Nitroreductase-mediated Gonadal Dysgenesis for Infertility Control of Genetically Modified Zebrafish

Shao-Yang Hu · Pei-Yu Lin · Chia-Hsuan Liao · Hong-Yi Gong · Gen-Hwa Lin · Koichi Kawakami · Jen-Leih Wu

Received: 5 April 2009 / Accepted: 22 September 2009 / Published online: 27 November 2009
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Abstract Genetically modified (GM) fish with desirable features such as rapid growth, disease resistance, and cold tolerance, among other traits, have been established in aquaculture. However, commercially available GM fish are restricted because of global concerns over the incomplete assessments of food safety and ecological impact. The ecological impact concerns include gene flow and escape of the GM fish, which may cause extinction of wild natural fish stocks. Infertility control is a core technology for overcoming this obstacle. Although polyploidy technology, GnRH-specific antisense RNA, and RNAi against GnRH gene expression have been used to cause infertility in fish, these approaches are not 100% reliable and are not heritable. In the present study, zebrafish was used as a model to establish an inducible platform of infertility control in GM fish. Nitroreductase, which converts metronidazole substrate into cytotoxin, was fused with EGFP and expressed specifically by oocytes in the Tg(ZP:NTR-EGFP) by a zona pellucida promoter. Through consecutive immersion of metronidazole from 28 to 42 days posthatching, oocyte-specific EGFP expression was eliminated, and atrophy of the gonads was detected by anatomical analysis. These findings reveal that oocyte-specific nitroreductase-mediated catalysis of metronidazole blocks oogenesis and leads to an undeveloped oocyte. Furthermore, oocyte cell death via apoptosis was detected by a TUNEL assay. We found that the gonadal dysgenesis induced by metronidazole resulted in activation of the ovarian killer gene bok, which is a proapoptotic gene member of the Bcl-2 family and led to infertility. These results show that oocyte-specific nitroreductase-mediated catalysis of metronidazole can cause reliable infertility in zebrafish and could potentially be used as a model for other aquaculture fish species.

Keywords Genetically modified fish · Infertile control · Nitroreductase · Oocyte

Introduction

Marine animals are an important source of food and income for many people in developing countries. While many wild captured species are approaching their exploitation limits, there is a considerable potential to expand aquaculture in order to meet the global food demand. Since the first transgenic fish with growth hormone (GH), gene expression was successfully established in 1985; transgenic fish of various species have been actively investigated worldwide in order to improve the
economic benefits of aquaculture. For example, the exogenous GH gene has been transferred into salmon, tilapia, and common carp to enhance growth (Zhu et al. 1985; Dunham et al. 1992; Chen et al. 1993; Devlin et al. 1994; Rahman et al. 1998); an antimicrobial peptide from flounder or insects was expressed in salmon or striped bass to prevent pathogen infection (Jia et al. 2000; Shike et al. 2002), and an antifreeze protein gene from ocean pout was introduced into Atlantic salmon to increase cold tolerance (Hew et al. 1992). The gene transfer technologies applied in the aforementioned transgenic fish have been successfully applied in aquaculture; however, no transgenic aquatic animals, except for transgenic ornamental fish, have been commercialized. The main impediment to the commercialization of genetically modified (GM) fish is the incomplete assessment of food safety and the ecological impacts. The ecological impacts encompass global issues concerning gene flow and the escape of transgenic fish, which may pose a threat to wild natural fish stocks. Infertility control may be a core technology to solve the potential problem of GM fish release into the natural environment.

