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Short communication

Improving the efficiency for generation of genome-edited zebrafish by labeling primordial germ cells

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ABSTRACT

Although CRISPR/Cas, a new versatile genome-editing tool, has been widely used in a variety of species including zebrafish, an important vertebrate model animal for biomedical research, the low efficiency of germline transmission of induced mutations and particularly knockin alleles made subsequently screening heritable offspring tedious, time-consuming, expensive and at times impossible. In this study, we reported a method for improving the efficiency of germline transmission screening for generation of genome-edited zebrafish mutants. Co-microinjecting *yfp-nanos3* mRNA with Cas9 mRNA, sgRNA and single strand DNA donor to label the distribution of microinjected nucleotides in PGCs (primordial germ cells), we demonstrated that founders carrying labeled PGCs produced much higher numbers of knockin and knockout progeny. In comparison with the common practice of selecting founders by genotyping fin clips, our new strategy of selecting founders with tentatively fluorescent-labeled PGCs significantly increase the ease and speed of generating heritable knocking and knockout animals with CRISPR/Cas9.

1. Introduction

Gene targeting, a genetic technique that uses homologous recombination (HR) to modify an endogenous gene, is a powerful tool to uncover gene functions. Traditionally, it relies on cultured embryonic stem cells (ESCs). However, the lack of ESCs in animals other than mouse and rat (Jacob et al., 2010) and very low frequency of HR (about 1×10^{-6}) between an artificial donor and its genomic recipient in ESCs have become the bottle neck of the technology, preventing from its application to other animals (Johnson and Jasin, 2001). The frequency of HR occurring nearby double strand breaks (DSB) is significantly increased (Johnson and Jasin, 2001). Therefore, increasing DSB would improve the efficiency of gene targeting. CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated 9), a newly established engineered endonuclease (EEN) (Cong et al., 2013; Mali et al., 2013), has been demonstrated to create genome-edited animals independent of ESCs by generating DSB with high efficiency in a variety of animals. It has been used to produce not only knockout animals including zebrafish (Hruscha et al., 2013; Hwang et al., 2013), mouse (Li et al., 2013; Mashiko et al., 2013; Sung et al., 2014; Zhou et al., 2014), rat (Hu et al., 2013; Li et al., 2013), fruit fly (Bassett et al., 2013a, Gratz et al., 2013; Kondo and Ueda, 2013; Ren et al., 2013; Yu et al., 2013), silkworm (Ma et al., 2014), *Caenorhabditis elegans* (Chen et al., 2013; Cho et al., 2013; Friedland et al., 2013; Katic and Großhans, 2013; Tzur et al., 2013), and frog (Guo et al., 2014) when the induced DSB was repaired by NHEJ, an error-prone repair pathway, but also knockin (via homology-mediated repair) animals including *C. elegans* (Chen et al., 2013; Tzur et al., 2013; Zhao et al., 2014) and fruit fly (Bassett et al., 2013b; Dickinson et al., 2013; Gratz et al., 2013, 2014) when the induced DSB was repaired by HR with an artificial DNA donor.

Zebrafish, a vertebrate animal model, is widely used in various genetic studies and biomedical researches. Although knockout zebrafish mutants have been created by CRISPR/Cas9 (Hruscha et al., 2013; Hwang et al., 2013), the low efficiency of germline transmission of induced mutations and particularly knockin alleles made subsequently screening heritable offspring tedious, time-consuming, expensive and at times impossible. For example, no heritable zebrafish carrying a knockin allele via gene targeting has been created to date though point mutations (Hwang et al., 2013), loxP (Chang et al., 2013) and HA tag (Hruscha et al., 2013) were integrated into zebrafish somatic genome by CRISPR/Cas9-triggered HR. In this study, we developed a method to improve the screening efficiency of germline transmitted CRISPR/Cas9-based gene targeting in zebrafish by screening founder embryos carrying primordial germ cells (PGCs) tentatively labeled with enhanced yellow fluorescent protein (eYFP). Using this method, we demonstrated that

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founders carrying labeled PGCs produced much higher numbers of knockin and knockout progeny. In comparison with the common practice of selecting founders by genotyping fin-clips, our new strategy of selecting founders with tentatively fluorescent-labeled PGCs significantly increase the ease and speed of generating genome-edited animals with CRISPR/Cas9. Particularly, we generated gene targeted zebrafish carrying a loxP in intron 3 and intron 4 of *aldh1a2*, respectively, laying the foundation for creating a conditional knockout zebrafish.

2. Materials and methods

2.1. Animals

Tubingen zebrafish used in this study are housed in the zebrafish facility of Model Animal Research Center, Nanjing University. The research protocol was approved by the Institutional Animal Care and Use Committee of Model Animal Research Center, Nanjing University.

2.2. Determination of indel rate in zebrafish embryos during early development after CRISPR/Cas9 injection by sequencing

Capped Cas9 mRNA was transcribed from plasmid pXT7-Cas9 (Chang et al., 2013) and tailed with mMessage mMachine T7 Ultra Kit (Ambion, USA). sgRNAs were transcribed from templates prepared by PCR with gene specific primers and a universal reverse primer gRNAR (Table 1) using plasmid pT7-gRNA as template (Chang et al., 2013).

