Generation of all-male-like sterile zebrafish by eliminating primordial germ cells at early development

Li Zhou¹, Yongyong Feng¹, Fang Wang¹, Xiaohua Dong², Lan Jiang¹, Chun Liu¹, Qinshun Zhao² & Kaibin Li¹

Production of all-male and sterile fish may not only substantially improve yield but also be crucial for the application of genome modified species in aquaculture. Previously, it was reported that the fish lacking primordial germ cells (PGCs) becomes infertile, and nitroreductase, an enzyme converting non-toxic metronidazole (MTZ) into toxic metabolites, induces targeted toxicity to kill the cells expressing it. In this study, we generated a transgenic zebrafish line of Tg(nanos3:nfsB-mCherry-nanos3 3′UTR) in which the NfsB nitroreductase is solely expressed in PGCs. Treating the embryos derived from the female transgenic zebrafish with MTZ from 0 through 2 dpf (days post fertilization), we found that the germ cells were completely eliminated in the ones older than 2.5 dpf. At 20 dpf, the MTZ-treated juvenile had no germ cells in their gonads. At 100 dpf, the MTZ-treated adult exhibited male-like morphology and showed normal mating behaviors although they had no germ cells but only supporting cells in their gonads. Taken together, our results demonstrated that conditional elimination of PGCs during early development make the zebrafish male-like and infertile. It may provide an alternative strategy to make sterile and all-male farmed fish that is good for increasing aquaculture yield and preventing the genome modified species from potential ecological risks.

Infertility treatment of farmed fish is a promising strategy for increasing aquaculture production as well as mitigating the potential ecological risks from biological invasion by escaped farmed species¹. In addition, inhibiting the development of sex organs can improve fish growth, enhance the quality of aquaculture products, increase the utilization rate of feed, and reduce production costs. Species-specific infertile techniques are therefore desirable for efficient large-scale farming².

Fish are sterilized by eliminating germ cells or interfering with reproductive function. The polyploid induction technique is frequently utilized in aquaculture, especially for rainbow trout (Oncorhynchus mykiss)³ and crucian carp (Carassius carassius)⁴. The sterile individuals obtained by this technique are the offspring of multiple parental hybridizations with different ploidy levels. The germ cells may become infertile due to chromosome synapsis disorder during meiosis⁵, which can be maintained by “fertile parent and infertile filial generation”¹. Surgery, drug treatment, and morpholino targeting reproductive cells have also been utilized to make fish infertile². However, these strategies have poor specificity, low efficiency, and are thus unsuitable for large-scale aquaculture production.

The germ cells of fish are derived from primordial germ cells (PGCs), so loss of PGCs impedes gonadal development⁶. Transgenic techniques causing stagnation of PGC development offer an alternative sterilization strategy. The Maternal Sterility Technology (MST) employs maternal expression of a pro-apoptotic protein to eliminate PGCs and obtain infertile individuals², but the efficacy of this pro-apoptotic protein on early development has not been assessed in practice. It is also challenging to maintain infertile parant⁶. Disrupting PGC migration during the early stage of development is another promising infertility technique for industrialized application. For instance, Wong et al. overexpressed stromal cell-derived factor 1a (Sdf1a), a chemokine vital for PGC homing, in zebrafish by heat induction, resulting in mis-migration of the PGCs and the development of sterile fish³. Nitroreductase (NTR), an enzyme derived from Escherichia coli, can convert non-toxic metronidazole (MTZ) into toxic metabolites, thereby inducing targeted toxicity in fish cells expressing NTR⁷. Indeed, this NTR/MTZ