The traditional approach to infertility control in GM fish is polyploidy technology. This technology causes infertility by changing the number of chromosomes in an individual. Temperature-shock or pressure–stress treatment of freshly fertilized fish eggs to induce triploidy is another common practice for infertility, but unfortunately triploid infertility is rarely 100% effective (Razak et al. 1999). An alternative method to induce infertility in fish is the blockade of gonadotropin-releasing hormone (GnRH) expression by antisense RNA, ribozymes, or siRNA (Uzbekova et al. 2000; Maclean et al. 2002; Wong and Van Eenennaam 2008). However, although undeveloped or deficient gonads result from a knockdown of GnRH, the presence of antisense GnRH in fish do not result in 100% infertility (Hu et al. 2007). Furthermore, even if such a treatment was successful in achieving 100% infertility, the transgenic stocks with superior characteristics are notheritable. For this reason, infertility would need to be inducible; otherwise, this infertility would be equivalent to ‘killing the goose that lays the golden eggs.’

Zebrafish have become the most important vertebrate model organism for studying the genetic control of development, human disease, and aquaculture research (Dahm and Geisler 2006). Several inducible methods designed to ablate cells have been used in zebrafish. For instance, the expression of diphtheria toxin A chain (DTA) in a tissue-specific manner can lead to the effective ablation of target cells, but it can also cause undesired damage to other cells due to its toxicity. Furthermore, a stable zebrafish line carrying the DTA transgene has not been successfully established, so this approach has been restricted to transient transgenesis (Wan et al. 2006). Another approach is the prokaryotic parD system, which consists of a toxin, kid, and an antidote, kis. Briefly, the bacterial toxin, Kid, is expressed in the target cell, while its antidote, Kis, is expressed in the rest of the organism to protect it from the cell death induced by Kid (Slanchev et al. 2005). Although this method has been practiced in zebrafish, it has only been used in embryos that transiently express Kid/Kis. Given that the methods previously described have various limitations and that an inducible infertility technique is needed, we evaluated the ability of the Escherichia coli nitroreductase (NTR)/metronidazole (Mtz) system to genetically destroy reproductive cells in a specific and inducible manner in zebrafish. This system depends on NTR-mediated conversion of a nontoxic metronidazole substrate, such as Mtz, into a cytotoxin. The NTR enzyme is first reduced by NADH or NADPH, and upon binding, Mtz is reduced by NTR and thereby, converted into a potent DNA interstrand cross-linking agent that causes cell death (Lindmark and Muller 1976; Edwards 1993). In the present study, the oocyte reproductive cell was specifically ablated by NTR-mediated catalysis of Mtz to establish an inducible platform for infertility control in GM zebrafish. We expect that our results can be considered as representing a useful model to solve the issue of GM fish being released into a natural environment.

Materials and Methods

Zebrafish Line and Maintenance

AB strain zebrafish were purchased from the zebrafish international resource center. The fish were raised and maintained in a freshwater recirculating tank with a controlled light cycle of 14 h light/10 h dark at 28°C. The intercrossed progeny from the AB strain zebrafish were used for microinjection. The age of the juveniles is indicated as the days posthatching.

Construction of Transgenic Constructs

The T2KXIGΔIN vector backbone, including the expression cassette (ef1α promoter, egfp gene, SV 40 polyadenylation) and flanks to the expression cassette of Tol2 transposon arms (500 bp left arm and 500 bp right arm), was kindly provided by Dr. Kawakami (Kawakami 2004). The nitroreductase (ntr) gene was cloned directly from E. coli BL21 genomic DNA by PCR using the forward primer ntr(f) 5′-ACTACCCGGTATGGATATCATTTCGTC GCTTTA and the reverse primer ntr(r) 5′-ACTACCGGT GTCACTCGGTTAAGGTGATGTTTTG. The primer design enabled the creation of an AgeI restriction site. The ntr gene PCR product (~671 bp) was digested with AgeI and cloned into the T2KXIGΔIN vector, downstream of the

