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

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Doctoral Dissertation

APPLICATION OF *dead end*-KNOCKOUT ZEBRAFISH TO RECIPIENTS OF GERM CELL TRANSPLANTATION

September 2017

Graduate School of Marine Science and Technology
Tokyo University of Marine Science and Technology
   Doctoral Course of Applied Marine Biosciences

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Abstract

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 \textit{in situ} hybridization (ISH) suggested that there were no expression of transcripts for the \textit{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 \textit{dnd} homozygous mutants that were germ cell-less.

In the second chapter, we examined 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 \textit{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 \textit{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 (\textit{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 \textit{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 \textit{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 \textit{dnd} homozygous mutant recipients showed green fluorescence in the germ cells and amplification of \textit{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 \textit{vasa-gfp} gametes and then produce donor-derived offspring entirely.
Introduction

We previously established surrogate broodstock technology wherein immature germ cells isolated from target fish species were transplanted into a closely related species, thereby allowing the surrogate species to produce eggs and sperm of the target species (Takeuchi et al., 2004; Okutsu et al., 2006; Okutsu et al., 2007). For example, when spermatogonial stem cells from rainbow trout (*Oncorhynchus mykiss*) were transplanted into embryonic male and female recipient masu salmon (*Oncorhynchus masou*), the recipients generated donor-derived, functional sperm and eggs, respectively (Okutsu et al., 2006; Okutsu et al., 2007). The spermatogonial transplantation technique has also been expanded to other species, such as Nile tilapia (Farlora et al., 2014), medaka (Seki et al., 2017), and several marine fish species, including members of the Sciaenidae (Takeuchi et al., 2009; Higuchi et al., 2011; Yoshikawa et al., 2017), Scombridae (Yazawa et al., 2010), as well as Carangidae (Morita et al., 2012; 2015; Bar et al., 2015).

Although the technique for interspecies spermatogonial transplantation was successful, the recipients also produced large quantities of autologous gametes in addition to the donor-derived gametes. Indeed, only a small portion of the F1 offspring from these recipients was genetically derived from the donors (Okutsu et al., 2006). Based on these outcomes, we explored the possibility of creating surrogate recipients that only produce donor-derived offspring (Okutsu et al., 2007; Yoshizaki et al., 2016). We were able to produce recipients that produced only donor derived trout
gametes by transplanting spermatogonia into sterile, triploid masu salmon (Okutsu et al., 2007). Triploids are generally sterile due to irregular meiotic division of chromosomes, resulting in retarded gonadal development (Piferrer et al., 2009). Although sterilized triploid recipients possess the ability to nurse donor-derived germ cells (spermatogonia and oogonia) into fully functional spermatozoa or eggs in the presence of recipient supporting cells in their gonads (Okutsu et al., 2007; Yoshikawa et al., 2017), endogenous germ cells (mostly mitotic germ cells) are also present in the gonads of triploid individuals. These undifferentiated, triploid germ cells are predicted to compete with transplanted germ cells for the germ cell niche, decreasing transplantation efficiency. Therefore, it is preferable to dislodge endogenous germ cells so that all the supporting cells and niche can be utilized for exogenous germ cells, resulting in increased colonization and proliferation efficiency of the donor-derived germ cells.

An alternative approach to obtain germ-cell-less gonads is through germ-cell-deficient recipients produced by dead end (dnd) gene knockdown, a phenotype previously achieved in zebrafish, sturgeon, and rainbow trout (Weidinger et al., 2003; Linhartová et al., 2015; Yoshizaki et al., 2016). The dnd gene is specifically expressed in primordial germ cells (PGCs), and is essential for their survival and migration to the gonadal anlagen (Weidinger et al., 2003). Zebrafish embryos injected with a dnd antisense morpholino oligonucleotide that blocked PGC development were devoid of endogenous PGCs as adults, and could serve as recipients for the transplantation of germ cells and subsequent production of gametes.
derived entirely from the donor germ cells (Ciruna et al., 2002). Similar results were obtained by xenotransplantation of PGCs into dnd-knockdown zebrafish (Saito et al., 2008). In previous research, we obtained germ-cell-deficient masu salmon by injecting dnd antisense morpholino oligonucleotide into 1-cell-stage embryos, and then transplanting rainbow trout germ cells, resulting in dnd-knockdown salmon that produced 100% rainbow trout offspring (Yoshizaki et al., 2016). Unfortunately, the sterility phenotype of dnd-knockdown recipients was never passed to the next generation, making the knockdown approach labor-intensive since fertilized eggs need to be injected individually to produce each knockdown fish.

Previous studies demonstrated that dnd-knockouts possess germ-cell-less, sterile gonads – as reported for mouse (Youngren et al., 2005) and Atlantic salmon (Wargelius et al., 2016). We therefore attempted to use dnd-knockout technology to generate genetically deficient, sterile zebrafish that could be mated to mass-produce sterile populations. To do so, we established procedures for zinc finger nuclease (ZFN)-mediated gene disruption of the dnd locus in zebrafish.

ZFNs are artificial nucleases used to generate DNA double-stranded breaks at a specific position in the genome (Carroll, 2011), allowing for genome editing and for loss-of-function studies. ZFNs were successfully employed for the production of gene knockout organisms, including zebrafish (Doyon et al., 2008; Ekker, 2008; Meng et al., 2008), medaka (Ansai et al., 2012), rat (Geurts et al., 2009), and mouse (Carbery et al., 2010; Meyer et al., 2010).

In this study, homozygous dnd mutants were obtained in the F2 generation, and
were confirmed as germ-cell-less in histological and molecular biological studies. Further, we examined the suitability of germ-cell-less zebrafish as recipients of spermatogonial transplantation in order to obtain basic information to support the application of \textit{dnd}-knockout recipients to spermatogonial transplantation of endangered or farmed fish species.
References


Yoshizaki G, Takashiba K, Shimamori S, Fujinuma K, Shikina S, Okutsu T, Kume S,

Chapter 1

Production of germ cell-less zebrafish by knockout of *dead end* (*dnd*) gene using artificial nuclease of zinc finger nuclease (ZFN)
1.1 Introduction

Zinc finger nuclease (ZFN) technology is one of the powerful tool for editing the genome and loss-of-function studies. ZFN is a kind of artificial nucleases to generate DNA double-stranded breaks (DSBs) at a specific position in the genome (Carroll, 2011). ZFN-induced DSBs accomplish gene editing through cellular repair mechanisms involving error-prone non-homologous end joining (NHEJ) with imperfect fidelity (Urnov et al., 2010). ZFN is a chimeric nuclease composed of custom-designed DNA binding domain fused to the DNA cleavage domain from the $Fok\ I$ endonuclease that upon dimer formation cleaves the DNA (Figure 1). ZFN has been successfully utilized for production of gene knockout organisms, such as zebrafish (Doyon et al., 2008, Meng et al., 2008), medaka (Zhang et al., 2014; Guan et al., 2014), rat (Geurts et al., 2009; Mashimo et al., 2010) and mouse (Carbery et al., 2010; Meyer et al., 2010). Further, the targeted inactivation of $sdY$ in male rainbow trout using ZFN could induce ovarian differentiation (Yano et al., 2014).

Therefore, the first chapter of this study was aimed at the establishment of procedures for gene knockout at $dnd$ locus in zebrafish using ZFN and the production of germ cell-less zebrafish using ZFN.