¹Pearl River Fishery Research Institute, Chinese Academy of Fishery Sciences, No. 1 Xingyu Road, Xilang, Liwan District, Guangzhou, Guangdong, 510380, China. ²MOE Key Laboratory of Model Animal for Disease Study, Model Animal Research Center, Nanjing University, 12 Xuefu Road, Pukou High-tech Development Zone, Nanjing, Jiangsu, 210061, China. Li Zhou and Yongyong Feng contributed equally to this work. Correspondence and requests for materials should be addressed to Q.Z. (email: qingshun@nju.edu.cn) or K.L. (email: kbli@prfri.ac.cn)
system is frequently employed for targeted ablation in studies of organ regeneration and function. For instance, it has been applied to conditionally eliminate ventricular cardiomyocytes\(^1,2\), hepatocytes\(^3\), and pancreatic β cells\(^4\) in zebrafish (Danio rerio) to investigate the mechanism underlying organ repair following injury. In addition, it can be applied for the targeted elimination of gonadal cells. Hsu et al. utilized the promoters of asp, odf, and nanos to specifically drive the expression of the NTR coding gene nfsB in zebrafish gonadal tissues and found that MTZ treatment could induce male infertility\(^5\). Dai et al. employed dnd promoter to construct NTR transgenic fish and demonstrated that MTZ treatment could induce male transformation and severely impair reproduction capability\(^6\).

A series of marker genes, including the maternal genes vasa\(^7\), piwi\(^8\), and dazl\(^9\), have been identified that allow for study of fish PGC origin, migration, and differentiation. The maternal gene nanos encoding the RNA-binding protein is also specifically expressed in PGCs. In this study, we used the zebrafish nanos regulatory sequences driving the expression of NfsB-mCherry specifically in PGCs and examined the effect NTR/MTZ system on zebrafish gonad development. Our results showed that conditionally eliminating PGCs during early development completely ablated the development of germ cells in gonad, resulting in sterile and male-like adult zebrafish.

**Results**

**Generation of Tg(nanos3:nfsB-mCherry-nanos3 3’UTR) zebrafish.** To generate the transgenic zebrafish of Tg(Tol2-nanos3:nfsB-mCherry-nanos3 3’UTR), we microinjected the transgenic construct (Fig. 1A) together with transposase mRNA into zebrafish fertilized eggs. Totally, we obtained 80 F0 zebrafish. To obtain the germline transmission transgenic zebrafish, we screened the F1 embryos using the strategy described in Fig. 1B. Briefly, 20 sexual mature females of the F0 were mated with wild-type males to produce F1 embryos. When reaching 42 hpf, the F1 embryos were examined whether they had the mCherry expression under fluorescence microscopy. Among the founders, 18 did not produce any fluorescent embryos. Only Founder #9 and Founder #13 produced 54 of 228 and 24 of 159 embryos carrying the fluorescent signals respectively. Among the embryos, the ones derived from Founder #9 had stronger fluorescent intensity than the ones from Founder #13. We therefore raised the fluorescent F1 embryos produced from Founder #9 for setting up the germline transmission transgenic zebrafish.

At 2 months after fertilization, the caudal fins of F1 zebrafish derived from Founder #9 were clipped to prepare the genomic DNA template for genotyping with PCR amplification using nfsb gene-targeted specific primers. Results from agarose gel electrophoresis of the PCR products revealed that 19 of 24 F1 zebrafish were positive for the expected 602 bp product (Fig. 1C) and the transgene identities were further confirmed by Sanger sequencing. The transgenic zebrafish were named Tg(nanos3:nfsB-mCherry-nanos3 3’UTR) or prfri001. That the other 5 F1 did not carry the transgene but exhibit fluorescent at early development is due to the expression of maternal nsfb-mCherry transcript.

To examine the expression of the transgene, the female F1 carrying transgene nfsB-mCherry was selected to mate with male wild type for producing F2 embryos. As shown in Fig. 1D–F, the localized mCherry fluorescence was found in the genital ridge in the F2 embryos at 24 hpf. Fluorescent cells were arranged and aggregated in two lines (Fig. 1G).

We next performed quantitative PCR to determine how many copies of transgene were recombined into the transgenic zebrafish genome using the method as described previously\(^10\). Briefly, we first set up the standard curve comparison between the copies of housing keeping actb DNA and the transgene Tg(Tol2-nanos3:nfsB-mCherry-nanos3 3’UTR) versus the actb cycle (Fig. 1H). We then performed the quantitative PCR using the template isolated from the F2 zebrafish carrying the transgene. The experiments were repeated three times and resulted in an average Ct with 21.773 for the transgenic nsfb. Calculated with the standard curve, we found the copy number of the transgenic nsfb is about 0.55. Therefore, we concluded that only one copy of transgene was inserted into the genome of transgenic zebrafish prfri001.