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In order to screen the F₀ transgenic founders, the F₁ offspring from each injected fish were used for the detection of germ line transmission and expression of ntr-egfp. The embryos were dissolved in 1.2 ml of extraction buffer (0.5% SDS; 100 mM NaCl; 10 mM Tris-Cl; pH 8.0; 25 mM EDTA, pH 8.0; 0.1 mg/ml proteinase K) at 55°C overnight and centrifuged at 12,000 rpm for 1 min. An equal volume of a saturated phenol solution was added to the supernatant and mixed gently by inversion. After centrifugation at 12,000 rpm for 10 min, the upper layer of the solution was carefully transferred to clean microcentrifuge tubes. The above steps of phenol extraction were repeated several times, and then the phenol was replaced by chloroform (1:1) solution the final time and centrifuged again to collect the supernatants in clean microcentrifuge tubes. The same volume of a saturated phenol solution was added to the pellet, mixed, and then mounted on poly-L-lysine coated slides. The histological analysis was carried out by hematoxylin–eosin staining using standard protocols. To evaluate the reproductive capacity, female Tg(ZP:NTR-EGFP) with Mtz treatment, the fertilized egg of a female Tg(ZP:NTR-EGFP) crossed with a male AB strain wild-type zebrafish was used as a reference.

Detection of Genomic Integration and NTR-EGFP Expression

Metronidazole (Mtz) [1-(2-hydroxyethyl)-2-methyl-nitroimidazole] (Sigma M3761) was dissolved in standard zebrafish embryo medium containing 0.1% DMSO with vigorous shaking and was placed in a dark place to prevent photoinactivation of Mtz. The intercrossed F₂ progeny from the F₁ Tg(ZP:NTR-EGFP) were collected and incubated at 28°C in a aquarium. The F₂ female Tg(ZP:NTR-EGFP) with NTR-EGFP expression were screened at 28 days posthatching under fluorescent microscopy and incubated in a dark tank with embryo medium containing 0.1% DMSO and 5 mM Mtz (n=20/per tank; duplicate). The embryo medium with 0.1% DMSO and 5 mM Mtz was renewed every week to maintain medium quality and sufficient Mtz substrate. The adult Tg(ZP:NTR-EGFP) with Mtz treatment were used to observe oocyte development by histological analysis and to evaluate the reproductive capacity.

Histological Analysis and Evaluation of Reproductive Capacity

Detection of Bcl-2 Gene Family by Real-time Quantitative PCR

In order to screen the F₀ transgenic founders, the F₁ offspring from each injected fish were used for the detection of germ line transmission and expression of ntr-egfp. The embryos were digested with Xhol and SalI restriction sites, respectively. The zp3 promoter PCR product was digested with Xhol and SalI and cloned into a pT2-EF1α-NTR-EGFP vector to replace the ef1α promoter, thereby, generating the expression construct pT2-ZP-NTR-EGFP. For genomic integration and generation of the stable transgenic line Tg(ZP-NTR-EGFP), this construct was coinjected with Tol2 transposon mRNA into single-cell stage embryos.

Detection of Genomic Integration and NTR-EGFP Expression

Metronidazole (Mtz) [1-(2-hydroxyethyl)-2-methyl-nitroimidazole] (Sigma M3761) was dissolved in standard zebrafish embryo medium containing 0.1% DMSO with vigorous shaking and was placed in a dark place to prevent photoinactivation of Mtz. The intercrossed F₂ progeny from the F₁ Tg(ZP-NTR-EGFP) were collected and incubated at 28°C in a aquarium. The F₂ female Tg(ZP:NTR-EGFP) with NTR-EGFP expression were screened at 28 days posthatching under fluorescent microscopy and incubated in a dark tank with embryo medium containing 0.1% DMSO and 5 mM Mtz (n=20/per tank; duplicate). The embryo medium with 0.1% DMSO and 5 mM Mtz was renewed every week to maintain medium quality and sufficient Mtz substrate. The adult Tg(ZP:NTR-EGFP) with Mtz treatment were used to observe oocyte development by histological analysis and to evaluate the reproductive capacity.

Histological Analysis and Evaluation of Reproductive Capacity

Ovary tissues were harvested from adult zebrafish and fixed overnight in PBS buffer containing 4% paraformaldehyde (PFA) at 4°C. The samples were dehydrated in methanol and embedded in agarose. The samples were sectioned at 8 μm and then mounted on poly-L-lysine coated slides. The histological analysis was carried out by hematoxylin–eosin staining using standard protocols. To evaluate the reproductive capacity of female Tg(ZP:NTR-EGFP) with Mtz treatment, the fertilized egg of a female Tg(ZP:NTR-EGFP) crossed with a male AB strain wild-type zebrafish was used as a reference.