ZFNs were designed to target the exon 2 of zebrafish $dnd$ gene. Synthetic cRNAs at different concentrations were microinjected into 1-cell stage of zebrafish embryos. The doses of ZFN cRNAs were optimized after comparison of larval survival rate. The ZFN-induced mutations in fin clips and gametes of founder were then detected using mismatch-specific DNA endonuclease (SURVEYOR nuclease) digestion and
direct sequencing analyses. Further, *dnd* homozygous F2 generation were obtained by crossings and their characteristics were analyzed by histological and molecular methodologies.
1.2 Materials and Methods

Animal husbandry and handling

All experiments using live fish were performed in accordance to the Guide for the Care and Use of Laboratory Animals, Tokyo University of Marine Science and Technology. Wild-type zebrafish were maintained under an artificial photoperiod of 14-h/10-h light/darkness at 28°C. Fish were fed 2-3 times daily with a commercial diet (Hikari Labo 130 [Kyorin, Hyougo, Japan]; Otohime A, B1, and B2, [Nisshin Marubeni Feed, Tokyo, Japan]) and Artemia nauplii. Zebrafish embryos were obtained by natural spawning, and held at 28°C.

Cloning of zebrafish dnd cDNA

Gonadal RNA was isolated by ISOGEN (Nippon Gene, Tokyo, Japan), and any trace of DNA was degraded with 3 U of RQ1 RNase-free DNase (Promega, Madison, Wisconsin, USA) for 30 min at 37°C in 450 µl of buffer containing 40 mM Tris-HCl, 11.1 mM NaCl, 6 mM MgCl₂, and 1 mM DTT. After phenol-chloroform extraction and ethanol precipitation, pellets were dissolved in diethylpyrocarbonate (DEPC)-treated water. Single-stranded cDNA was synthesized using Ready-to-Go™ You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK). dnd cDNA was amplified by PCR with primers dnd-FW and dnd-RV (Table 1), designed according to a sequence in the GenBank database (AY225448.1) (Weidinger et al., 2003).

PCR amplification was conducted for 30 cycles in a 10-µl reaction volume
containing 1× Ex Taq Buffer, 200 μM of dNTPs, 0.125 U of Ex Taq polymerase (Takara, Shiga, Japan), 50 ng of cDNA as the template, and 1 pM of each primer (Table 1). PCR reaction program was shown in Table 2. One microliter of each PCR product was run on a 0.7% Tris-borate-EDTA (TBE) agarose gel, and the amplified products were recovered from the gel using UltraClean® 15 DNA Purification Kit (Mo Bio, Carlsbad, CA, USA). Zebrafish dnd cDNA (1242 bp) was direct-sequenced with either dnd-FW or dnd-RV (Table 1) using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA), according to manufacturer instructions. The sequences were then visualized using the Sequence Scanner Software v1.0.

ZFN constructs

ZFN (5’-CTCCATCCTTTAACCAAGTCAATGGGCAGAGGAA) was designed to target the exon 2 of zebrafish dnd gene (Figure 2). Platform ZFN-vectors and ZFN-template vectors were designed and synthesized using the method described by Fujii et al. (2013). ZFN Vectors were constructed by overlap PCR and TA-cloning, as previously described (Fujii et al., 2013).

One subunit (left-ZFN) of ZFN, which targets exon 2 of the dnd gene in zebrafish, contained an array of six zinc fingers that recognizes the 18-bp nucleotide sequence 5’-TGGGTTAAAGTGATGGAG, and the other (right-ZFN) contained an array of four zinc fingers that recognizes the 12-bp sequence 5’-TGGGCAGAGGAA. DNA-recognition helix sequences of these two ZFN subunits are shown in Table 5. These zinc-finger arrays were fused in-frame with FokI endonuclease-forming ZFN
(Figure 2). The target site of this ZFN pair is separated by a 6-bp spacer, 5’-AGTCAA, allowing for dimer formation.

Preparation of ZFN cRNA

Left-ZFN and right-ZFN vectors were linearized by XhoI, and in vitro transcribed using T3 mMessage mMachine Transcription Kit (Ambion, Austin, TX, USA) to generate capped RNA transcripts. The resulting transcripts were purified by phenol-chloroform extraction and ethanol precipitation. RNA pellets were then dissolved in DEPC-treated water. Left- and right-ZFN cRNAs were mixed in a 1:1 ratio, and stored at -80°C until injection.

Microinjection

Zebrafish embryos at the 1-cell stage were injected with 2 nl of ZFN cRNAs or DEPC-treated water (as a control). ZFN cRNAs was injected at concentrations ranging from 10 to 100 ng/μl (0, 10, 20, 40, 60, 80, and 100 ng/μl) using the method described by Alimuddin et al. (2005). Survival rates of embryos were monitored until hatching.

DNA Extraction

Genomic DNA was isolated from the caudal fin, sperm, or eggs of adults using Puregene Core Kit A (Qiagen, Hilden, Germany). Caudal fins were cut into small pieces with dissecting scissors, and eggs were briefly homogenized with a plastic
pestle. Homogenized samples were placed in a 1.5-ml tube with 300 μl of cell lysis solution and 0.1 mg/ml proteinase K solution (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 55°C for 3-4 hr. Then, 20 μg/ml RNase A was added, and the samples were incubated at 37°C for 15 min. Protein was removed by adding 100 μl of protein-precipitation solution, and placing the reaction on ice for 15 min. DNA was then precipitated from the protein-depleted supernatant with 300 μl of 100% isopropanol, pellets were rinsed with 300 μl of 70% ethanol, and dissolved in 50 μl of distilled water.

Detection of ZFN-induced mutation using SURVEYOR nuclease (CEL 1)

Mutations in the region around the ZFN cleavage site were detected by amplifying the 308-bp region by PCR with primers dnd-ZFN-FW and dnd-ZFN-RV (Table 1), followed by SURVEYOR nuclease treatment. Genomic DNA (50 ng) was used as a template in each PCR reaction with amplification for 35 cycles (Table 2). SURVEYOR Mutation Detection Kit (Transgenomic, Omaha, NE, USA) was used to detect ZFN-induced mutations within exon 2 of the dnd gene. PCR products were hybridized and digested following the manufacturer’s instructions. Formation of cleavage products due to the presence of mismatches was observed by the presence of cleaved bands on 2% TBE agarose gels following electrophoreses. Direct sequencing was performed by the methods used for sequencing dnd cDNA, described above, in order to double-check for mutations in the founder generation. Mutations could be identified as dual peaks in sequence data when using primers dnd-ZFN-FW or
dnd-ZFN-RV (Table 1).

PCR products were TA-cloned into pGEM-T Easy vector (Promega). Genotypes associated with the DNA fragments were identified by sequencing with primers dnd-ZFN-FW or dnd-ZFN-RV (Table 1). Founders carrying mutations were crossed with wild-type zebrafish. The obtained F1 generation individuals were screened for \textit{dnd} heterozygous mutants using the SURVEYOR Mutation Detection Kit. Heterozygous mutants were then used to produce the F2 generation, 25\% of which were expected to be \textit{dnd} homozygous mutants. Homozygous mutants were distinguished in two steps, both conducted with SURVEYOR Mutation Detection Kit. First, heterozygous mutants were identified based on electrophoretic patterns showing cleaved fragments. Then, equal amounts of original PCR products from uncleaved and wild-type samples were hybridized and digested with SURVEYOR nuclease. From the resulting electrophoresis, PCR products with cleaved bands were identified as homozygous mutants.

Sequence analysis of mutation induced by ZFN at \textit{dnd} exon 2 in zebrafish larvae

DNA-positive PCR products were sub-cloned into pGEM-T Easy vector (Promega) and were transformed into DH5\(\alpha\) cells. After plating and incubating overnight at 37\(^\circ\)C, white clones were used for cracking (Jeffery et al., 2002). From the electrophoresis gel, clones which carry bigger than non-insert plasmid DNA were identified. PCR tests were performed to amplify insert DNA using the same primers used for SURVEYOR nuclease analysis. Fifty times diluted cracking DNA were used as the
template DNA. Genotypes of insert DNA were identified by direct sequencing with
the primers of ZFN-FW and ZFN-RV (Table 1).

