Targeted mutagenesis in medaka using targetable nuclease systems

（ゲノム編集ツールを用いたメダカにおける標的遺伝子破壊）

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2016

1. General Introduction

Genetics is the study to know the relationships between genes and phenotypic traits in living organisms. Recent advances in DNA sequencing technologies have increased the importance of reverse genetics for understanding of functions of individual genes found in the sequenced genome. Of course, a number of genomes have been sequenced in teleost fish species including both models for basic research fields and fishes for aquaculture and the fishing industry. However, available techniques for genetic manipulation in teleost fishes, especially loss-of-function approaches, has been restricted, and thus, understanding of each gene function identified in the genome sequencing projects by the reverse genetics approach has been hindered. In this study, I intended to establish the efficient analytical techniques for gene functions by the reverse genetics approach in medaka (*Oryzias latipes*), a small fish model suitable for genetics, which could be easily applied to a wide range of teleost fish species. I focused on genome editing using targetable nuclease systems that has become a powerful tool for approaches involving reverse genetics in a wide range of organisms. These systems efficiently induce site-specific DNA double-strand breaks (DSBs), resulting in targeted gene disruptions by insertions and deletions (indels) or targeted gene integrations by homologous recombination. Firstly, I demonstrated targeted mutagenesis by small insertions and deletions (indels) in medaka using three classes of targetable nuclease systems: zinc-finger nucleases (ZFNs), transcription activator-like (TAL) effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system-based RNA-guided endonucleases (RGENs). Subsequently, to assess the usefulness of targeted mutagenesis using the nucleases in loss-of-function analysis, I also demonstrated targeted disruption of medaka *tph2* gene that is involved in serotonin synthesis in the brain stem and examined the neurobehavioral phenotypes in the homozygous mutant by a newly developed behavioral testing system for medaka.

2. Targeted mutagenesis using zinc-finger nucleases

ZFNs are artificial nucleases consisting of engineered zinc fingers fused to the nuclease domain of FokI. In this chapter, I demonstrated successful gene disruption in somatic and germ cells of medaka using ZFN to target exogenous *EGFP* genes. Embryos that were injected with an RNA sequence pair coding for ZFNs showed mosaic loss of green fluorescent protein fluorescence in skeletal muscle. A number of mutations that included both deletions and insertions were identified within the ZFN target site in each embryo, whereas no mutations were found at the non-targeted sites. Additionally, ZFN-induced mutations were introduced in germ cells and efficiently transmitted to the next generation. The mutation frequency varied (6–100%) in the germ cells from each founder, and a founder carried more than two types of mutation in germ cells.

3. Targeted mutagenesis using custom-designed transcription activator-like effector nucleases

TALENs consist of a fusion between a FokI nuclease domain and a transcription activator-like (TAL) effector DNA recognition domain found in plant pathogenic bacteria. In this chapter, I demonstrated efficient targeted mutagenesis in medaka using TALENs. I designed and constructed a pair of TALENs targeting the medaka *DJ-1* gene, a homolog of human *DJ-1/PARK7*. These TALENs induced a number of insertions and deletions in the injected embryos with extremely high efficiency. This induction of mutations occurred in a dose-dependent manner. All screened G0 fish injected with the TALENs transmitted the TALEN-induced mutations to the next generation with high efficiency (44–100%). These TALENs induced site-specific mutations because none of the mutations were found at potential off-target sites. Additionally, the DJ-1 protein was lost in *DJ-1*$^{\Delta 7/\Delta 7}$ fish that carried a TALEN-induced

frameshift mutation in both alleles. I also investigated the effect of the N- and C-terminal regions of the TAL effector domain on the gene-disrupting activity of DJ1-TALENs and found that 287 amino acids at the N terminus and 63 amino acids at the C terminus of the TAL domain exhibited the highest disrupting activity in the injected embryos. These results suggest that TALENs enable us to rapidly and efficiently establish knockout medaka strains.

4. Design, evaluation, and screening methods for efficient targeted mutagenesis with TALENs

In this chapter, I have described efficient detection methods for TALEN-induced mutations at endogenous loci and presented guidelines of TALEN design for efficient targeted mutagenesis in medaka. I performed a heteroduplex mobility assay (HMA) using an automated microchip electrophoresis system, which is a simple and high-throughput method for evaluation of in vivo activity of TALENs and for genotyping mutant fish of F1 or later generations. I found that a specific pattern of mutations is dominant for TALENs harboring several base pairs of homologous sequences in target sequence. Furthermore, I found that a 5′ T, upstream of each TALEN-binding sequence, is not essential for genomic DNA cleavage. These findings provide information that expands the potential of TALENs and other engineered nucleases as tools for targeted genome editing in a wide range of organisms including medaka and other teleost fishes.

