Gene-Editing Technology of Zebrafish

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As a vertebrate model, zebrafish (Danio rerio) plays a vital role in the field of life sciences. Recently, gene-editing technology has become increasingly innovative, significantly promoting scientific research on zebrafish. However, the implementation of these methods in a reasonable and accurate manner to achieve efficient gene-editing remains challenging.

Keywords: zebrafish ; Danio rerio ; gene editing ; double-stranded break ; nick ; genome modification ; fixed-point orientation transformation

1. Introduction

Zebrafish (*Danio rerio*) is genetically similar to humans [1] and has unique advantages in the field of vertebrate development, organ formation, gene function, and organ regeneration, mainly because of its small body size, transparent embryos, ex vivo fertilization and embryogenesis, rapid embryological development, low breeding cost, and high fecundity [2]. In addition, zebrafish is currently the only vertebrate suitable for microplate high-throughput drug screening, the application of which can be promoted if zebrafish are used to create different disease models. Numerous zebrafish models of human diseases can be constructed through drug immersion or physical injury. However, genetic zebrafish models of human diseases associated with gene mutations must be created through genetic modification. With the development of gene editing technology, an increasing number of gene-editing methods have been proposed and applied. Continuous exploration and improvement of gene-editing methods are important for the advancement of life science research and the promotion of gene therapy.

2. Transgenic Technology

DNA recombination technology started developing in the 1970s, marking a new era in biology. In the 1980s, with the successful application of transgenic technology in fish, a milestone in fish gene-editing technology was achieved [3]. Originally, the target gene, including plasmid DNA and bacterial artificial chromosomes, was introduced into the organism to meet human demands [4][5]. Traditionally, linearized exogenous DNA was injected into fertilized eggs alone to realize the transgene. However, the integration efficiency of foreign genes into the genome and the probability of transmitting the DNA to offspring is very low [5]. Moreover, the technique is non-directional, and the presence of multi-exogenous plasmid copies may lead to abnormal development of fertilized eggs, a large number of malformed offspring, and difficulty in integration site detection. Therefore, establishing transgenic lines in zebrafish was a difficult task.

More recently, transposase or macronuclease I-SceI-mediated transgenic methods have been developed, which can significantly improve the efficiency of transient and stable expression of zebrafish genes [6]. When a transposon, reporter gene, and promoter with a transposase are co-injected into the embryo, researchers can successfully insert the reporter gene into the genome [7][8].

DNA transposons are mobile genetic elements whose insertion positions can change within the host genome [9]. The transposon can be cut off from its original position and inserted into a new genomic position through catalysis by a transposase, forming a forward repeat at the target point.

In addition to the advantages of high transgenic efficiency, transposons can also be used as sequence tags for inserted genes. Reverse PCR can facilitate the detection of insertion sites of exogenous genes, and Cre/LoxP technology can be used for efficient site-specific operations [10]. With the advancement in research, transposons have been widely employed for gene trapping. For example, Suster and colleagues have reported the use of the Tol2 transposon to capture genes in zebrafish [11]. The genomic sequences upstream and downstream of the insertion site of the trap vector can be identified by reverse PCR, and fusion transcripts of reporter genes and endogenous genes can be obtained by rapid amplification of

cDNA ends (RACE) [12]. By using integrase (ex. phiC31), researchers can avoid the positional effects and multiple insertion of exogenous DNA into the genome [13].

The use of transposons has significantly improved the efficiency of transgenes in zebrafish, and a large amount of transgenic zebrafish strains have been developed accordingly. Tol2 and *Sleeping Beauty* transposons, found in fish, can form a genetically modified zebrafish strains [12]. For instance, some transgenic zebrafish lines show cell- or tissue-specific expression of commonly used fluorophores, such as green fluorescent proteins (GFP), which can provide valuable insights into gene function, organ formation, and cell behavior during development [14][15].

3. Targeting Induced Local Lesions in Genomes (TILLING)

The approaches in the above studies represent forward genetics. Given that zebrafish can also be used to establish a specific gene disease model to screen drugs for related diseases, reverse genetics approach would be more suitable for pre-clinical research. In recent years, the zebrafish has become a reliable model for reverse genetic analysis of vertebrate development and human diseases [16][17].

TILLING is a reverse genetics strategy that identifies mutations in specific genes of interest in chemically mutagenized populations [18]. The method was first described in *Arabidopsis thaliana* in 2000 [19] but was rapidly implemented in other organisms including zebrafish [20]. The approach consists of screening individual genomic DNA samples from a cohort of ENU-mutagenized F1 zebrafish to identify mutations that alter a chosen gene, while the sperm of the corresponding fish is cryopreserved for subsequent reconstitution of the mutant line by in vitro fertilization once desired mutations are identified.

