Title

Establishment of a practical gene knock-in system and its application in medaka

Author

Yu Murakami

Chapter2

An efficient gene knock-in system mediated by homologous recombination

Abstract

The CRISPR/Cas system is a powerful genome editing tool that enables targeted genome modifications in various organisms. In medaka (*Oryzias latipes*), targeted mutagenesis with small insertions and deletions using this system have become a robust technique and are now widely used. However, to date there have been only a small number of reports on targeted gene integration using this system. I thus sought in the present study to identify factors that enhance the efficiency of targeted gene integration events in medaka. I showed that longer homology arms (ca. 500 bp) and linearization of circular donor plasmids by cleavage with bait sequences enhances the efficiency of targeted integration of plasmids in embryos. A new bait sequence, BaitD, which I designed and selected by *in silico* screening, achieved the highest efficiency of the targeted gene integration *in vivo*. Using this system, donor plasmids integrated precisely at target sites and were efficiently transmitted to progeny. I also reported that the genotype of F_2 siblings, obtained by mating

of individuals harboring two different colors of fluorescent protein genes (e.g. GFP and RFP) in the same locus, can be easily and rapidly determined non-invasively by just visual observations. I reported that the efficiency of targeted gene integration can be enhanced by using donor vectors with longer homologous arms and linearization using a highly active bait system in medaka. These findings contribute to the establishment of more efficient systems for targeted gene integration in medaka and other fish species.

Chapter3

Targeted gene integration into a lethal gene using Cas9 nickase

Abstract

The CRISPR/Cas system has evolved as the most powerful approach to generate various genetic models both for fundamental and applied research. In medaka (*Oryzias latipes*), although the CRISPR/Cas9 system have enabled the HDR-mediated targeted gene integration, the conventional knock-in method using Cas9 inevitably activates both NHEJ and HDR pathway, which induces the unintended NHEJ-mediated gene disruption on the targeted locus. Because the gene disruption due to NHEJ may make lethal effects when lethal genes are targeted, the new knock-in method that can eliminate the undesirable side-effects was required. Then, I focused on Cas9 nickase (Cas9n) that can hardly cause NHEJ and mainly induce HDR by generating the site-specific nick on genome. So far, no studies have demonstrated the successful gene knock-in using Cas9n in teleost fish, thus, I aimed to establish an efficient gene knock-in system with Cas9n in medaka by designing a novel donor plasmid (p2BaitD). The p2BaitD plasmid containing two pairs of bait sequences can doubly enhance the efficiency of targeted gene integration events on 4 days post injection comparing to the conventional plasmid pBaitD containing one pair of bait sequences. Moreover, the gene knock-in method by combining p2BaitD and Cas9n allows to generate F_0 founders with germline transmission of the HDR-induced transgene integration on a lethal gene. In summary, I have developed an efficient procedure using Cas9n for the gene knock-in targeting lethal genes in medaka, which would contribute to

achieve flexible modifications of lethal genes in a wide range of organisms.

Chapter4

A useful system for transporting foreign proteins into eggs with vitellogenin signal

Abstract

In this study, I developed a novel bioreactor system to deliver and accumulate foreign proteins in eggs using medaka fish *Oryzias latipes* with the aid of a partial sequence of vitellogenin (Vtg). In teleost fish, Vtg, the hepatically generated precursor of egg yolk proteins, is secreted into the bloodstream and then taken up into eggs. I predicted in silico a probable region (Vtg signal) of Vtg that mediates transportation of proteins from the liver into eggs. Then, I established two transgenic lines expressing the fused proteins including the Vtg signal and each reporter gene, *enhanced green fluorescent protein* (*EGFP*) or firefly *luciferase* (*LUC*)-fused *EGFP*, in the liver driven by a liver-specific *choriogenin*H (*chg*H) promoter. Each reporter signal was detected from the fertilized eggs spawned by the transgenic females, showing successful transportation of the proteins into the eggs with the Vtg signal. This is the first report demonstrating that the Vtg signal has capability to deliver exogenous proteins into eggs. Because Vtg is a highly conserved protein among most of oviparous organisms, our findings hold promise for establishing bioreactor systems viable in a wide range of organisms.