**The transgenic zebrafish embryos treated with MTZ exhibit ablation of PGCs.** After treating the embryos derived from the female transgenic zebrafish of Tg(nanos3:nfsB-mCherry-nanos3 3’UTR) with 10 mM MTZ for 48 h from 0 hpf through 48 hpf (referring as MTZ-treated embryos hereafter), we examined the number and distribution of PGCs under fluorescence microscopy. When observed at 24 hpf, the control embryos without MTZ treatment (referring as control embryos hereafter) displayed most labeled PGCs colonizing the genital ridge (Fig. 2A) whereas the MTZ-treated embryos had obviously fewer PGCs colonizing in the genital ridge and some labeled cells were incomplete migration towards the genital ridge (Fig. 2B). Throughout development, the most of labeled cells were arranged around the genital ridge in the control embryos (Fig. 2C) whereas fewer cells were seen in the genital ridge and the labeled cells were incomplete migration towards the genital ridge of the MTZ-treated embryos at 36 hpf (Fig. 2D). When observed at 48 hpf, almost no fluorescent cells can be seen in the MTZ-treated embryos (Fig. 2E) while the PGCs labeled by mCherry fluorescent were rich around the genital ridge of the control transgenic embryos (Fig. 2E). By 60 hpf, the fluorescent intensity of PGCs was slightly reduced and dispersed in the control embryos (Fig. 2G), and this became more evident at 72 hpf (Fig. 2F). However, no fluorescent cells or PGCs were found in the MTZ-treated embryos at neither 60 hpf (Fig. 2H) nor 72 hpf (Fig. 2I). Taken together, the results suggest that MTZ treatment not only affected early migration but also targeted cells for elimination of the PGCs.

**No germ cells are present in the gonads of 20-dpf juvenile developed from the MTZ-treated transgenic zebrafish embryos.** The differentiation and development of gonad in zebrafish are relatively distinctive during early stage. Both male and female zebrafish undergo a juvenile ovary stage and subsequently...
proceed into the gonadal differentiation stage. Histological examination of H&E-stained sections showed that the gonadal tissues of 20-dpf juvenile derived from control embryos mainly localized in the upper posterior cavity, specifically nearby the posterior chamber of the swim bladder, the hepatopancreas, and intestinal tract (Fig. 3A), where numerous perinucleolar oocytes in the gonad indicated the juvenile ovary stage (Fig. 3C). However, the 20-dpf juvenile derived from the MTZ-treated embryos displayed a gonad-like structure beneath the swim bladder (Fig. 3B), primarily consisting of supporting cells with no detectable perinucleolar early oocytes or other germ cells (Fig. 3D). Thus, MTZ treatment appears to completely eliminate the germ cells in the juvenile zebrafish developed from the female transgenic zebrafish Tg(nanos3:nfsB-mCherry-nanos3 3’UTR), while somatic cells adjacent to the gonadal tissues normally differentiates into supporting structures in the absence of germ cells.

