Exogenous gene integration mediated by genome editing technologies in zebrafish

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ABSTRACT

Genome editing technologies, such as transcription activator-like effector nuclease (TALEN) and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) systems, can induce DNA double-strand breaks (DSBs) at the targeted genomic locus, leading to frameshift-mediated gene disruption in the process of DSB repair. Recently, the technology-induced DSBs followed by DSB repairs are applied to integrate exogenous genes into the targeted genomic locus in various model organisms. In addition to a conventional knock-in technology mediated by homology-directed repair (HDR), novel knock-in technologies using refined donor vectors have also been developed with the genome editing technologies based on other DSB repair mechanisms, including non-homologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ). Therefore, the improved knock-in technologies would contribute to freely modify the genome of model organisms.

KEYWORDS

genome editing; knock-in; MMEJ; NHEJ; TALEN; CRISPR/Cas9; zebrafish

Insertion of exogenous genes into the genome of model organisms

Sophisticated technologies for genome engineering have been developed during the past few decades, resulting in the improvement of our understanding of developmental and physiologic molecular functions in organogenesis of various model organisms. Insertion of exogenous DNA into the genome of model organisms is a powerful genetic tool in genome engineering with which one can induce the expression of exogenous genes or disrupt the function of endogenous target genes. In fact, the insertion of fluorescent protein genes driven by well-characterized tissue-specific promoters enables us to monitor the expression and behavior of the reporter genes in predictable tissues and organs.1

In the vertebrate model zebrafish (Danio rerio), genome insertion of a donor vector containing a tissue-specific promoter and a fluorescent protein gene has been achieved either by using the I-SceI meganuclease or the Tol2 and Sleeping Beauty transposon systems.2,3 These methods allow efficient integration of exogenous genes into the genome that can be judged by the expression of the reporter gene in the progeny and have been used to generate transgenic zebrafish lines. Unpredictable ectopic expression of the reporter gene is often observed in these transgenic lines,4 presumably due to the transcriptional influence around the insertion site. Because the donor vector usually does not contain the entire enhancer/promoter region of the target gene, the expression of the reporter in these lines partially overlaps the expression of the endogenous target gene. Recent innovation of the genome editing technologies, including transcription activator-like effector nuclease (TALEN) and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas), enables us to perform the targeted integration (knock-in) of the reporter gene in addition to the targeted gene disruption (knockout), presenting valuable applications in genome engineering.5 In this review, we briefly overview methods for gene insertions that have been used in zebrafish and intensively introduce recent improved knock-in technologies using the CRISPR/Cas9 system.
Insertion of exogenous genes using the meganuclease and transposon systems

The I-SceI meganuclease has been used as a tool for integration of a reporter into the genome of model organisms. The donor vector containing a reporter gene driven by a tissue-specific promoter plus a fluorescent protein gene flanked by the recognition site of I-SceI is co-injected with the meganuclease into the embryos. The I-SceI meganuclease, which recognizes the long recognition motif, rarely cuts the genome, leading to the reduction of the mosaicism of reporter expression (Fig. 1A). It has been reported that co-injection of the donor vector and I-SceI increased the incidence of the construct insertion into the genome. It is still not clear how the linearized construct can be integrated into the genome.

Transposon systems have improved the integration efficiency of the reporter gene compared with the I-SceI meganuclease-mediated insertion. Two transposable elements, Tol2 and Sleeping Beauty, were found in fish and have been applied to generate transgenic zebrafish lines. The transposon vector containing Tol2 transposable elements, a tissue-specific promoter, and a reporter gene is co-injected with Tol2 transposases into the embryos, often resulting in the insertion of the reporter into the genome (Fig. 1B). Because both the integration frequency and germline transmission rate are relatively high compared with those of the meganuclease method, the transposon-mediated insertion methods have been widely used to generate transgenic zebrafish lines. Unpredictable insertion events of these methods often cause different expression profiles of the reporter compared with those of individual endogenous genes because the expression of the inserted reporter gene could be influenced by the transcriptional regulatory region around the insertion site (e.g., enhancers and repressors). Thus, the targeted knock-in technology of the reporter construct is desired in genome engineering.