TILLING is a powerful technology that raises zebrafish as a pertinent model in gene function research. However, the procedure of TILLING is costly, labor intensive, and cannot be implemented in most individual labs. The most important thing is that the identification of mutant genes is very troublesome, and whether there are multiple mutation sites cannot be well determined [21].

4. Discovery and Application of Fixed-Point Shear Enzymes

In 1983, the first zinc finger protein (ZFP) domain was identified in the transcription factor IIIA (TFIIIA) from *Xenopus laevis* [22]. In 2008, a zinc finger nuclease (ZFN) was designed to recognize the homologous sequence of *vascular endothelial growth factor receptor 2* (*kdrl*) in zebrafish [23]. The technique using ZFN relies on the specific recognition and binding of DNA ZFP, and the cleavage domain of FokI endonuclease enables reverse genetics in zebrafish [23][24][25][26][27]. Gene mutation was successfully induced by injecting ZFN mRNA into the one-cell stage zebrafish embryos to generate double-strand breaks (DSBs) at the target site. However, the design of ZFN is difficult, expensive, and inefficient for certain targets [28][29][30], which hinders the development of this technology in zebrafish. Designing and screening specific ZFNs requires time and numerous experiments. If the specific binding site of DNA is invalid or FokI homodimerization cleavage is difficult, an off-target effect may occur, thus limiting the large-scale application of this process [24][31].

The transcription activator-like effector nuclease (TALEN) is another type of genome targeting nuclease after ZFN, which is more flexible and efficient [32][33]. In 2007, a novel DNA-binding protein called the transcription activator-like effector (TALE) was identified in a Gram-negative plant pathogen. TALE is a class of protein effectors that can be injected into host cells by *Flavobacterium* through the secretion system [34]. TALEN is constructed using the same method of constructing ZFN and can target and modify genomes conveniently and efficiently. ZFN and TALEN are artificial nucleases composed of specific DNA-binding proteins and non-restricted nuclease FokI [35][36]. Their mechanism of action is similar: FokI exhibits endonuclease activity by forming dimers, causing DSBs in the target DNA sequence and subsequently inducing endogenous repair mechanisms of cells. This activates non-homologous end joining (NHEJ) or homologous recombination (HR) in vivo that leads to endogenous gene knockout or exogenous fragment knock-in (KI) at the target site [37]. In 2011, RNA encoding different TALEN pairs was injected into one-cell stage zebrafish embryos for the first time [31]. All four pairs of TALENs induced targeted indels with high mutation frequency ranging from 11% to 33%. These mutations were caused by TALEN-induced DSB repair through NHEJ, which resulted in effective indels at the breaking site. These indels can cause frameshift or knockout mutations that can be passed on to the next generation [31]. ZFN and TALEN can thus mediate targeted genomic modifications in vivo, enabling the development of genetic studies and disease models. The emergence of targeted ZFN and TALEN technology has improved the efficiency and success rate of targeted gene modification as well as recognition specificity [38].

The gene tool clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (CRISPR/Cas) is economical, convenient, and efficient [39][40][41] and requires only one guide RNA (gRNA) to be

customized for a specific sequence [42] rather than two ZFN or TALEN proteins that must be designed and assembled for each site. gRNA is approximately 100 bp, and it is thus easier to construct compared with that of ZFN or TALEN. Moreover, because of its short length, complications caused by the long encoding vector can be avoided. The CRISPR/Cas repeating structure was first discovered in 1987 in the flanks of the *iap* gene sequence from *Escherichia coli* [43] and was named short regularly spaced repeats in 2000 [44]. By 2013, an expression vector to produce Cas9 mRNA by a SP6 RNA polymerase and a customizable single guide RNA (sgRNA) that consists of a 20 bp nucleotide sequences complementary to the target site was used to construct the CRISPR/Cas9 genome editing technology in zebrafish [32]. The Cas9 capped mRNA and sgRNA were co-injected into one-cell stage embryos to effectively introduce somatic indel mutations at 8 out of 10 sites in the zebrafish genome, and the average mutation frequency of these eight loci ranged from 24.4% to 59.4% (Table 1) [32]. Therefore, CRISPR ushered in a new chapter for zebrafish gene-editing technology. Nevertheless, CRISPR/Cas9 also faces a major challenge regarding off-target effects that result from the tolerance of several base mismatches between the targeted DNA and the 20 bp sgRNA [45]. For clinical application, complete accuracy is required; therefore, it is particularly important to improve the specificity of sgRNA and change the targeted cutting mode [46][47][48]. In addition, donor type and targeted editing efficiency are important factors that limit zebrafish gene editing [49].

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