Production of stable lines

The founders carrying mutation were crossed with wild type zebrafish. F1 obtained
from the cross were used for screening of the dnd mutation. F1 offspring carrying the
mutation were then used to produce F2 generations by intercrosses. Production and
screening procedures for F1 and F2 were the same as the method used for founders.

Characterization of dnd-knockout zebrafish

First, histological analyses of gonads were performed. Whole adult fish were fixed
in Bouin’s solution at 4°C overnight, embedded in paraffin wax, and then sliced into
4-µm thick cross serial sections. The paraffin sections were dewaxed and rehydrated
by passing them through a xylene-ethanol series, followed by hematoxylin and eosin
staining (HE staining). Images of sections were taken under a light microscope
(BX-53; Olympus, Tokyo, Japan) and a digital camera (DP-72; Olympus).

Reverse transcription PCR (RT-PCR) was also performed to examine the
expression of vasa (Yoon et al., 1997), a germ cell marker, in the gonads of wild-type
zebrafish, dnd heterozygous mutants and homozygous mutants (dnd-knockouts).
Gonadal RNA extraction and reverse-transcription were performed as described above.
RT-PCR was performed as shown in Table 2 using primers for vasa (Table 1). The
β-actin gene was also amplified as an internal control by primers for β-actin-FW and
-RV (Table 1).

Further, *in situ* hybridization (ISH) was carried out to characterize cellular-level the expression profiles of *vasa* gene in *dnd* mutants. cDNA fragments of zebrafish *vasa* (2501-bp, BC129275.1 in GenBank) were sub-cloned into pGEM T-easy vector. Sense and antisense RNA probes were transcribed *in vitro* using digoxigenin-labeled uridine triphosphate (UTP; Roche, Mannheim, Germany) and T7 or SP6 RNA polymerase (Promega), respectively. Whole bodies of wild-type zebrafish, *dnd* heterozygous mutants and *dnd* homozygous mutants were fixed and sliced following the methods used for HE staining. ISH was performed with methods modified from those of Hoshino et al. (1999). Following ISH, the sections were counterstained by nuclear fast red, and photographs were taken using a digital camera (DP-72; Olympus) under a light microscope (BX-53; Olympus).
1.3 Results

Survival after injection of ZFN RNAs

We first examined the effect of various doses of ZFN RNAs on the survival rate of injected embryos. Zebrafish embryos at the 1-cell stage were injected with ZFN RNAs at different doses, and their survival rates were monitored at the gastrula and hatching stages. The hatching and survival rates following injection of various concentrations of ZFN cRNAs into 1-cell-stage embryos decreased compared to those of uninjected controls. Control embryos showed a 96.2% hatching rate, but the rate decreased to 32.0%, 21.5%, and 15.3% for the injection of ZFN cRNAs at 10, 20, and 40 ng/μl, respectively; no embryos to survive to gastrulation following injection of higher ZFN cRNA concentrations (60, 80, and 100 ng/μl ZFN cRNAs) (Table 3). Embryos injected with 20 and 40 ng/μl ZFN RNAs often showed malformation (Figure 3). All of the malformed embryos died before they reached to the hatching stage. These results indicate that an excess of ZFN cRNAs has an adverse effect on embryogenesis and survival. Injection of ZFN cRNAs at 10 ng/μl ensured a satisfactory survival rate for larvae production, so we used this concentration for the remainder of this study.

Mutagenesis in founder generation by ZFN

SURVEYOR nuclease analysis revealed that among the 20 samples which received ZFN RNAs, four adults (#5, #6, #8, and #13) showed cleaved fragments which were
about 150 bp (Figure 4). Results of direct sequencing were showed in Figure 5. Sequence analysis showed that in the 3’-side of the cleavage site of ZFN in #5, dual peaks were identified. By reading the secondary peaks, a dnd sequence lacking 12 bp region around the cleavage site was identified. The sequences of the other three adults (#6, #8 and #13) were the same as wild type.

Genomic DNAs from the four adults were subjected to PCR followed by cloning into plasmid vectors. A total of 160 recombinant (40 for each) clones were sequenced, which led to the identification of 3 different gene disruption alleles (Figure 6). The first and second are the simple deletion of 6 and 5 bp nucleotides, respectively. The third has 31 bp insertion and 4 bp deletion. The second 5 bp-mutation caused frameshift and eventually truncation of dnd polypeptides by stop codon. The third mutation created a stop codon within the insertion sequences, resulting also truncation (Figure 7).

Germline transmission of the ZFN-induced mutations

Among the four founder adults (#5, #6, #8, and #13) described above, one (#13, female) was confirmed to be sterile, given that no eggs were produced. The remaining three founder adults (#5, #6, and #8) were examined for germ-line transmission of the knocked-out dnd gene. Genomic DNA from eggs of founder #5, eggs of founder #6, and sperm of founder #8 were analyzed using the same method as for analysis of genomic DNA from the fin clips. Direct sequencing showed dual peaks at and after the ZFN-cleavage site in gametes from founders #5 and #8 (Figure 8), but a single
peak in eggs from founder #6 (Figure 8). Therefore, the PCR-amplified fragments of the gamete DNA from founders #5 and #8 were subjected to cloning and sequencing; a total of 80 recombinant clones of gametes (40 for each founder) were analyzed. Eggs of founder #5 contained two different types of mutant alleles (Figure 8): a 12- or 13-bp deletion around the ZFN cleavage site. Sperm of founder #8 had a mutation that was a combination of an 11-bp deletion and an 8-bp insertion (Figure 8).

Offspring from crosses of founders #5 and #8 with wild-type individuals showed that founder #8 (male) had germ-line transmission whereas founder #5 (female) did not. SURVEYOR nuclease analysis of 20 F1 progenies of founder #8 revealed that 12 had gene disruption events with distinctly cleaved bands (Figure 9), indicating a germ-line transmission rate of 60%. Cloning and sequencing of the ZFN target region suggested that gene-disrupted alleles had a single mutation pattern around the cleavage site of the dnd locus that was identical to the mutation (11 bp deletion and 8 bp insertion) detected in the sperm of founder #8 (D11I8) (Figure 8e).

The F2 generation of founder #8 was produced by intercrossing dnd heterozygous mutant F1 individuals. A total of 20 F2 progeny were genotyped; 14 showed nuclease-cleaved bands, indicating that these 14 zebrafish are dnd heterozygous mutants (Figure 11a). The remaining six PCR products were determined to be homozygous mutants for dnd (n=4, 20%) or wild-type (n=2, 10%) (Figure 11b) by SURVEYOR nuclease analysis, using original PCR products hybridized with wild-type DNA. Cloning and direct sequencing of PCR products of dnd homozygous mutants showed that the gene-disrupted alleles carried only one type of mutation: an
11-bp deletion and 8-bp insertion around the cleavage site of the dnd locus, which was identical to the gene disruption observed in the sperm of founder #8 and F1 progeny of founder #8 (D11I8) (Figure 8e). This mutation translated to amino acid mutations of a single amino acid deletion and three substitutions (Figure 10).

Molecular and histological characteristics of gonads in the mutant zebrafish

All of the 24 homozygous mutants (dnd-knockout) showed male phenotype. RT-PCR revealed that transcripts for the germ cell marker vasa were not expressed in the testes of dnd-knockout zebrafish. However, wild-type zebrafish and dnd heterozygous mutants showed clear expression (Figure 12a).