Genome editing technologies and DNA repair mechanisms

DNA double-strand breaks (DSBs) induced by genome editing technologies at the targeted locus enable us to disrupt the function of the gene of interest. TALEN technology utilizes a chimeric molecule consisting of the transcription activator-like effector (TALE) and the FokI nuclease catalytic domains (Fig. 2A). The TALE domain, which was originally found in the plant pathogenic bacteria Xanthomonas, binds to the targeted genomic sequences through its approximately 34-amino acid DNA recognition motifs, each of which possesses repeat variable diresidues (RVD) essential for individual nucleotide recognition. The FokI nuclease domain functions as a

Figure 1. I-SceI- and Tol2-mediated insertion of exogenous genes. (A) I-SceI-mediated insertion of an exogenous gene. I-SceI recognition sites (orange rectangle; 5'-TAGGGATAACAGGGTAAT-3') in the donor vectors (light blue lines) can be cleaved by the I-SceI meganuclease. Co-injection of the donor vectors and I-SceI increases the incidence of the integration of the promoter (pink box) and the reporter (green box) constructs into the genome. (B) Tol2-mediated insertion of an exogenous gene. The reporter construct flanked by the Tol2 inverted terminal repeat (Tol2 ITR, yellow boxes) sites is excised by Tol2 transposase, leading to the integration of the construct containing the promoter (pink box) and the reporter (green box) into the genome.

Figure 2. TALEN and CRISPR-Cas9 systems. (A) TALEN-mediated disruption of a targeted gene. A chimeric molecule consisting of a TALE nuclease (green) and a FokI nuclease (red) binds to the target site (dark blue) and cleaves the genomic DNA (light blue) at the recognition sites (orange rectangles). (B) CRISPR-Cas9-mediated gene disruption. A single guide RNA (sgRNA, light blue) binds to the target site (dark blue) and recruits a Cas9 nuclease (red) to cleave the genomic DNA (light blue) at the recognition site (orange rectangle).
dimer; therefore, using the forward and reverse TALENs at the targeted locus, DSBs can be generated by the dimerized FokI catalytic domains at the spacer region between the TALEN recognition sites.

The CRISPR/Cas9 complex composed of CRISPR RNA (crRNA), trans-activating CRISPR RNA (tracrRNA), and Cas9 nuclease recognizes the targeted genomic sequences and induces DSBs at the targeted locus (Fig. 2B). The spacer domain (20 nucleotides) of the crRNA:tracrRNA duplex specifically associates with the complementary DNA sequences followed by the protospacer adjacent motif (PAM). The crRNA:tracrRNA duplex has recently been modified into a single guide RNA (sgRNA) that can be functional with Cas9, making it easier to perform the genome editing.

DSBs induced by the TALEN or CRISPR/Cas9 system can be repaired by at least 3 DSB repair mechanisms: (1) homology-directed repair (HDR), (2) non-homologous end joining (NHEJ), and (3) micro-homology-mediated end joining (MMEJ) (Fig. 3). HDR-mediated DSB repair is a precise repair pathway, as it uses a sister chromatid or a donor vector that has long homologous sequences (> 400 bp) complementary to the targeted genomic sequences as a template. In the process of NHEJ, various insertion and/or deletion (indel) mutations can be introduced at the target site, resulting in the frameshift-mediated gene disruption. When microhomology sequences (3–30 bp) exist near the target site, the microhomology sequences exposed near the DSBs site anneal with each other and delete excess DNA in the process of the MMEJ-mediated DSB repair, often leading to the predictable deletion mutations at the target site.

DNA repairs following the CRISPR-mediated DSBs in the targeted locus and in the donor vector are critical for the efficient targeted knock-in of the reporter genes. In fact, the HDR-mediated knock-in of a reporter has been improved by adding the recognition motifs cleaved by genome editing technologies in the donor vector containing long homologous sequences to the targeted locus. Simultaneous cleavages of the endogenous target site and the donor vector by sgRNAs/Cas9 increase the efficiency of the HDR-mediated integration of the reporter gene. It has recently been shown that the donor vector lacking homologous sequences, but possessing the CRISPR target site, can be integrated by NHEJ-mediated DNA repair at the DSBs induced by CRISPR/Cas9. By using the donor vector containing microhomology sequences (20–40 bp) homologous to the targeted genomic sequences, we and others have recently reported that MMEJ could efficiently induce the targeted integration of exogenous genes into the genome.