Detection of Bcl-2 Gene Family by Real-time Quantitative PCR

Total RNAs from ovary tissues of a 3-month-old AB strain wild type, transgenic line Tg(ZP:NTR-EGFP), and Mtz-treated 6-week-old Tg(ZP:NTR-EGFP) transgenic zebrafish were extracted, respectively, using TRIzol (Invitrogen). For real-time quantitative PCR, first-strand cDNAs were synthesized by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers. Quantitative PCR was performed by using Power SYBR Green PCR Master Mix in LightCycler 480 System (Roche). Gene-specific primers of Bcl-2 gene family were designed by Roche ProbeFinder software for real-time quantitative PCR. The transcript of ef-1α gene was used as endogenous control. Primer sets used in quantitative PCR are listed in
follows: bcl-2 forward, 5'-tggctgctccaggtgataaa t-3' and reverse, 5'-acctgacttccc gaagg-3'; bcl-xl forward, 5'-ggctgtttctgtttgtaagc and reverse 5'-tggtgctagctctc-3'; mcl-1a forward, 5'-ggatcaggtggttggctcgtg-3' and reverse, 5'-tcggagatcaggtggttggctcgtg-3'; bax-1 forward, 5'-gcccagtccttctcttcgtaa-3' and reverse, 5'-ctcaggaaccctggttgaaat-3'; bok-1 forward, 5'-cgaaaggtttg caaataaca-3' and reverse, 5'-ccgaacagctccagc-3'; bok-2 forward, 5'-gtggcttctcactttacctgta-3' and reverse, 5'-ctctgttgccacttgcttga-3'; bax-2 forward, 5'-gttccaagatggcagatc-3' and reverse, 5'-gctgatcctacagctcct-3'; mcl-1b forward, 5'-tctgaaacaattctgggtaggc-3' and reverse, 5'-tctgaaacaattctgggtaggc-3'; bcl-xl forward, 5'-gtggtgcaatggctcatacc-3' and reverse, 5'-tggtgcaatggctcatacc-3'; bcl-xl forward, 5'-gtggtgcaatggctcatacc-3' and reverse, 5'-tggtgcaatggctcatacc-3'; bcl-xl forward, 5'-gtggtgcaatggctcatacc-3' and reverse, 5'-tggtgcaatggctcatacc-3'; bcl-xl forward, 5'-gtggtgcaatggctcatacc-3' and reverse, 5'-tggtgcaatggctcatacc-3';

TUNEL Cell Death Assay

The TUNEL cell death assay was performed using the In Situ Cell Death Detection Kit (Roche number 11684817910). After the fixation of ovary tissue in 4% PFA and sectioning, the ovary sections were preincubated in PBST and then washed with PBST and visualized by microscopy.