ISH was also performed to confirm the expression of vasa. Although positive signals were detected on sections of the testes from dnd heterozygous mutants and wild-type individuals, no signals were observed in the testes of dnd-knockout zebrafish by ISH with vasa antisense probes (Figure 12b-12d and Figure 12b'-12d'). Notably, the testes of dnd-knockout zebrafish were vacuolate and extremely small, having diameters of approximately 100 μm (Figure 12d”).
1.4 Discussion

In this study, the feasibility of ZFN-mediated gene disruption at dnd locus was clearly demonstrated in zebrafish embryos. Injection of ZFN cRNA that targeted the dnd gene into fertilized eggs could induce various mutations including both insertions and deletions around the ZFN-cleavage site. We also discovered the mutations found in gametes of the founders were transmitted to progenies. Especially, they were inherited from F1 to F2 generations followed by Mendelian fashion.

Although the mutant strain obtained in this study did not show frameshift or truncations on the dnd gene, the one amino acid deletion and three amino acid substitutions were enough to produce a germ cell-less phenotype, suggesting that amino acid sequences in the location of No. 36-39 (Weidinger et al., 2003) are essential for its function.

Dnd is an essential gene for germ cell development in various vertebrates including zebrafish (Weidinger et al., 2003). In the previous study, Weidinger knocked down this dnd gene by antisense morpholino oligonucleotide and successfully produced sterile zebrafish (Weidinger et al., 2003). In this study, we used dnd gene knockout, instead of knock down to establish a more efficient method to facilitate production of sterile fish, which can be used as recipients for germ cell transplantation. Homozygous mutants, which are sterile, were easily mass-produced by brother-sister mating of heterozygous mutants. Therefore, ZFN-induced mutants are more convenient method to produce sterile fish compared with AMO injection, which
requires a lot of microinjection.

RNA injection at a low dose of 10 ng/μl (2pl, 20 pg each) ensured a high survival rate of zebrafish embryos. We revealed that the doses of ZFN RNAs correlate negatively with the survival rate, which ranges from 32.0 to 15.3% when the ZFN RNAs were injected at 10, 20 and 40 ng/μl. The previous ZFN-mediated gene disruption case in zebrafish reported that embryos injected with 5 pg each of ZFN RNAs displayed a highest survival rate of 88%. However, highest lesion rate of approximately 20% was detected in the embryos injected with 10 pg each of ZFN RNAs (Meng et al., 2008). Another case in zebrafish showed that embryos injected with ZFN RNA of 20 pg each led to the highest lesion rate of 15% (Gupta et al., 2011). In medaka, ZFN RNA injection at a low dose of 10 ng/μl ensured a high survival rate of 77% in fry development, whereas RNA injection at a high dose of 40 ng/μl gave rise to nearly 100% gene disruption (Zhang et al., 2014). In the further study, the lesion efficiency at different doses of ZFN RNAs should be compared each other, together with their survival.

Zebrafish embryos injected with ZFN RNAs, especially with high dose of RNAs, might cause malformations with high frequency and some of them were even lethal. It was also reported in zebrafish that increasing ZFN RNAs caused nonspecific developmental malformations and that lesions in off-target sites occurred with greater frequency in morphologically abnormal embryos relative to normal embryos (Meng et al., 2008). Thus, one explanation of malformation is that ZFN may cause toxicity to embryos, leaving many embryos malformation or lethality. The other explanation is
that malformation or lethality are caused by nonspecific cleavage at off-target sites. Extra DSBs have been correlated to increase dose-dependent cytotoxicity in primary cultured human cells (Pruett-Miller et al., 2008).

In the study of founder #5, we found that the mutation patterns detected in caudal fin and eggs were different. Also, even in one founder, varieties of subtle allelic alterations could be detected in its progenies. These phenomenon can be explained by mosaicism. Injected ZFN RNAs were distributed to blastomere mosaically, resulting in the independent mutations in each blastomere. Therefore a single fertilized egg develops into doughter cells carrying different genotypes in an organism. Amanda and Cecilia (2006) also demonstrated mosaicism, they injected RNA into the yolk of one-cell stage embryos of zebrafish, during the early cleavage stages, streams of cytoplasm move from the yolk into the blastomeres, distributing the injected RNA, which was inherited to the next generation mosaically. Furthermore, the transmission rate from founder to F1 generation was approximate 50%, one explanation about it was the timing of mutation formation. In zebrafish, vasa homologue RNA which is located to the cleavage planes of 2- and 4- cell-stage embryos and expressed in primordial germ cells (PGCs) condenses into four subcellular clumps by the 32-cell stage. These 4 clumps are segregated to four cells through the 1000-cell stage. At the 4000-cell stage, the PGCs begun to divide (Yoon et al., 1997). If the ZFN RNAs distributed into all four cells at 1000-cell stage, mutation can occur in all germ-cell lineage. Consequently, mutation can inherit to the next generation at the percentage of 50% after meiosis.
In this study, our ultimate goal of genome editing is germline transmission of the mutation and to produce zebrafish containing a defined genetic alteration and a stable phenotype, which is sterile, to serve as recipients for germ cell transplantation. We noticed that, among four adults which contained detectable proportions of gene disruption alleles in caudal fin, only one was capable of germline transmission with a high frequency of 60% (12 out of 20 screened fish). The discrepancy between presence of gene disruption in the soma of founder and a lack of gene disruption in offspring perhaps resulting from mosaicism described above. The germline transmission rate we obtained was equal or superior to those determined in the previous ZFN-mediated gene disruption studies in zebrafish (4.0 to 32%, Meng et al., 2008; 4.9 to 31.9%, Doyon et al., 2008; 9.0 to 60%, Foley et al., 2009; 0.5 to 31%, Sood et al., 2013). In the case of medaka, the rates were ranging from 6.25 to 50% (Ansai et al., 2012). Also, in rainbow trout the rates ranged from 3.0 to 17% (Yano et al., 2014). These results demonstrated that ZFN-induced mutations can be introduced into the zebrafish at a high efficiency using our method established in this way.

As mentioned in the result, the dnd-knockout zebrafish had extremely small gonads lacking any germ cells. It was confirmed by RT-PCR and ISH that the testes of dnd-knockout zebrafish did not express the vasa gene, which is a molecular marker for germ cells (Yoon et al., 1997). These observations are consistent with findings in previous gene knockdown studies using AMOs against the dnd gene (Weidinger et al., 2003). Although the mutant strain obtained in this study did not show frameshift or truncations on the dnd gene, the one amino acid deletion and three amino acid
substitutions were enough to produce a germ cell-less phenotype, suggesting that amino acid sequences in the location of No. 36-39 (Figure 10) (Weidinger et al., 2003) are essential for its function.

In this chapter, we demonstrated that ZFN can be a method used for knockout if dnd gene in zebrafish, and for production of germ cell-less zebrafish. Further, the expression of gsdf gene, which is the marker of Sertoli cells and is essential for germ cell development, should be identified. Moreover, germ cell transplantation should be performed to evaluate the applicability of germ cell-less zebrafish larvae using as recipients in surrogate broodstock technology.
1.5 References


Sood R, Carrington B, Bishop K, Jones M, Risson A, Candotti F, Chandrasekharappa


1.6 Figure Legends

Figure 1.

Principle of ZFN-mediated gene knockout.

ZFN induced double-stranded breaks (DSBs), which accomplish gene editing through cellular repair mechanism of error-prone non-homologous end joining (NHEJ) with imperfect fidelity. ZFN is a chimeric nuclease composed of custom-designed DNA binding domain fused to the DNA cleavage domain from the \textit{Fok} I endonuclease that upon dimer formation and cleaves the target DNA.

Figure 2.

Zinc finger nuclease target site on \textit{dead end (dnd)} cDNA sequences.

