using two pairs of TALENs. To delete a large DNA fragment, two DSBs were introduced simultaneously on each side of the targeted genomic fragment using two pairs of TALENs. Repair of the DSB by ligation of the broken ends will lead to deletion of the flanked genomic fragment. The genomic deletion could be detected by PCR using properly designed primers.