To confirm the above observation, we performed in situ hybridization on the sections of gonad with the antisense RNA probe of vasa. The results showed that no vasa positive cells were found in the gonad tissues of the 20-dpf juvenile derived from control embryos (Fig. 3E) while the vasa expression was obviously present in the gonad of the juvenile derived from the control embryos (Fig. 3E). Moreover, results from RT-PCR analysis revealed that the 20-dpf juvenile derived from MTZ-treated embryos exhibited no expressions of vasa and ziji that were normally expressed in the juvenile developed from control embryos (Fig. 3G). And the 20-dpf juvenile neither from MTZ-treated embryos nor from control embryos had the expressions of sox9a or foxl2 (Fig. 3G). The results suggest that no germ cells were present in the 20-dpf juvenile developed from MTZ-treated embryos.
Adult transgenic zebrafish derived from MTZ-treated embryos exhibit male-like morphology.
The appearances of male and female adult zebrafish differ substantially. Females are plumper than males due to expansion of the abdomen with ovarian development. In contrast, males are longer and slender with larger fins.
especially the anal fin, than females. Sexually mature males are bright yellow-brown (the normal nuptial color), especially in the anal region and caudal fin. The abdominal regions adjacent to each fin are also brightly colored (Fig. 4A), especially at daybreak. Mature males are observed chasing females counterparts, a normal mating behavior. Interestingly, the adult zebrafish derived from MTZ-treated embryos exhibited no observable disparities in feeding, behavior, and growth. The majority of them looked long and slender at 60 dpf, and displayed obvious male characteristics (Data not shown). Moreover, no evident abdominal expansion was observed in 124 of the adult zebrafish derived from the MTZ-treated embryos. They all displayed the normal nuptial color (Fig. 4B,D). In contrast, about 39% (52 of 133) of the adult zebrafish derived from the control embryos appeared to be females and the other 61% (81 of 133) exhibited male morphology (Fig. 4A,C) when reaching 100 dpf.
Effect of MTZ treatment on reproductive capability. The adult zebrafish derived from the MTZ-treated embryo showed normal mating behavior (chasing), and stimulated females to lay eggs. However, at approximately 4 h after spawning, the eggs, presumably fertilized by 10 of the different adult zebrafish with male appearances, all gradually became white and died. In the control group, the eggs fertilized by the adult zebrafish derived from control embryos developed normally, and the hatching rate all exceeded 90%. The results demonstrate that the eggs were not able to be fertilized by the adult zebrafish derived from the MTZ-treated embryos though they exhibited male-like morphology and normal reproductive behaviors.
Effect of MTZ on the gonad structure of mature zebrafish. The testes of the sexually mature male zebrafish locate bilaterally at the dorsal side of the digestive tract, closely adhered to the abdominal wall and the ventral side of the swim bladder, occasionally surrounded by adipose tissues. Both cylinder-shaped testes extend from the middle abdominal cavity to the anal fin, converging to form a V shape at the end of the posterior abdominal cavity with separation at the gonopore. Bilateral gonads were fully developed in the adult zebrafish derived from the control embryos, and were cream white in color and plump in shape (Fig. 4E). The gonadal position of the adult zebrafish derived from MTZ-treated embryos was similar to that of control males, and their gonadal tissues still formed a bilateral structure. Additionally, their general shape of the testis was preserved, and bilateral gonadal tissues also converged to the gonopore. However, after removal of the adipose tissues, the gonadal tissues appeared completely withered and shrunken (Fig. 4F) compared to testes of the adult zebrafish developed from the control embryos (Fig. 4E).

The fine structural features of mature zebrafish testes were then examined in thin sections throughout the transverse axis (Table 1). In the adult zebrafish developed from the control embryos, the testis was likely divided into two functional parts including bilateral testes and intersection point. Bilateral testes are responsible for sperm production. They were surrounded by membrane structures and both consisted of a large quantity of irregularly shaped seminiferous tubules, which were interleaved and intimately arranged. Cross sections of seminal vesicles, the central structure of the seminiferous tube, were generally circular or oval. The seminal vesicles consisting of germ cells were enveloped by Sertoli cells. Sperm were formed within the seminal vesicles, and the germ cells within it were derived from the same spermatogonia with nearly synchronous development. However, the germ cells between adjacent seminal vesicles differed in developmental phases. Spermatogonia, primary spermatocytes, Sc I, secondary spermatocytes, Sc II, spermatids, and spermatozoa could be observed within a single cross section (Fig. 5A,C,D). In contrast, the adult zebrafish developed from MTZ-treated embryos exhibited neither germ cells at any developmental phases nor seminiferous tubule-like structures. Rather, the gonadal structure was composed of grid-shaped supporting tissues consisting of Sertoli cells and connective tissues (Fig. 5B,E,F). Sertoli cells were seen in multi-porous structures due to the lack of filling by functional sperm cells, consistent with the gross withering and shrinkage of the gonad.

