Application of dead end-knockout zebrafish to recipients of germ cell transplantation

［課程博士・論文博士共通］

博士学位論文内容要旨
Abstract

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論文題目 Title	Application of *dead end*-knockout zebrafish to recipients of germ cell transplantation （*dead end* ノックアウトゼブラフィッシュの生殖細胞移植用宿主への利用）		

Germ cell transplantation can be a promising technology for propagation of endangered or valuable fishes. In this technology, sterile fish are widely used as recipients and injected with donor germ cells and produce viable gametes derived from the donors. Although triploid fish can be used as recipients, the mitotic germ cells in the gonads of triploids often compete the niche with the transplanted germ cells, which decreases the transplantation efficiency. Knockdown of the *dead end* (*dnd*) gene, which is involved in the migration of primordial germ cells, would produce germ cell-deficient fish, which can provide open niche to transplanted germ cells. However, as the sterile phenotype obtained from *dnd* gene knockdown was not passed to the next generation, microinjection into fertilized egg individually is necessary. It makes gene knockdown technology very laborious and time-consuming. Also, *dnd* knockdown fish sometimes carry small numbers of endogenous germ cells, which can proliferate later and eventually shows normal fecundity. Therefore, we aimed at genome editing method of zinc finger nuclease (ZFN), which is a kind of artificial nuclease used to generate DNA double-stranded breaks as a specific position in the genome, to knockout *dnd* gene to produce recipient fish that are genetically germ cell-less. We attempted to use *dnd*-knockout technology followed by mating heterozygous mutant male and female individuals for mass production of sterile fish. In this study, *dnd* gene knockout was performed to examine fertility of *dnd* homozygous mutants and their suitability as recipients for germ cell transplantation. There are two chapters in this study. The first chapter is production of germ cell-less zebrafish by knockout of *dnd* gene using artificial nuclease of ZFN. The second chapter is germ cell transplantation using germ cell-less zebrafish as recipients.

In the first chapter, we demonstrated that ZFN is a functional genome editing method in knockout of *dnd* gene in zebrafish. ZFN were designed to target the exon 2 of zebrafish *dnd* gene. Two nanoliters of the synthetic cRNAs at different concentrations (10, 20, 40, 60, 80 and 100 ng/μl) were microinjected into blastodisc of zebrafish embryos before the cell cleavage stage. We optimized the dose of ZFN cRNAs after comparison of larval survival rate under the different concentrations of cRNAs. The larvae survival rate decreased to 32.0%, 21.5% and 15.3% when for the injection of ZFN cRNAs at 10, 20 and 40 ng/μl, respectively. For higher concentrations of ZFN cRNAs (60, 80 and 100 ng/μl), there was no survival as gastrulation. These results indicated that zebrafish embryos microinjected with cRNA at 10 ng/μl showed the highest larval survival rate and an excess of ZFN cRNAs has an adverse effect on survival. The ZFN-induced mutations in fin clips and gametes of founder were then detected using mismatch-specific DNA endonuclease, CEL I digestion, and direct sequencing analyses. Four out of twenty founder zebrafish, which were detected, carried mutations of deletions or the combination of deletion and insertion. One male founder fish carried mutation of the combination of 11 bp deletion and 8 bp insertion in *dnd* gene of its sperm. *dnd* heterozygous F1 generation were obtained by crossing the male founder that carried mutation with wild-type female zebrafish. This male founder was capable of transmitting the mutation of 11 bp deletion and an 8 bp insertion to the *dnd* heterozygous F1 generation and the germline transmission rate was 60%. Crossing of the *dnd* heterozygous F1 males and females, which carried mutation, revealed that approximately 25% of the F2 generations were *dnd* homozygous mutants (*dnd*-knockout) which carried the same mutation of 11 bp deletion and an 8 bp insertion with the sperm of the founder and the *dnd* heterozygous F1 generation. All of the *dnd*-knockouts showed male phenotype. HE staining of gonadal section

suggested that all of the *dnd*-knockout zebrafish carried testis-like gonads. Molecular marker and histological analyses were performed to examine the existence of germ cells in the testes of *dnd*-knockout zebrafish. No germ cells were histologically detected in the testes of *dnd*-knockout zebrafish. Further, RT-PCR and *in situ* hybridization (ISH) suggested that there were no expression of transcripts for the *vasa* gene, which is the marker of germ cells, in the gonads of *dnd*-knockouts, demonstrating that *dnd*-knockouts were germ cell-less. Thus, we could successfully produce *dnd* homozygous mutants that were germ cell-less.

In the second chapter, we examined that whether germ cell-less zebrafish can be used as a more efficient recipient in germ cell transplantation. Firstly, as Sertoli cells play important roles for supporting and nursing germ cells, expression of *gsdf* gene, which is the marker of Sertoli cells, in the gonads of germ cell-less individuals were detected. Results of RT-PCR and ISH suggested that *gsdf* gene expresses in the testes of germ cell-less zebrafish, demonstrating that germ cell-less testes carry Sertoli cells, which are important for germ cell development. Then, to explore if the Sertoli cells in the empty testes can also nurse the transplanted germ cells, germ cell-less zebrafish larvae together with control recipients (*dnd* heterozygous mutants and wild-type individuals) carrying germ cells at the stage of 9-10 days post fertilization (dpf) were intraperitoneally transplanted with testicular cells which were isolated from the testes of *vasa-gfp* transgenic zebrafish. Fluorescence observation of the recipients at 50 days post-transplantation revealed that GFP-positive germ cells can be incorporated into both the control group and the germ cell-less recipients comparing with GFP-positive testes. Among the germ cell-less recipients, 2 out of 40 recipients carried green germ cells. Further, the germ cell-less recipients receiving GFP-positive germ cells could mature and produce sperm. Germ-line transmission rates of donor-derived genotype were examined by progeny tests. Offspring were produced by mating mature *dnd*-knockout zebrafish receiving donor germ cells (male) with female wild-type zebrafish. The fertilization rate of eggs inseminated with sperm obtained from recipients and wild-type individuals were similar. Donor-derived GFP-positive offspring were identified by fluorescence observation. Most importantly, all resulting offspring from *dnd* homozygous mutant recipients showed green fluorescence in the germ cells and amplification of *gfp* DNA fragments by PCR. On the other hand, control recipients produced both GFP-positive and GFP-negative offspring, and the germline transmission rates were 3.9-7.3%. Thus, in this chapter, we could demonstrate that germ cell-less zebrafish possessed an ability to nurse donor-derived germ cells, produce functional *vasa-gfp* gametes and then produce donor-derived offspring entirely.