Results and Discussion

Expression of NTR-EGFP Controlled by Zona Pellucida Promoter

NTR-mediated cell and tissue ablation has been previously applied in zebrafish developmental and regeneration studies (Curado et al. 2007; Pisharath et al. 2007), and manipulation of NTR/Mtz system-mediated cell ablation has been described (Curado et al. 2008). In order to cause Mtz-dependent oocyte death, NTR must be specifically expressed in the oocyte. Vertebrate zona pellucida (zp) proteins, which are encoded by multiple zp genes family, are glycoprotein and extracellular matrix structures that surround the membrane of an oocyte (Harris et al. 1994). In zebrafish, the zp2 and zp3 genes have ovary-specific expression, and the zp3 promoter, which is oocyte-specific, has been cloned (Wang and Gong 1999; Mold et al. 2001; Liu et al. 2006). In the present study, a 2.2-kb promoter upstream of the zp3 ATG start codon was amplified by PCR and cloned into T2KX1GΔ1N to generate the plasmid pT2-ZP-EGFP for expression of the NTR-EGFP reporter protein, which enables simple observation of oocyte ablation by Mtz. The Tg(ZP::NTR-EGFP) fish were established by Tol2 transposon-mediated DNA integration and characterized by EGFP expression analyses (Fig. 1a). Eight female and eight male transgenic founders were screened from fifty pT2-ZP-NTR-EGFP plasmid-injected fish. Germ-line transmission of the NTR-EGFP expression cassette in each F1 Tg(ZP::NTR-EGFP) was confirmed by PCR using the genomic DNA as template (Fig. 1b). Because zona pellucida proteins only exist in female fish, mRNA expression of ntr-egfp in the F1 progeny was confirmed by RT-PCR in each transgenic female founder (Fig. 1c). The F1 progeny from intercrossed transgenic founders were maintained to generate the F2 progeny. Similar to a previous report, which mentioned that zebrafish zp3 mRNA expression, was initiated at 21 days posthatching (Liu et al. 2006); specific expression of EGFP in the gonads was first detectable through the body in the juvenile female F1 Tg(ZP::NTR-EGFP) at 21–28 days posthatching (Fig. 1c). Certainly, the F2 fertilized eggs from the intercrossed F1 Tg(ZP::NTR-EGFP) fish contain maternally expressed NTR-EGFP fusion protein and are bright green when visualized by fluorescence illumination.

The EGFP fluorescence triggered by the zp3 promoter during embryogenesis is strongest within the cytoplasm of the eggs (Fig. 1d) and then gradually disappears during the successive stages of somitogenesis.

Oocyte Dysgenesis Induced by Mtz in a NTR-specific Manner

In order to determine the tolerable concentration of Mtz for zebrafish, AB strain zebrafish embryos at the single-cell stage were incubated at 28°C in a range of Mtz concentrations (0–20 mM). We observed obvious abnormal development of larvae at 3 days postfertilization (dpf). The larvae were dead at 5 dpf after consecutive immersions in 15 mM Mtz (100%, n = 26/26) and 20 mM Mtz (100%, n = 30/30). The nonspecific death of the larvae was first observed at 6 dpf after consecutive immersions in 10 mM Mtz (23%, n = 7/30). In contrast, we observed that no larvae died after at least 10 dpf of consecutive immersion in 5 mM Mtz (0%, n = 30). Therefore, the optimal Mtz concentration used in the present experiment was 5 mM.

To evaluate the ability of NTR to induce Mtz-susceptibility in oocytes, stable F2 female Tg(ZP::NTR-EGFP) fish with gonad-specific EGFP expression were screened at 28 days posthatching and then shifted to 5 mM Mtz cultured medium. In contrast to the female Tg(ZP::NTR-EGFP) fish without Mtz treatment, expression of EGFP in the gonads of female Tg(ZP::NTR-EGFP) fish with Mtz treatment was eliminated gradually and disappeared completely at 6 weeks posthatching (Fig. 2a and b). Obviously, the elimination of EGFP expression was induced by Mtz treatment due to the specific expression of NTR in the gonads. It is well known that zp proteins are the major component of the extracellular structural coat of mature oocytes. According to a previous report, the zp genes are abundantly transcribed during

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Fig. 1 The establishment of the Tg(ZP:NTR-EGFP) fish with oocyte-specific expression of NTR-EGFP fusion protein. 

- **a**: A diagram illustrating the main features of the pT2-ZP-NTR-EGFP plasmid. The expression of NTR-GFP fusion is controlled by the oocyte-specific ZP promoter (the bent arrow). Two Tol2 transposon elements (black box) flank the expression cassettes. The SV 40 polyadenylation site is indicated by the gray box.