To examine the activities of sgRNAs, 1 nl solution containing 250 pg Cas9 mRNA and 50 pg sgRNA (Supplementary Table 1) was microinjected into 1-cell zebrafish embryos (Each sgRNA was tested separately). When reaching 24 hpf, 20 of the microinjected embryos were randomly selected for genomic DNA isolation using the method described previously (Dong et al., 2011). The genomic fragment containing the sgRNA (a3gR11) binding site in intron 3 of *aldh1a2* was amplified with primers a3F2 and a3R2, while the genomic fragment containing the sgRNA (a4gR05) binding site in intron 4 of *aldh1a2* was amplified with primers a4F3 and a4R3 (Table 1). The PCR program was 95 °C 2 min, 35 cycles of (95 °C 30 s, 56 °C 30 s, and 72 °C 1 min), and a final extension at 72 °C for 5 min. The PCR products were cloned into pGEM-T easy vector (Promega, USA). Forty positive transformants from each group of embryos were sequenced to determine the indel mutations. The sgRNAs with the highest activity in the two different sites were chosen for subsequent experiments.

To determine the rate of indel mutations induced in zebrafish embryos at different developmental stages, 1 nl solution containing 250 pg Cas9 mRNA and 50 pg sgRNA a4gR5 was microinjected into 1-cell embryos. Thirty embryos at 8-cell, 16-cell and 32-cell stages and 20 embryos at 1 K-cell, 30% epiboly, 50% epiboly, 6-somite and prim-5 were collected for indel mutation determination, respectively. The experiment was performed 3 times independently and the indel ratios were shown in mean ± standard error of the mean (SEM).

2.3. Observation of yfp-nanos3 distribution in zebrafish PGCs after microinjection by fluorescence photography

yfp-nanos3 cDNA was constructed by overlapping PCR. Briefly, *yfp* was amplified using primers YFPF and nos3′U-YFPR from plasmid pycbeta-actinpr_eYFP (Ge et al., 2012). The 3′ untranslated region (UTR) of zebrafish *nanos3* (GenBank Gene ID 140631) was amplified using primers YFP-nos3′UF and nos3′UR (Table 1) from cDNA reverse transcribed from the total RNA isolated from zebrafish ovaries. The two overlapping fragments were purified, mixed and then used as templates to amplify the *yfp-nanos3* DNA fragment using primers YFPF and nos3′UR (Table 1). The resultant product was cloned into pGEM-T easy vector (Promega, USA), transcribed, capped, and tailed with mMessage mMachine T7 Ultra Kit (Ambion, USA).

A mixture containing 500 pg *yfp-nanos3* mRNA (Fig. 1A) and 50 ng Rhodamine B isothiocyanate (RITC)-dextran (Sigma, USA) was microinjected into 1-cell zebrafish embryos. The microinjected embryos were observed under an Olympus DVX10 stereo fluorescence microscope when they reached 48 hpf. RITC signal was captured in RFP channel, while YFP signal was observed in YFP channel.

2.4. Measuring the effects of Cas9 mRNA, sgRNA and ssODN at different concentrations on knockin efficiency by polymerase chain reaction

Single stranded oligodeoxynucleotide (ssODN) a4gR5ssD harboring a 20-bp left homology-arm, a 19-bp right homology-arm and a modified loxP sequence (Bedell et al., 2012), synthesized commercially and dissolved to a 1 μg/μl stock solution, was used as a homologous donor. 1× (containing 15.625 pg Cas9 mRNA, 3.125 pg gRNA a4gR5 and 3.125 pg ssODN donor a4gR5ssD, Table 1 and Supplementary Table 1), 4× and 16× doses were microinjected into 1-cell zebrafish embryos, respectively. Knockin event was detected by nested PCR. The first round PCR was performed in the same way as the detection of indel mutation described above. The second round PCR detected mloxP integration using forward primer mloxPf complementary to mloxP and gene specific reverse primer a4R12 (Table 1), and 1 μl of 1st round PCR product as template. The PCR program was 94 °C 2 min, 30 cycles of (95 °C 30 s, 57 °C 30 s, and 72 °C 35 s), and a final extension at 72 °C for 5 min. 4 μl of the 2nd round PCR was then analyzed on 1.1% agarose gel. The images of the gels were analyzed with ImageJ (http://imagej.nih.gov/ij/). The experiment was performed 3 times independently and the relative knockin efficiency was shown in mean ± SEM. The results were subjected to Student's *t*-test.

2.5. Selecting founder embryos carrying PGCs labeled with enhanced yellow fluorescent protein via fluorescence photography

To perform gene targeting in zebrafish *aldh1a2*, 1 nl solution containing 500 pg *yfp-nanos3* mRNA, 250 pg Cas9 mRNA, 50 pg sgRNA a3gR11, 50 pg sgRNA a4gR5, 50 pg donor a3gR11ssD and 50 pg donor a4gR5ssD (Table 1 and Supplementary Table 1, Supplementary Protocol) was co-microinjected into 1-cell embryos. At 48 hpf, the embryos were observed for yellow fluorescence as described above. Embryos exhibiting YFP signal in their PGCs were selected as founders (on-PGC founders) and grown to sexual maturity. The embryos microinjected with the same solution but not selected by yellow fluorescence were raised as control founders.

Gene targeting (knockin) efficiency was first detected in the oocytes isolated from adult founder ovaries using nested PCR as described above. To detect knockin events in intron 3, the gene specific reverse primer used in 2nd round PCR was a3R3 (Table 1). The relative knockin efficiency in founder ovaries was measured as described above and normalized to the average ratio of knockin at a3gR11 locus of control founders. The results were subjected to Student's *t*-test. To determine knockin and knockout efficiencies in the F1 progeny, 35 of on-PGC founders and 60 of control founders were allowed to mate ad libitum, respectively. 16 of 24 hpf embryos derived from on-PGC founders and 93 of 24 hpf embryos derived from control embryos were then randomly selected to examine their genotypes by directly sequencing the 1st round of PCR products amplified from the 24 hpf embryos using the method

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