This target site resides within the coding sequence of \textit{dnd} exon 2. The ZFN pair was designed to recognize the sequence containing two recognition sites and a 6-bp spacer. One zinc finger nuclease has 6 zinc fingers and the other has 4. Each zinc finger recognizes 3 bp of DNA. So this ZFN pair can recognize 30 bp of \textit{dnd} exon 2 and cleave the DNA sequences, located between the two recognition sites.

Figure 3.

External views of ZFN-injected embryos at the age of one day post fertilization.

a, Embryos injected with 20 ng/μl ZFN RNAs developed into malformed embryos.

b, Embryos injected with 40 ng/μl ZFN RNAs developed into malformed embryos.
c, Embryos injected with water as control.

**Figure 4.**

ZFN-mediated gene knockout in founder generation.

Amplicon of *dnd* fragment (308 bp) including ZFN cleavage site was cleaved by SURVEYOR nuclease. After the digestion, four founders (#5, #6, #8 and #13) out of twenty showed two bands which were about 150 bp, demonstrating that they carried heteroduplex DNA. Since cleaved fragments were similar sizes (153 and 155 bp), only one band was visible on the gel. The other two bands indicated fragments caused by single nucleotide polymorphism between two alleles. “WT”, wild-type.

**Figure 5.**

Results of direct sequencing of ZFN-target sites in #5, #6, #8 and #13 founders.

In #5, 3′-side of ZFN cleavage site had clear dual peaks. Twelve base pair deletion were observed here. The sequences of the other three founders (#6, #8 and #13) were the same as wild type samples.

**Figure 6.**

Nucleotide sequences of ZFN-target site in founder #5 and #6.

Three different gene disruption alleles: deletions of 5 and 6 bp within the cleavage site and complex modification including 4 bp deletion and 31 bp insertion which was
27 bp longer than the wild type allele were observed. “D”, deletion; “I”, insertion; “WT”, wild-type.

**Figure 7.**

Frameshift mutation caused by ZFN in the *dnd* coding region.

a, Wild type *dnd* cDNA and amino acid sequences.

b, *dnd* cDNA and amino acid with mutations found in founder #5. Five bp deletion induced frameshift and truncation occurred by stop codon located on nucleotide of No.238 (*).

c, *dnd* cDNA and amino acid with mutations found in founder #5. Mutation of 4 bp deletion and 31 bp insertion induced frameshift and truncation occurred by stop codon located on nucleotide of No.179 (*).

**Figure 8.**

Genotype of gametes obtained from founders.

a, PCR amplicons from gametes of three founders (eggs from #5 and #6; sperm from #8) generated cleaved fragments after the SURVEYOR nuclease treatment. Approximately 80 eggs from #5 and #6 were pooled and used for DNA extraction. Milt (5 µl) from #8 was used for DNA extraction. The resulting DNA was used for PCR followed by the SURVEYOR nuclease treatment. “WT”, wild-type.

b, In #5-eggs, dual peaks are presented around and 3′-side of the cleavage site.

c, In #6-eggs, single peak presented around and 3′-side of the cleavage site.
d, In #8-sperm, single peak presented around and 3’-side of the cleavage site.

e, Mutations were detected and genotyped from the gametes of founder #5 and #8. Three different kinds of mutations were detected: 12-bp deletion, 13-bp deletion and a complex modification including 11-bp deletion and 8-bp insertion (red frame). The number in the parenthesis shows the occurrence frequency of each type of mutation.

**Figure 9.**

Inheritance of knockout alleles to F1 generation of #8 founder.

Twelve (60%) out of 20 F1 larvae from founder #8 had a gene disruption (surrounded by dotted lines). “WT”, wild-type.

**Figure 10.**

*dnnd* cDNA and amino acid with mutations found in sperm, F1 generation and F2 generation of founder #8. Mutation of 11-bp deletion and 8-bp insertion (red frame) translates to amino acid mutations of a single amino acid deletion and three substitutions.

**Figure 11.**

Germline transmission of ZFN-mediated gene disruption from F1 to F2 generation derived from founder #8.

a, Fourteen (70%) out of 20 F2 larvae from founder #8 had a gene disruption that was heterozygous (surrounded by dotted lines). “WT”, wild-type.
b, SURVEYOR nuclease cleavage of PCR products for #1, #3, #7, #10, #16 and #17 mixed with equal proportions of WT indicated that 2 (#3 and #7; 10%) out of 20 products were WT and 4 (#1, #10, #16 and #17; 20%) out of 20 were homozygous. “WT”, wild-type.

Figure 12.

Vasa gene expression in WT zebrafish, dnd heterozygous and homozygous mutants.

a, RT-PCR using primer sets specific to a germ cell marker (vasa) were performed on template gonadal cDNA from three individuals. The negative control is distilled water instead of template cDNA.

b-d, Histological characteristics of the gonads of dnd-knockout zebrafish. ISH micrographs of WT zebrafish (b), dnd heterozygous mutants (c) and dnd homozygous mutants (d) using vasa probe. d and d’ show no expression of vasa in germ cell-less gonads. b’-d’ are magnified views of b-d, respectively. b”-d”: Hemotoxylin and eosin-stained views of b’-d’, respectively. “WT”, wild-type. Scale bars, 500 μm (b-d) and 30 μm (b’-d’, b”-d”).
1.7 Figures

Figure 1.
Figure 2.

dnd cDNA sequence

CTT*TAAATGAACCTTTTTGACTTTTCCACAAAATTTACAGGTGTGTCTATCATCATACAT

ACAGATGGTCGGAGACATGGATGCCAGACAGGAGCTTGCTACAGATCGAGAGCTGAG
GCAGAGACTAAGCTCTGACAGAAGATTTCCAGGATGGTCTACAGATG

CTTGTCATCTCTCTCCACACAGATCGAGAGCTGAG

TTGTCGCTGAGTTTTCATCAGCTCATCCCAGAGAGCTGAG

CTCAGAGACTAAGCTCTGACAGAAGATTTCCAGGATGGTCTACAGATG

CCATTACGCCACCATCCATGGCTAAGAAAGATGGCTGAGGTAAGGTTTTCCAGGACCGGA

CGCATTTCTCATACCCATAGTATGGTCTTCTCTTCTCCTAGTCAAGATCAG

CAGCTCTGAGATAGCGATTTCTCCAGAGCTGAG

GAGGAGCTGAG

ATG

GAATGTCAGATTATGGCTTGATCGA

ATGTGATTGTGATCAGTTTTACGTTCAGTATTATGTACTGTTCCGGTTATAGATGATGA

ATATGTGGAAATGTAATGAA

AAATAAGCATTTAGTTTACTGTTGATGAAGAAAA

AAAAAGGTGACCAAGGCAGTATTACTTTTATTTGATTTTATTTTTTTCAAGCTCTTGAA

TTTAGTGGTTTGAAGTTTTATGTTCTCGTCGTTTTATAATATTTTAACTATGTAATATTA

ATAATTGAGTTGTATTAGTCACGCTCATATTAGGATGACTGCTGTTTTTCACAGCTT

TTCTTTGAGTGTATTTTCTCATGATTTTATGTTCTCGTCTCATTTTATATTTTAACTAATATT

ATAATGAGTTGTATTAGTCACGCTCATATTAGGATGACTGCTGTTTTTCACAGCTT

TTCTTTGAGTGTATTTTCTCATGATTTTATGTTCTCGTCTCATTTTATATTTTAACTAATATT

ATAATGAGTTGTATTAGTCACGCTCATATTAGGATGACTGCTGTTTTTCACAGCTT

TTCTTTGAGTGTATTTTCTCATGATTTTATGTTCTCGTCTCATTTTATATTTTAACTAATATT

ATAATGAGTTGTATTAGTCACGCTCATATTAGGATGACTGCTGTTTTTCACAGCTT

ATGACTTTTGTACAAATAAATAGTTGTT
Figure 3.
Figure 4.
Figure 5.