In the adult zebrafish developed from control embryos; however, the testes intersection point stored the sperm. It consisted mainly of seminal vesicle-like structures distinct from those of bilateral testes. No spermatogonia or spermatids were seen; rather, the structure consisted of a large quantity of honeycomb-shaped tissues composed of multiple irregular cavities forming ridge structures. These cavities were full of sperm, similar to functional seminal vesicles (Fig. 5G,H). We proposed that the sperm were generated in the seminiferous tubules and transferred from the seminal duct for temporary storage. Cross-sections close to the intersection point also revealed long strips of tissue in a dumbbell-shape. There was no significant change in the structures adjacent to the cloacal opening, whereas the cross-sections gradually became round, consistent with the overall appearance of the testes at this position (Fig. 5G,H). The gonadal structures of the adult zebrafish developed from the MTZ-treated embryos were similar to those of controls, but no sperm were detected within the seminal vesicles. Cavities consisting of Sertoli cells were seen, which subsequently formed network structures (Fig. 5I,J). In the adult zebrafish developed from control embryos, the seminal vesicles were full of semen to nourish the sperm and maintain ionic equilibrium (Fig. 5G,H). In the adult zebrafish developed from MTZ-treated embryos, however, the testis lacked germ cells, failed to produce and store sperm, and were withered, all manifested as collapsed network structures under the microscope (Fig. 5I,J).

To confirm the histological observation, we performed in situ hybridization on the sections of gonads with the antisense RNA probe of vasa. The results revealed that no vasa positive cells were found in the gonad tissues of adult zebrafish (100-dpf) derived from MTZ-treated embryos (Fig. 5M) while the vasa expressions were obviously present in the primary spermatocytes (Sc I) and secondary spermatocytes (Sc II) of the testis in the adult male zebrafish derived from control embryos (Fig. 5K), and in the primary oocyte (Po) of the ovary in the adult female zebrafish derived from control embryos (Fig. 5L). Moreover, results from RT-PCR analysis showed that the gonads of 100-dpf adult zebrafish developed from MTZ-treated embryos had no expressions of vasa and ziwi that are normally expressed in the ovary or testis in the adult zebrafish derived from control embryos (Fig. 5N). Consistently, foxl2, expressed in ovary but not in testis of the adult zebrafish developed from the control embryos, was not found to express in the gonads of adult zebrafish derived from the MTZ-treated embryos. In contrast, sox9a, strongly expressed in testis but weakly in ovary of the adult zebrafish developed from the control embryos, was also found to express weakly in the gonads of adult zebrafish developed from the MTZ-treated embryos (Fig. 5N). Taken together, the adult zebrafish developed from the MTZ-treated embryos had no germ cells at all.

Discussion
Fish gonads are derived from two types of cells: PGCs and somatic cells adjacent to the genital ridge. PGCs are critical determinants of zebrafish sex as they can differentiate into either ovogonium or spermatogonia. During early embryonic development, PGCs aggregate at a specific position, undergo oriented migration to the gonadal

| Cells detected | Numbers of fish | Primary spermatocyte | Secondary spermatocyte | Spermatid | Spermatozoa | Sertoli cell |
|----------------|-----------------|----------------------|------------------------|-----------|------------|-------------|
| Control        | 10              | 10                   | 10                     | 10        | 10         | 10          |
| MTZ treatment  | 10              | 0                    | 0                      | 0         | 0          | 0           |

