Application of genome editing to marine aquaculture as a new breeding technology

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Chapter 1

An effective microinjection method for genome editing of marine aquaculture fish: tiger pufferfish *Takifugu rubripes* and red sea bream *Pagrus major*

Abstract

Genome editing technology is getting accepted as a way to improve traits in marine fish aquaculture. In fish, microinjection is a major method for introducing RNA or protein into eggs for genome editing; however, this method has not been established yet in aquaculture fish. I successfully established microinjection methods achieving high survival rates for tiger pufferfish *Takifugu rubripes* (fugu) and red sea bream *Pagrus major* by optimizing the following three parameters: (1) the soaking solution of fertilized eggs during microinjection, (2) the elapsed time from *in vitro* fertilization to microinjection, (3) the elapsed time from stripping to microinjection. In fugu, Iwamatsu solution or diluted sea water is effective as the soaking solution. *In vitro* fertilization can be performed at intervals of 15 minutes from fertilization until 2.5 hours after stripping. Similarly, in red sea bream, Leibovitz's L-15 medium or Iwamatsu solution is effective as the soaking solution and *in vitro* fertilization can be performed at intervals of 10 minutes from fertilization until 2.5 hours after stripping. I anticipate that the findings in the present study will contribute to effectively establish genome edited aquaculture breeds.

Chapter 2
Production of a breed of red sea bream *Pagrus major* with an increase of skeletal muscle mass and reduced body length by genome editing with CRISPR/Cas9

Abstract

Genome editing is a powerful tool as a new breeding technology including for aquaculture because of the high efficiency of gene targeting without the requirement for exogenous gene integration. CRISPR/Cas9 system, a genome editing tool, has been widely used in various species due to its efficiency and flexibility. I demonstrated the establishment of a new breed of myostatin (*Pm-mstn*) complete knockout red sea bream (*Pagrus major*) using CRISPR/Cas9. This is the first report of the establishment of a new breed in aquaculture marine fish using genome editing. The mutations were formed by deletions in the first exon of the *Pm-mstn*, which cause disruption of the C-terminal active domain of MSTN. The breed exhibited a 16% increase of skeletal muscle, that is, an increase of edible parts. The breed showed the phenotype of short body length and small centrum, which is not observed in mice and other teleost fish. I established the homozygous gene disrupted breed in 2 years, which is far shorter than the conventional breeding method. The present study indicates that genome editing can accelerate the speed of aquaculture fish breeding.

Chapter 3
The establishment of myostatin knockout tiger pufferfish *Takifugu rubripes* with CRISPR/Cas9 genome editing for a breed improvement in aquaculture and an investigation of the gene function in fugu

C3.1 Abstract

Tiger pufferfish *Takifugu rubripes* (fugu) is an important fish for aquaculture breeding and genetics. Therefore, the method of mutagenesis in fugu is necessary to add valuable traits for aquaculture and to investigate gene function. In the present study, genome editing technology CRISPR/Cas9 was utilized to establish the fugu breed exhibiting high meat production by myostatin gene (*mstn*) knockout. Furthermore, the *mstn* gene function on skeletal muscle and bone structure was investigated in fugu. Two kinds of sgRNAs were designed on *mstn* gene for gene knockout to produce mutants. The mutations were successfully induced in the target *mstn* locus in G_0 fish and these G_0 mutants showed increased skeletal muscle mass. Moreover, I successfully established F_1 homozygous mutants harboring 8 base deletions in *mstn* which cause knockout of the gene. The homozygote exhibited 20-32% increase of skeletal muscle in cross-sectional areas, short body length, and short length of centrums in the direction of anterior-posterior and dorsal-ventral, in comparison with wild type. It was revealed by off-target analysis that the sgRNAs had high specificities for target *mstn* loci. The present results indicated that CRISPR/Cas9 genome editing enables rapid breeding with certainty and reverse genetics analysis in fugu, and that the new fugu breed with skeletal muscle mass is valuable for aquaculture. Furthermore, it is suggested that it is important to investigate a gene function in each fish species of interest because the function and phenotype may be different from the results obtained by model animals.