Thus, both NHEJ- and MMEJ-mediated knock-in technologies are functional during zebrafish embryogenesis in addition to HDR-mediated knock-in technology.
NHEJ-mediated reporter knock-in to visualize and disrupt the target gene

Two groups have recently reported the NHEJ-mediated integration of a reporter gene at the targeted locus. Kimura et al. designed a reporter construct containing the target site for sgRNA/Cas9, the heat shock protein (hsp) 70 promoter, and the GAL4 gene. By using the reporter integration upstream of the transcription start site, they tried to visualize the expression of the evx2 gene, which is expressed in specific subsets of neurons in the central nervous system (CNS) during zebrafish embryogenesis. After the GAL4 reporter construct, sgRNAs targeting for the evx2 gene promoter, and Cas9 mRNA were injected into Tg[UAS:RFP] transgenic zebrafish embryos, they established a transgenic line expressing RFP in CNS. Because both reporter expression and Evx2 protein expression visualized by anti-Evx2 antibody were almost overlapped, the reporter integrated in the promoter region would receive the enhancer and repressor activity in the evx2 locus. Thus, the targeted integration of the reporter into the promoter is a very useful genetic tool to visualize the expression of target genes during embryogenesis.

We anticipated that if insertion of the reporter gene into the start codon of a target gene suppresses the expression of a functional protein, the loss-of-function phenotypes of the target gene should be observed in the homozygous embryos. To test this possibility, we...
tried to integrate the reporter construct into the start codon region of the *paired box 2a* (*pax2a*) gene because *pax2a* is expressed in the midbrain-hindbrain boundary (MHB), and its disruption causes the loss of the MHB.26,27 The donor vector possessed the hsp70 promoter and *eGFP* gene (Mbait-hs-eGFP) that were flanked by sgRNA-target sites (Fig 4A). When the donor vector was co-injected with Cas9 mRNA and 2 sgRNAs (targeting for the *pax2a* locus and for the donor vector) into zebrafish embryos, the eGFP expression in some of the injected F0 embryos partially overlapped with the *pax2a* expression domains.28 We established the transgenic line Tg*pax2a-hs:eGFP*, showing that the eGFP expression in the heterozygous embryos completely overlaps the endogenous *pax2a* expression domains28 (Fig. 4B (i)). We found that all the homozygous embryos exhibited the loss of the MHB identical to the

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**Figure 4.** NHEJ-mediated integration of a reporter gene at the targeted genomic locus. (A) A schematic of NHEJ-mediated integration steps of a reporter gene into the start codon region of the *pax2a* gene. Two distinct sgRNAs (sgRNA-1 and -2) targeting the *pax2a* locus (pink box) and the donor vector (red box) containing the hsp70 promoter and *eGFP* gene were designed. Simultaneous cleavages of the targeted genomic locus and the donor vector by the sgRNAs induce an efficient integration of the reporter gene into the genome. The white and light blue boxes indicate the 5′ untranslated region and the exons of the *pax2a* gene, respectively. (B) Morphological phenotypes caused by the integration of the reporter gene into the *pax2a* locus. Upper schematics illustrate the genomic alleles of the *pax2a* locus. The *eGFP* expression domains in the heterozygous embryo completely overlap the *pax2a* expression domains (i, green in middle panel), whereas the homozygous embryo lost the MHB structure (ii, lower panel), which was closely reminiscent of the *noi/pax2a* mutant embryo.26 The asterisk indicates the MHB. The middle and bottom panels show lateral views of the anterior left and the dorsal view of the embryo at 30 hours post fertilization (hpf), respectively.
noi/pax2a mutant (Fig. 4B (ii)), while all the heterozygous embryos exhibited normal MHB. We have also reported that the NHEJ-mediated integration of the reporter gene into the start codon is useful to monitor the expression pattern of an uncharacterized gene (*epdr1*: ependymin related 1) in the heterozygous embryos and to analyze the loss-of-function phenotypes in the homozygous embryos.28

Recently, Hoshijima et al. have demonstrated reporter gene insertion just downstream of the start codon of the potassium voltage-gated channel subfamily H member 6a (*kcnh6a*) gene in zebrafish using TALEN system.29 The *kcnh6a* gene is exclusively expressed in the heart, and its disruption causes a cardiac disorder.30-32 The authors used a promoter-less donor vector containing the eGFP gene flanked by approximately 1 kb homology arms homologous to the *kcnh6a* gene and the recognition motifs of I-SceI meganuclease. The donor vector was injected with TALEN mRNA and I-SceI into zebrafish embryos. They found that the eGFP expression in the heterozygous embryos recapitulated the expression of endogenous *kcnh6a*, while the cardiac deficiencies identical to the *kcnh6a* null mutant were observed in the homozygous embryos.29

Taken together, these studies suggest that targeted knock-in of the reporter gene around the start codon of a target gene allows the visualization of target gene expression in heterozygous embryos and the appearance of loss-of-function phenotypes in homozygous embryos.