#5

#6

#8

#13
Figure 6.

<table>
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<tr>
<th>Allele</th>
<th>Founder</th>
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<tr>
<td>...AACCCAAGTCAATGGGCAG...</td>
<td>WT</td>
</tr>
<tr>
<td>...AACCCA----------------TGGGCAG...</td>
<td>#5</td>
</tr>
<tr>
<td>...AACCCA----------------TGGGCAG...</td>
<td>#5, #6</td>
</tr>
<tr>
<td>...AACCCA----------------ATGGGCAG...</td>
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</table>

CAATGAGGGAGAGGAAGATGGGAAGAGGGAG
Figure 7.
Figure 8.

(e) Sequencing traces showing the insertion of a single nucleotide at different positions in the Allele Gamete WT D12 #5-eggs (1/40) D13 #5-eggs (1/40) D11B #8-sperm (21/40)
Figure 9.
Figure 10.

a

... ATTCTGACCCGCAGAAAATCGATCTGTGTGCAAGGAGTGCAGAGAATCTCAACTCTGCTGAGGATGGATGACAGAGAACTCCTACCTTAAACC
... I L N P Q K L K S L Q E W M Q R N S I T L T

180 190 200 210 220 230 240

CARGTCATGGGAGGAAATATGGTGTTGGCTCTCCTCCAGGGGATGGTGCTGAGAGGCTCTTGTCGGCGGCG
Q V N G Q R K Y G G P P G W Q G P A P G S G

b

... ATTCTGACCCGCAGAAAATCGATCTGTGTGCAAGGAGTGCAGAGAATCTCAACTCTGCTGAGGATGGATGACAGAGAACTCCTACCTTAA
... I L N P Q K L K S L Q E W M Q R N S I T L

180 190 200 210 220 230 240

GCCATATTGGGCAAGGAAATATGGTGTTGGCTCTCCTCCAGGGGATGGTGCTGAGAGGCTCTTGTCGGCGGCG
A I F G Q R K Y G G P P G W Q G P A P G S G

cleavage site
Figure 11.
Figure 12.

[Image of a gel electrophoresis blot showing bands for vasa and β-actin under different conditions (dnd+/+, dnd+/-, dnd-/-, and negative control). Below the gel, images labeled b, b', c, c', d, and d' showing histological sections with measurements.]
### 1.8 Tables

**Table 1.** Primer Sequences Used in This Study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′-3′)</th>
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<tr>
<td>dnd-FW</td>
<td>CAGGTGTGTCTATCATCATCATCA</td>
</tr>
<tr>
<td>dnd-RV</td>
<td>TTCGATCAAGCCATAATCTGACA</td>
</tr>
<tr>
<td>dnd-ZFN-FW</td>
<td>TGTCGGTTTGACACTTGAAAGG</td>
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<td>dnd-ZFN-RV</td>
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</tr>
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<td>vasa-FW</td>
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<tr>
<td>vasa-RV</td>
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<tr>
<td>β-actin-RV</td>
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Table 2.
PCR Reactions Performed in This Study

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<th>Gene name</th>
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<th>Primer name</th>
<th>Denature</th>
<th>Annealing</th>
<th>Extend</th>
<th>Cycle No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>dnd</td>
<td>dnd-FW</td>
<td>dnd-RV</td>
<td>94°C (30s)</td>
<td>57°C (30s)</td>
<td>72°C (30s)</td>
<td>30</td>
</tr>
<tr>
<td>dnd-ZFN</td>
<td>dnd-ZFN-FW</td>
<td>dnd-ZFN-RV</td>
<td>94°C (30s)</td>
<td>59°C (30s)</td>
<td>72°C (30s)</td>
<td>35</td>
</tr>
<tr>
<td>vasa</td>
<td>vasa-FW</td>
<td>vasa-RV</td>
<td>94°C (30s)</td>
<td>63°C (30s)</td>
<td>72°C (130s)</td>
<td>30</td>
</tr>
<tr>
<td>β-actin</td>
<td>β-actin-FW</td>
<td>β-actin-RV</td>
<td>94°C (30s)</td>
<td>62°C (30s)</td>
<td>72°C (20s)</td>
<td>30</td>
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</tbody>
</table>
Table 3.
Survival rates of zebrafish embryos injected with different concentrations of ZFN RNAs.

<table>
<thead>
<tr>
<th>ZFN cRNA (ng/µl)</th>
<th>No. of injected embryos</th>
<th>Survior No. (%)</th>
<th>Gastrula</th>
<th>Hatching</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>212</td>
<td>206 (97.2)</td>
<td>204 (96.2)</td>
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<td>10</td>
<td>81</td>
<td>59 (73.0)</td>
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<td>93</td>
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<td>80</td>
<td>169</td>
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<tr>
<td>100</td>
<td>202</td>
<td>0 (0)</td>
<td>0 (0)</td>
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</tbody>
</table>

Table shows the relationship between survival rate and ZFN cRNAs with various concentrations. A series of concentrations of ZFN cRNAs were applied: 10, 20, 40, 60, 80 and 100 ng/µl with DEPC-water (0 ng/µl) as negative control. Survival rates regarding two stages of gastrula and hatching were recorded. After ZFN cRNAs injection, with the decrease of concentration, survival rate increased.
Chapter 2

Germ cell transplantation using germ cell-less zebrafish as recipients
2.1 Introduction

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 injected with donor germ cells so they can produce viable gametes derived from the donor cells. In the first chapter of this study, we have demonstrated that knockout of the dnd gene, which is involved in the migration of primordial germ cells, produced genetically germ cell-less fish in the F2 generation of mutated founder. In this chapter, we aimed to examine the suitability of germ-cell-less zebrafish as recipients of spermatogonial transplantation in order to obtain basic information to support the application of dnd-knockout recipients to spermatogonial transplantation of endangered or farmed fish species.

Firstly, as Sertoli cells play important roles for supporting and nursing germ cells, histological and expression analysis of gsdf gene, which as is the marker of Sertoli cells, were performed to examine the existence of Sertoli cells in the gonads of dnd homozygous mutants. Then, germ cell-less zebrafish larvae were intraperitoneally transplanted with testicular cells of vasa-gfp transgenic zebrafish to explore whether they can produce functional GFP-positive sperm and produce donor-derived offspring entirely.
2.2 Materials and methods

Characterization of dnd-knockout zebrafish

Reverse-transcription PCR (RT-PCR) was performed to examine the expression of gsdf (Sawatari et al., 2007), a Sertoli cell marker, in the gonads of wild-type zebrafish, dnd heterozygous mutants, and homozygous mutants (dnd-knockouts). Gonadal RNA extraction and reverse-transcription were performed as described above. PCR amplification was performed as shown in Table 1 using primers for gsdf (Table 2). actb was also amplified as an internal control using primers for β-actin-FW and -RV (Table 2).

ISH was carried out to characterize cell-level expression of gsdf gene in dnd mutants. cDNA fragments of zebrafish gsdf (788-bp, GenBank accession NM_001114668.1) were subcloned into pGEM T-easy vector. The same method was used as described above (ISH using vasa probe).