- **b**: The genomic integration and germ line transmission of the NTR-EGFP expression cassette in the F1 Tg(ZP:NTR-EGFP) inherited from the transgenic founder was confirmed by PCR. C the PCR was performed using the pT2-NTR-EGFP as a positive control, W the AB strain zebrafish, Numbers 1~8 each transgenic male and female founder. e The mRNA expression of NTR-EGFP in the F1 progeny was confirmed by RT-PCR. Ef-1α was used as an internal control.

- **d**: The expression of the NTR-EGFP fusion was observed as early as 28 days posthatching in the gonads (arrow indicated) of the F1 Tg(ZP:NTR-EGFP) female fish under fluorescent microscopy.

- **e**: Newly fertilized F2 Tg(ZP:NTR-EGFP) embryos possess maternally expressed NTR-EGFP fusion protein in the cytoplasm.

Fig. 2 Oocyte dysgenesis was induced by Mtz in a NTR-dependent manner. The oocyte-specific expression of NTR-EGFP is shown in a a 6-week-old and e an adult female Tg(ZP:NTR-EGFP) fish. The expression of NTR-EGFP eliminated by Mtz immersion is shown in b a 6-week-old and d an adult female Tg(ZP:NTR-EGFP). e The complete ovary structure made up of oocytes is shown in an adult female Tg(ZP:NTR-EGFP) by anatomical analysis. f The ovarian atrophy without oocyte formation is shown in an Mtz-treated adult female Tg(ZP:NTR-EGFP).
oogenesis and zp mRNA accounts for 10.3% of the total transcripts expressed in the ovary (Zeng and Gong 2002). It is apparent that the expression of the NTR-EGFP fusion was strongly interspersed in the ovary of the adult female Tg(ZP:NTR-EGFP) fish (Fig. 2c). Morphological observation after continuous Mtz treatment indicated that the protuberant shape of the ovary was completely formed in the adult Tg(ZP:NTR-EGFP) female fish, whereas, a flat abdomen without EGFP expression was observed in the adult Tg(ZP:NTR-EGFP) female fish (Fig. 2c and d). In order to evaluate the anatomical changes in the ovary induced by Mtz treatment, the ovarian tissue was harvested and dissected. The anatomical analysis showed that the entire ovary was made up of sufficient oocytes and developed normally in the Tg(ZP:NTR-EGFP) female fish (Fig. 2e); however, the ovary was lacking oocytes due to atrophy and dysgenesis in the Mtz-treated fish (Fig. 2f). These results indicate Mtz-mediated oocyte dysgenesis through NTR.

Oogenesis is Blocked by NTR Catalysis of Mtz Substrate

To further understand the effect of Mtz treatment on oocyte maturation, the formation of oocytes was detected by HE staining. Oogenesis in zebrafish has been described to occur in five stages according to oocyte size and certain morphologic features. In stage IA (7~20 μm), the oocyte lies within a nest and the nucleus fills most of the oocyte compared to the cytoplasm at the prefollicle primary growth stage. In stage IB (20~140 μm), the oocyte is surrounded by the follicle at the follicle primary growth stage. In stage II (140~340 μm), there is an increasing number of yolk vesicles at the yolk-vesicle stage. In stage III (340~690 μm), there is an accumulation of yolk bodies, which obscures the germinal vesicles at the vitellogenesis stage. In stage IV (690~730 μm), the oocyte initiates meiosis and becomes transparent at the oocyte maturation stage. In stage V (730~750 μm), the mature egg leaves the follicle during ovulation (Selman et al. 1993). Oocytes with normal development are similar between the 6-week-old AB strain and the Tg(ZP:NTR-EGFP) fish, and histological analysis showed that the main oocytes were proceeding as stage II and stage III (Fig. 3a and b). In contrast, ovary development was incomplete in the Mtz-treated 6-week-old Tg(ZP:NTR-EGFP) fish due to blockade of oogenesis (Fig. 3c). The zp3 gene that encodes the zp protein is a main structure in the fish chorion and its expression accompanies the development of oocytes. Because ablation of the oocytes induced by Mtz treatment in the Tg(ZP:NTR-EGFP) female fish began at 28 days posthatching, the time at which oogenesis is initiated and is in its early stage, no mature oocytes formed in the Mtz-treated Tg(ZP:NTR-EGFP) female fish, and there were few oocytes stuck at the prefollicle primary growth stage (Fig. 3d). These results show that the blockade of oogenesis at the early stage by