5. Targeted mutagenesis using CRISPR/Cas system

CRISPR/Cas system-based RGEN, consisting of the endonuclease Cas9 protein and small guide RNA to program the recognition specificity of the Cas9, has recently emerged as a simple and efficient tool for targeted genome editing. In this chapter, I showed successful targeted mutagenesis using RGENs in medaka. Somatic and heritable mutations were induced with high efficiency at the targeted genomic sequence on the *DJ-1* gene in embryos that had been injected with the single guide RNA (sgRNA) transcribed by a T7 promoter and capped RNA encoding a Cas9 nuclease. The sgRNAs that were designed for the target genomic sequences without the 5′ end of GG required by the T7 promoter induced the targeted mutations. This suggests that the RGEN can target any sequence adjacent to an NGG protospacer adjacent motif (PAM) sequence, which would occur once every 8 bp. The off-target alterations at two genomic loci harboring double mismatches in the 18-bp targeting sequences were induced in the RGEN-injected embryos. However, I also found that the off-target effects could be reduced by lower dosages of sgRNA. Taken together, these results suggest that CRISPR/Cas-mediated RGENs may be an efficient and flexible tool for genome editing in medaka.

6. Effects of chronic fluoxetine administration on anxiety-related and social behaviors in medaka

Medaka have used as a model in neurobehavioral research because of their complex social and/or visually-evoked behaviors; however, there have been few studies that comprehensively evaluated the behavioral effects of genetic manipulations by the multitiered phenotyping strategies that are used behavioral phenotyping of knockout mice. In this chapter, I established a behavioral testing system for assessment of the behavioral alterations in medaka by the multitiered strategy. To demonstrate the usability of the system, the behavioral alterations in medaka adult fish chronically administrated fluoxetine, a selective serotonin reuptake inhibitor (SSRI) class of anti-depressant, were assessed using five behavioral paradigms (diving, open-field, light-dark transition, mirror-biting, and social interaction) as indicators of anxiety-related and social behaviors. Fish chronically treated with fluoxetine exhibited anxiolytic responses such as an overall increased time spent in the top area in the diving test and an increased time spent in center area in the open-field test. Analysis of socially evoked behavior showed that chronic fluoxetine administration decreased the number of mirror biting times in the mirror-biting

test and increased latency to first contact in the social interaction test. Additionally, chronic fluoxetine administration reduced the horizontal locomotor activity in the open-field test but not the vertical activity in the diving test. These investigations are mostly consistent with previous reports in the other teleost species and rodent models. These results indicate that behavioral assessment in medaka adult fish will become useful for not only pharmaceutical and toxicological screening but also understanding the gene function in animal behaviors.

7. Behavioral phenotyping of tph2-deficient mutant fish generated by TALENs

In this chapter, to show the utility of the established techniques to understand the behavioral functions of genes, I demonstrated the neurobehavioral phenotyping of a medaka mutant that had a frame-shifted deletion in the *tph2* gene, encoding a tryptophan hydroxylase involved in the synthesis of serotonin (5-hydroxytryptamine; 5-HT), induced by TALENs. At first, I showed that the mutant fish were deficient in 5-HT of the raphe nuclei in the brain stem by immunohistochemical and mass spectrometric analysis. Subsequently, the behavioral alterations in the mutant fish were assessed using five behavioral paradigms (diving, open-field, light-dark transition, mirror approaching, and social interaction). The *tph2* mutant fish exhibited decreased locomotor activity and reduced the number of entries to the top area in the diving test. The *tph2* mutant female also showed a decreased mirror biting time in the mirror biting test and an increased contact number in social interaction test. In addition, the *tph2* mutant fish exhibited longer duration of freezing for the first several minutes of each test in all examined paradigms. These results indicate that the *tph2* gene will be involved in modulation of anxiety/fear responses and social behaviors in medaka. These facts also suggest that a combination of the genome editing technology established in the previous chapters and the behavioral testing system that was newly developed in the last chapter is an effective technique for understanding of gene functions in complex behaviors of medaka and other teleost fishes.

8. General Discussion

In this study, I demonstrated the successful targeted mutagenesis in medaka using targetable nuclease systems–ZFNs, TALENs, and CRISPR/Cas system. All three systems were introduced as RNA sequences into each fertilized egg of medaka by the microinjection method, thereby inducing small insertions and deletions on the target DNA sequence with high efficiency. The mutation events were occurred at the germ cells of the injected fish and therefore were efficiently transmitted to next generations. Subsequently, I showed that the frame-shifted mutation on the *tph2* gene by TALENs caused impairment of the 5-HT synthesis in the raphe nuclei of the brain stem, resulting in alterations of anxiety-related and social behaviors that were assessed with a newly developed behavioral testing system for small laboratory fish. These facts indicate that targeted mutagenesis using the targetable nuclease systems has become a powerful technology to elucidate gene functions within organisms, especially in adult phenotypes including complex behaviors, by reverse genetics approaches in medaka and other teleost fish species. These techniques will make a significant contribution in a broad range of studies including basic biology, biomedical research, toxicology, and aquaculture/fisheries.