Germ cell transplantation into dnd-knockout zebrafish

Mature vasa-gfp transgenic zebrafish (Krøvel and Olsen, 2002) were used as donors. Zebrafish larvae of the F2 generation (25% of which are expected to be dnd-knockout zebrafish) were used as recipients at 9-10 days post-fertilization. Freshly isolated donor testes were minced using dissecting scissors, and then incubated at 28°C for 1 hr in 500 μl of L-15/10% fetal bovine serum (Invitrogen, Waltham, MA) containing 2 μg/μl of Collagenase H (Roche), 1.7 μg/μl of Dispase II (Roche), and 5 μg/μl of DNase I (Sigma-Aldrich) with gentle pipetting at 0.5-hr
intervals. Resultant cell suspensions were rinsed with 1 ml of Leibovitz’s L-15 Medium/10% FBS, followed by filtration through a nylon screen with 42-μm pore size to remove non-dissociated cells clumps. Cells were collected by centrifugation at 190 g for 10 min at 4°C, then cell pellets were resuspended in Leibovitz’s L-15 Medium/10% FBS. Approximately 0.2 μl of cell suspension (containing approximately 3,000 GFP-positive cells) was transplanted into the peritoneal cavity of each recipient larva. Resultant larvae were reared at 28°C to 50 days post-transplantation, and then dissected. Incorporation of donor germ cells within the recipient gonads was analyzed under a BX-53 fluorescence microscope equipped with a GFP filter set (U-WIB2) (Olympus).

Mature recipient zebrafish were screened for dnd-knockout using genomic DNA from fin clips. Offspring were produced by mating dnd-knockout zebrafish recipients (male) with female wild-type zebrafish. The frequency of donor-derived offspring was determined by counting the number of larvae with green germ cells under a BX-53 fluorescence microscope with a GFP filter set (Olympus). PCR (Table 1) was performed using egfp primers (Table 2) to determine the genotype of offspring containing GFP-positive gonads.
2.3 Results

Molecular and histological characteristics of gonads in the mutant zebrafish

Transcription of gsdf (a marker of gonadal supporting cells) was confirmed in the gonads of all individuals, including dnd-knockout zebrafish, suggesting that supporting cells were present in dnd-knockout zebrafish (Figure 1a). This pattern of gene expression was recapitulated by in situ hybridization: gsdf antisense signal was present in sections of testes from all individuals including germ cell-less zebrafish, dnd heterozygous zebrafish and wild-type (Figure 1b-1d and 1b’-1d’).

Germ cell transplantation

Spermatogonia of transgenic, vasa-gfp zebrafish were injected into control (wild-type and dnd heterozygous mutants) or dnd-knockout (dnd homozygous mutants) recipient larvae at 9-10 days post-fertilization (Figure 2a-2e). Observation of recipients at 50 days post-transplantation revealed green-fluorescent germ cells in the testes of both the control (Figure 2d) and dnd-knockout recipients (Figure 2e): 4 of 60 control recipients carried green germ cells in their testes while 2 of 40 dnd-knockout recipients carried green germ cells (Table 3).

Production of donor-derived offspring using germ-cell-less zebrafish

Germ-line transmission rates of the donor-derived genotype were examined by progeny tests following the maturation of germ-cell-less recipients that received vasa-gfp germ cells. Mature, dnd-knockout male zebrafish receiving donor germ cells
were mated with wild-type female zebrafish. The fertilization rate of eggs inseminated with sperm obtained from germ-cell-less recipients was similar to that of wild-type individuals. Donor-derived, green fluorescent protein (GFP)-positive offspring were identified by fluorescence (Figure 2f-2h). Most importantly, all resulting offspring from germ-cell-less recipients possessed green fluorescence in their germ cells (Figure 2h; Table 3) as well as amplification of gfp DNA fragments by PCR (Figure 2i). On the other hand, control recipients produced both GFP-positive and GFP-negative offspring, and germ-line transmission rates were 3.9-7.3% (Table 3).
2.4 Discussion

Expression of the *dnd* gene is restricted to germ cells, and no expression is observed in the somatic cells of any fish species examined (Weidinger et al., 2003; Liu et al., 2009; Yazawa et al., 2013). Indeed, the loss of *dnd* functions by stable knockouts did not adversely affect somatic or gonadal somatic tissue development. For example, *dnd*-knockout zebrafish expressed *gsdf*, a marker of Sertoli cells (Sawatari et al., 2007), suggesting that although the *dnd*-knockout completely lacked germ cells, they still maintained functional testicular supporting cells needed to nurse male germ cells through spermatogenesis. More importantly, *dnd*-knockout zebrafish could support the migration of intraperitoneally transplanted spermatogonia toward the recipient gonadal anlagen and subsequent spermatogenesis and spermiogenesis. They also successfully produced functional sperm and donor-derived offspring, indicating that the *dnd*-knockout zebrafish produced in this study have functional somatic tissue in the testes (not limited to the Sertoli cells mentioned above) and endocrine systems necessary for their reproduction.

Phenotypes obtained in knockout studies are not always consistent with those obtained in knockdown studies (Kok et al., 2015; Rossi et al., 2015). In the case of *dnd*, however, our knockout phenotype was similar to those previously reported for *dnd*-knockdown zebrafish (Saito et al., 2008) and rainbow trout (Yoshizaki et al., 2016). The *dnd*-knockdown treatment eliminated all endogenous germ cells during early embryogenesis, and the resulting fish exhibited no expression of the *dnd* gene from any remaining somatic cells. In both approaches, the *dnd*-disrupted recipient
individuals supported migration of donor-derived germ cells to the recipient gonadal anlagen and complete gametogenesis. Although phenotypes of the dnd-knockout and -knockdown zebrafish were almost identical, there are two potential advantages of dnd-knockouts over individuals produced by knockdown treatment. First, dnd-knockouts can completely wipe out the endogenous germ cells, whereas dnd knockdown fish sometimes possessed small numbers of endogenous germ cells (Yoshizaki et al., 2016) that can proliferate and contribute to fecundity. This minor population could be a disadvantage since somatic resources would not be fully allocated to the production of donor-derived germ cells. Second, gene-knockdown technology with antisense oligonucleotides requires microinjection into each fertilized egg, making this early and transient approach laborious and time-consuming. By comparison, gene knockout requires only mating when a stable, heterozygous mutant line is established. Thus, the dnd-knockout zebrafish produced in this study provides a powerful tool for producing recipients for germ cell transplantation studies.

We previously reported that triploidization yields sterile recipients for germ cell transplantation in various fish species (Okutsu et al., 2007; Lee et al., 2013; Yoshikawa et al., 2017; Seki et al., 2017). Although production of triploid recipients is technically very easy to achieve, and can be applied to mass-scale production, triploid gonads carry endogenous germ cells (albeit they are unable to proceed to meiosis and mature) that can occupy the germ cell niche. Therefore, competition between endogenous triploid germ cells and transplanted diploid germ cells would occur within the germ cell niches. Conversely, the germ-cell-less zebrafish produced in this
study should have more available niches because their testes are completely devoid of endogenous germ cells. Another caveat of triploid recipients is their ability to produce abnormal, aneuploid gametes in some fish species – although their fecundity is significantly reduced (Hamasaki et al., 2013; Takeuchi et al., 2016). Such an outcome will not occur with the completely sterile dnd-knockout zebrafish, making them ideal recipients for effectively producing donor-derived sperm after germ cell transplantation.

In this study, only 25% of the F2 offspring produced by mating dnd-heterozygous mutants were dnd-knockout fish. Although this low frequency of dnd-knockout fish production is a potential disadvantage, the dnd-knockout larvae are easily selected using transgenic zebrafish carrying GFP-labeled germ cells at the post-hatching stage. Germ-cell-less dnd-knockout larvae are easily distinguishable from dnd heterozygous mutants as well as from wild-type fish by lack of fluorescent germ cells, allowing them to be separated as potential recipients.