![Fig. 3](https://example.com/fig3.jpg) **Fig. 3** The effects of the Mtz treatment on oocyte structure in the Tg(ZP:NTR-EGFP) was detected by hematoxylin–eosin (HE) staining. Normal development of the oocytes was detected in the adult a female AB strain zebrafish and b female Tg(ZP:NTR-EGFP) by HE staining. The stages of the developing oocytes are indicated by Roman numerals. c The dysgenesis of the oocytes in the gonads was shown in the adult Tg(ZP:NTR-EGFP) female fish with Mtz treatment. The arrow indicates the site of the gonad. d A few of the developmental oocytes are stuck at the IA stage of oogenesis. The arrows indicate that the nucleus filled most oocytes at the prefollicle primary growth stage. The black bar in each micrograph represents 100 μm.

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Oocyte cell death was induced by Mtz via apoptosis. Normal status of the oocyte was detected in the 3-month-old female a AB stain zebrafish and b Tg(ZP:NTR-EGFP) by a TUNEL cell death assay. The stages of the developing oocytes are indicated as Roman numerals. c, d An obvious signal of cell death (arrows indicated) induced by Mtz treatment was shown in the gonads of the Mtz-treated 6-week-old Tg(ZP:NTR-EGFP) female fish. The white bar in each micrograph represents 100 μm.

NTR oocyte-specific catalysis of Mtz causes infertility of the Mtz-treated 6-week-old female Tg(ZP:NTR-EGFP) and activates ovarian killer gene expression. a Proapoptotic and b antiapoptotic genes of the Bcl-2 gene family as detected by real-time PCR. c The ovarian killer genes bok-1 and bok-2 are upregulated by the NTR/Mtz oocyte-specific mediated system. The star symbols indicate significant differences (P<0.05). d The Tg(ZP:NTR-EGFP) lose their spawning activity, which leads to infertility by oocyte-specific NTR-mediated catalysis of Mtz. The number of fertilized eggs is shown as the mean ± S.D (n=20). The offspring from the AB strain zebrafish intercrossed or the female Tg(ZP:NTR-EGFP) crossed with the male AB strain zebrafish were used as controls. W the AB strain zebrafish, C the female Tg(ZP:NTR-EGFP), M the female Tg(ZP:NTR-EGFP) with the Mtz treatment.
NTR-mediated catalysis of Mtz leads to undeveloped oocytes.

Oocyte Cell Death Induced by Mtz via Activation of Apoptosis

NTR/drug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954), which is an analog of Mtz, has been proposed as an agent for cancer gene therapy (Greco and Dachs 2001; Plumb et al. 2001). Bacterial NTR converts the prodrug CB 1954 to an active drug, which thereby introduces interstrand cross-links into the DNA and leads to apoptosis via the activation of caspases 3, 8, and 9. These lesions kill dividing and nondividing tumor cells, such as the SKOV3 ovarian carcinoma cell line as well as colon cancer cells (Palmer et al. 2003; Lukashev et al. 2005). Mtz was chosen over CB1954 in this study due to its efficacy in destroying oocytes. In order to understand whether the mechanism of Mtz is similar to that of CB1954, which induces cell death via apoptosis, oocytes sectioned from adult female fish were evaluated by a TUNEL cell death assay. A significant signal of cell death was revealed in the ovaries of the adult Tg(ZP:NTR-EGFP) female fish with Mtz-induced atrophy (Fig. 4c and d), whereas, a normal status for oocyte formation was shown in the AB strain zebrafish and the Tg(ZP:NTR-EGFP) fish (Fig. 4a and b). These results show that Mtz-induced oocyte cell death at the early stage of oogenesis and led to gonadal dysgenesis.