**MMEJ-mediated precise integration of the reporter using CRISPR/Cas9**

It has recently been shown that DNA polymerase theta (Polq), which is a low-fidelity DNA polymerase

![Figure 5](image-url). MMEJ-mediated integration of a reporter gene at the targeted genomic locus. (A) A schematic of MMEJ-mediated integration steps of a reporter gene into the *krtt1c19e* locus. Two distinct sgRNAs (sgRNA-3 and -4) targeting the *krtt1c19e* locus (pink box) and the donor vector (red box) containing the eGFP gene were designed. Microhomology sequences complementary to the C-terminus of the *krtt1c19e* gene were located adjacent to the sgRNA-target site in the donor vector (yellow boxes with and without hatched lines). These constructs allowed a precise integration of the eGFP gene into the *krtt1c19e* locus when they were simultaneously cleaved by sgRNAs/Cas9. The white and light blue boxes indicate the untranslated region and the exons of the *krtt1c19e* gene, respectively. The green arrow indicates the stop codon of the *krtt1c19e* gene. (B) eGFP expression (green) in the epidermis was observed in the embryos at 2 d post fertilization (2 dpf) injected with sgRNAs/Cas9 mRNA and the donor vector, suggesting that MMEJ is functional in zebrafish embryos (Hisano et al. 2015).
required for MMEJ in mammals, is indispensable for the DNA repair mechanism during zebrafish embryogenesis because the maternal-zygotic (MZ) polq mutant (M2polq) exhibited severely deformed or dead embryos when DSBs were induced by CRISPR/Cas9 or ionizing radiation. This result suggests that Polq plays an important role in DSB repairs during zebrafish development. Consistent with this result, when the microhomology sequences existed around the target site, we often obtained the predictable deletion mutants presumably generated in the processes of CRISPR-induced MMEJ.

To take advantage of enough MMEJ activity in zebrafish embryo, we developed a novel knock-in method using a reporter construct containing short homologous sequences (40 bp) around the target site. We designed a sgRNA targeting the C-terminus of the keratin type 1 c19e (krtt1c19e) gene, which is highly expressed in basal keratinocytes, and another sgRNA targeting the reporter construct that possesses 40 bp homology arms matched to the C-terminal region of the krtt1c19e gene on both ends of the eGFP sequence (Fig. 5A). When the reporter construct was co-injected with the sgRNAs and Cas9 mRNA into zebrafish embryos, eGFP expression in keratinocytes was observed in one-third of the injected embryos at 2 dpf (Fig. 5B). DNA sequence analysis revealed precise knock-in of the reporter at the target site, suggesting that the MMEJ-mediated precise knock-in is functional in zebrafish. We further demonstrated the efficient germline transmission of the knocked-in gene and the expression of Krtt1c19e-eGFP chimeric molecules in keratinocytes of Tg[krtt1c19e:eGFP] embryos, presenting the usefulness of this method to generate precise knock-in lines in zebrafish.

**Conclusion remarks and future perspective**

Genome editing technologies, including TALEN and CRISPR/Cas9, should be powerful genetic tools for basic and applied researches, including medical science. The CRISPR/Cas9-mediated knock-in of exogenous genes in zebrafish should contribute to the replacement of a zebrafish functional gene with the human counterpart gene containing mutations found in human genetic disorders, leading to the establishment of a disease model organism for human genetic disorders. Such a zebrafish disease model may be useful to analyze the molecular function of the gene responsible for human disease. Furthermore, drug screening to suppress pathological phenotypes in the zebrafish disease model is promising to find effective drugs because it is relatively easy to test the effects of drugs using zebrafish embryos by administering them in the breeding water of the embryos. Innovation of knock-in technology as well as genome editing technologies in zebrafish would develop valuable medical applications to overcome human genetic disorders.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Funding**

This work was supported by the Japan Society for the Promotion of Science, Japan Agency for Medical Research and Development (AMED, 16km0210077j0001) and the Takeda Science Foundation.

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