Dnd-knockout females were never produced in this study (data not shown). In teleosts, the effects of germ cell numbers on sex differentiation vary by species. An absence of germ cells leads to exclusive male development in zebrafish and medaka (Slanchev et al., 2005; Siegfried and Nüsslein-Volhard, 2008; Kurokawa et al., 2007; Saito et al., 2008; Tzung et al., 2015), but not in loach, goldfish, Atlantic salmon, or rainbow trout (Fujimoto et al., 2010; Goto et al., 2012; Wargelius et al., 2016; Yoshizaki et al., 2016). In zebrafish, a certain number (3-29) of PGCs must be present at the gonadal anlagen of the 30-somite stage embryo in order for subsequent ovarian
development to proceed (Tzung et al., 2015). Indeed, feminization in individuals carrying a knockout of *dmrt1*, which plays essential roles in testicular development, does not occur if the fish does not have a high enough number of germ cells (Webster et al., 2017). We therefore hypothesize that all of our recipients were male because of the late timing of donor-derived germ cell transplantation (9-10 days post-fertilization) into the germ-cell-less zebrafish. One solution to this all-male yield could involve the application of exogenous estrogens to obtain female recipient zebrafish – albeit this outcome is inexplicably unpredictable (L. Orbán, personal communication). As mentioned above; however, the effects of germ cell numbers on sex differentiation vary depending on species. Therefore, this technology would be quite useful when applied to species with germ-cell-less mutants that can produce both females and males.

Finally, the low success rate of germ cell transplantation should be addressed in further studies. Among the 40 recipient *dnd*-knockout zebrafish, only two produced donor-derived sperm. By comparison, the frequency of recipient fish producing donor-derived gametes is generally 40%-55% in salmonids (Okutsu et al., 2006; Lee et al., 2013), 29%-37% in Nibe croaker, 77%-84% in medaka (Seki et al., 2017), and 100% in yellow tail (Morita et al., 2012). A 20% success rate in oogonial transplantations was reported for zebrafish using embryos at the same developmental stage as the ones in this study (Wong et al., 2011). According to previous research regarding intraperitoneally germ cell transplantation, post-migratory germ cells should be transplanted during the PGC migration period or right after the PGC
migration period (Takeuchi et al., 2003). PGC migration in zebrafish is completed at 24 hours post-fertilization (Weidinger et al., 2002). Therefore, delivering spermatogonia into earlier stage embryos may be one means to increase the success rate of transplantation – and may also rebalance the sex ratio closer to 1:1. Yet, transplantation into earlier stage embryos severely reduces survival rates of recipients (data not shown), so more work is needed to identify the developmental stage of recipients that will balance optimal survival and transplantation efficiency.
2.5 References


Dev 83: 298-311.
2.6 Figure Legends

**Figure 1.** *gsdf* gene expression in wild-type, *dnd* heterozygous, and homozygous mutant zebrafish. (a) Reverse-transcription PCR using primer sets specific to a supporting cell marker (*gsdf*) performed on gonadal cDNA from three individuals. The negative control is distilled water instead of template cDNA. (b-d) Histological characteristics of the gonads of *dnd*-knockout zebrafish. In situ hybridization micrographs for *gsdf* in wild-type zebrafish (b), *dnd* heterozygous mutants (c), and *dnd* homozygous mutants (d). Note the presence of *gsdf* (d and d') in germ-cell-less gonads. b'-d' are magnifications of their respective images. b''-d'' are hemotoxylin-and-eoxin-stained views b'-d', respectively. WT, wild-type. Scale bars, 500 μm (b-d) and 30 μm (b'-d', and b''-d'').

**Figure 2.** Germ-cell-less zebrafish recipients support donor-derived gamete development. Fluorescence microscopy of zebrafish gonads isolated from (a) *vasa-gfp* transgenic donor, (b) wild-type, (c) *dnd*-knockout, (d) wild-type injected with donor-derived germ cells, and (e) *dnd*-knockout injected with donor-derived germ cells. Juveniles produced by (f) wild-type, (g) *vasa-gfp*-transgenic, and (h) *dnd*-knockout zebrafish injected with donor-derived germ cells. The right panels show magnified views of each gonadal area. (i) Gel electrophoresis of PCR products for *gfp* in offspring produced by *dnd*-knockout zebrafish recipients. Larvae were randomly selected for genomic DNA extraction, and used as templates in the PCR reactions. The positive-control reaction used template DNA of *vasa-gfp* zebrafish; recipient
zebrafish sample had template DNA of a recipient zebrafish; and the negative-control reaction was performed with distilled water instead of template DNA. WT, wild-type. Scale bar, 500 µm (a-e); 1000 µm (f-h, whole fish); and 200 µm (f-h, gonadal areas).
2.7 Figures

Figure 1.
## 2.8 Tables

### Table 1.
PCR Amplification conditions

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<th>Gene</th>
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<th>Primer name</th>
<th>Denature</th>
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<td>62°C (30s)</td>
<td>72°C (20s)</td>
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Table 3.
F1 larvae production by recipient zebrafish

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<tr>
<th>Recipient</th>
<th>Number of F1 larvae</th>
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<td>204</td>
<td>204/229 (89.1%)</td>
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<td>Control recipient 4</td>
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<td>86 (100%)</td>
</tr>
<tr>
<td>Gem-cell-less recipient 2</td>
<td>105</td>
<td>105/121 (86.8%)</td>
<td>105 (100%)</td>
</tr>
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</table>
Summary

In this study, we aimed at production of germ cell-less zebrafish as recipients in germ cell transplantation. In the first chapter, we established the procedures for gene knockout at dnd locus in zebrafish using ZFN and produced germ cell-less zebrafish. In the second chapter, we applied the germ cell-less zebrafish larvae as recipient of germ cell transplantation.

In the first chapter, we suggested that a low concentration of ZFN cRNAs (10 ng/µl) injected into 1-cell stage zebrafish embryos induced the highest survival rate of zebrafish larvae and various mutations around the target site of the ZFN. Some mutations detected in founder individuals could also be detected in the gametes of founder, and then were transmissible to their F1 progeny and subsequently passed from F1 to F2 generations along a Mendelian pattern of inheritance. As a result, nearly 25% of F2 generation were dnd homozygous mutants (dnd-knockout). These resulting dnd-knockout zebrafish had extremely small gonads that lacked germ cells.

In the second chapter, we demonstrated that dnd-knockout zebrafish expressed gsdf, a marker of Sertoli cells (Sawatari et al., 2007), suggesting that although the dnd-knockout completely lacked germ cells, they still maintained functional testicular supporting cells needed to nurse male germ cells through spermatogenesis. Further, dnd-knockout zebrafish could support the migration of intraperitoneally transplanted spermatogonia toward the recipient gonadal anlagen and subsequent spermatogenesis and spermiogenesis. They also successfully produced functional sperm and
donor-derived offspring. Therefore, we concluded that germ cell-less zebrafish is a strong candidate as a recipient in germ cell transplantation.

Recently, cryopreservation of zebrafish bioresources is becoming more important since a large number of mutants and strains are being deposited to resource centers. Since cryopreservation of fish eggs remain difficult, maternally inherited genomic materials cannot be preserved in a frozen condition. We previously established a reproducible and simple method for cryopreserving fish spermatogonia (Lee et al., 2013; 2015; 2016a) and oogonia (Lee et al., 2016b) using salmonids. By combining cryopreservation of germ cells with the transplantation technology established in this study, long-term storage of zebrafish bioresources will be possible in near future.
References


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High tribute shall be paid to Dr. Hirono Ikuo, who comes from genome science laboratory, Dr. Sakamoto Takashi, who comes from fish culture laboratory, and Dr. Yazawa Ryosuke, who is the associate professor of our fish physiology laboratory. They gave me a lot of suggestions for this thesis.

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