NTR Oocyte-specific Catalysis of Mtz Regulates Activation of Ovarian Killer Gene

As indicated by the results presented in Fig. 4c and d, NTR catalyzes the Mtz substrate to cause oocyte death predominantly via apoptosis. Members of the Bcl-2 gene family are critical mediators of apoptotic cell death. We detected members of the Bcl-2 gene family, including proapoptotic and antiapoptotic genes, by real-time PCR, but only bok-1 and bok-2 are regulated by NTR/Mtz oocyte-specific mediated system (Fig. 5a, b, c). Bok, which is a proapoptotic member of the Bcl-2 family, was first isolated in a yeast two-hybrid screen of an ovarian cDNA library. It is also called the ovarian killer gene because of its primary expression in the reproductive tissues of mammals and zebrafish (Hsu et al. 1997; Suominen et al. 2001; Kratz et al. 2006). Bok contains the Bcl-2 homology domain (BH-1, -2, and -3) and can induce apoptosis in a variety of cell types; however, this activity can be inhibited by heterodimerizing with Mcl-1 (Hsu et al. 1997; Ha et al. 2001; Suominen et al. 2001; Brown et al. 2004). In the present study, expression of the antiapoptotic genes mcl-1a and mcl-1b is not affected by NTR/Mtz. Therefore, we assume that NTR/Mtz may mediate oocyte-specific cell death by disrupting the heterodimerization balance via activation of the bok ovarian killer gene.

Gonadal Dysgenesis Results in Infertility

A bloated ovary is an explicit feature in adult female zebrafish; however, this feature was nonexistent in the Tg(ZP:NTR-EGFP) female fish after Mtz treatment. This phenomenon resulted from gonadal dysgenesis, and it might reduce reproductive capacity. In order to know whether the Mtz-treated transgenic Tg(ZP:NTR-EGFP) female fish are infertile, the number of fertilized eggs from the Mtz-treated Tg(ZP:NTR-EGFP) female fish and the AB strain male fish was used as a reference for the evaluation of reproductive capacity. In contrast to the normal spawning activity in the control groups, all of the Tg(ZP:NTR-EGFP) female fish with Mtz treatment lost spawning activity and became infertile (Fig. 5d). This result shows that reliable infertility of transgenic zebrafish can be achieved by a NTR/Mtz oocyte-specific mediated system.

Platform Technology of Infertility Control in Transgenic Ornamental Zebrafish

It is well known that zebrafish and medaka ornamental fish with exogenous fluorescent protein expression are the only
two commercialized products of genetically modified aquatic animals to date. However, transgenic ornamental zebrafish that are able to reproduce will impede marketing and cause a decline in the price due to private breeding. In the present study, we established an inducible technology platform for the control of infertility, which can be immediately applied to transgenic ornamental zebrafish. The strategy for infertility control in ornamental zebrafish is shown in Fig. 6. Homozygous Tg(ZP:NTR-EGFP) female fish and homozygous transgenic ornamental male zebrafish were selected and crossed to generate progeny. The female progeny with EGFP expression in the gonads were screened at 28 days posthatching and then immersed in embryo medium containing 5 mM Mtz until the fish grew into adults. All of the adult transgenic ornamental zebrafish with Mtz treatment can be traded on the market, whereas, the breeders of the transgenic ornamental zebrafish can be maintained without Mtz treatment. The infertile platform strategy presented in this study not only has a high efficiency and can be easily manipulated; it also improves the disadvantages of traditional approaches for infertility, such as less than 100% reliability and irreversibility. We expect that the inducible platform developed in this work can be used as model of infertility control and will be applicable to other aquaculture fish species.

Acknowledgments We would like to thank Dr. Jenn-Kan Lu at the National Taiwan Ocean University and our colleagues for their helpful criticisms and suggestions. This work was supported by grants from the Academia Sinica, Taiwan (94S-1402), the National Science Council NSC 97-2317-B-001-012-CC1, and GeneReach Biotechnology Corporation.

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