Table 1. Summary of the histological results for different types of cells detected in adult zebrafish testis.
Figure 5. Histological analysis revealed the absence of germ cells at various developmental phases in the testis of MTZ-treated adult zebrafish. Transverse sections showing that the gonads of control zebrafish (A,C,D,G,H) and the ones of MTZ-treated zebrafish (B,E,F,I,J) at 100-dpf. At top of bilateral testis in the control adult zebrafish, germ cells with various developmental phases were found: spermatogonia, primary spermatocytes ScI, secondary spermatocytes ScII, spermatids and spermatozoa (A), whereas MTZ-treated zebrafishes were found to have only Sertoli cells which were derived from somatic cells (B). Testis (arrow) near the bilateral gonadal junction also carried numerous seminiferous tubules with irregular shape in control adult fish (C,D), but there was no germ cells in the gonad (arrow) of the MTZ-treated fish at any developmental phases observed (E,F, arrow). Seminal vesicle-like structures were found in cross-section of the testis adjacent to the gonopore. The organ (arrow) consisted of numerous cavities communicating with each other, that contained a large quantity of sperm cells in the control zebrafish (G,H). No sperm was detected within the seminal vesicle in gonads (arrow) of the MTZ-treated zebrafish, so the cells of cavities which derived from somatic cells formed net structures (I,J). Transverse sections showing that the gonads of control male zebrafish at 100-dpf (K, upper panel) carried normal testis (black arrow) with germ cells at different developmental phases and had weak vasa expression in gonads (K, lower panel, black arrow) detected by in situ hybridization, the gonads of control female zebrafish at 100-dpf (L, upper panel) carried normal ovary and had strong vasa expression in developing oocytes (L, lower panel, black arrow) detected by in situ hybridization, whereas the gonads of MTZ-treated zebrafish at 100-dpf had only somatic gonadal cells (M, upper panel) and had no vasa expression (F, lower panel) detected by in situ hybridization. (N) RT-PCR results showing the expressions of vasa and ziwi were detected in the gonads of control male and female zebrafish, but not in the zebrafish developed from the MTZ-treated embryos, and strong expressions of sox9a and foxl2 were detected in control male zebrafish and control female zebrafish respectively, weak expression of sox9a was found in control female zebrafish and MTZ-treated zebrafish, and no expression of foxl2 was detected in control male zebrafish and MTZ-treated zebrafish. Expression of actb was used as positive control. Sg: spermatogonia, Sc I: Primary spermatocytes, Sc II: Secondary spermatocytes, St: Spermatids, Sz: Spermatooza, SC: Sertoli cell, In: Intestine, M: Muscle, K: Kidney, Po: Primary oocyte, Vo: Vitellogenic oocyte. Scale bars = 20 µm.
and develop with surrounding somatic-derived cells to form a functional gonad where PGCs differentiate into two types of germ cells namely sperm and oocyte, and somatic cells adjacent to germinal primordium develop into the structures supporting the gonadal tissues. The NTR/MTZ system can specifically eliminate cells or tissues, and currently serves as an effective approach for investigating the mechanism of organ function and regeneration. Previously, Hsu et al. generated transgenic zebrafish Tg(asp:nsfB), Tg(odf:nsfB) and Tg(sam:nsfB) in which NsfB was expressed specifically in testis under the promoter of asp (A-kinase anchoring protein-associated protein), odf (outer dense fibers) and sam (sperm acrosomal membrane-associated protein). After treated with MTZ, 68%, 59%, 54% of the transgenic zebrafish displayed male sterile, respectively. Because asp, odf and sam were all expressed in spermatid or sperm, the transgenic zebrafish treated with MTZ still had spermatogonia in their testis although the spermatid was ablated by MTZ treatment. Therefore, it is reasonable to hypothesize that the normal sperm can be developed and the transgenic zebrafish are fertile again once MTZ is removed because the spermatogonia are not affected by MTZ at all. In contrast, two other groups generated transgenic zebrafish Tg(zp:nsfB) in which the expression of nsfB is specifically driven by the promoter of ovariian specific expression gene of zp (zona pellucida). When treated with MTZ, the transgenic zebrafish exhibited female sterile because the disrupted formation of zona pellucida blocked the oogenesis although oogonium were not affected at all. Once MTZ was removed, oogenesis was recovered and normal oocyte was developed from the unaffected oogonium, resulting in the female transgenic zebrafish fertile.

Unlike sperm and oocyte that are formed in testis and ovary respectively, PGCs are the germ stem cells that are specified in the embryos at early development and are fate to differentiate into gametes. Therefore, the fish would be sterile if their PGCs are eliminated in the embryos at early development. Actually, in the absence of PGCs, the somatic cells of zebrafish and medaka (Oryzias latipes) gonads tend to spontaneously differentiate into male characteristics, while gonadal differentiation with female characteristics requires PGCs in transgenic zebrafish, although the underlying mechanisms remain to be elucidated. Consistently, Wong and Collodi reported that overexpressing stromal-derived factor 1a (Sdf1a), providing the directional cue that guides the migration of PGCs to the gonadal tissue in a gradient, disrupted the normal PGC migration pattern, resulting that the embryos developed into sterile males. Additionally, microinjection of ndd morpholino into zebrafish fertilized eggs interfered with the migration and formation of PGCs, resulting in zebrafish with male characteristics. In this study, we set up a transgenic zebrafish line Tg(nanos3:nsfB-mCherry-nanos3 3’UTR). Driven by nanos3 promoter, the transgene was expressed during oogenesis but not spermatogenesis, and the mRNA message was deposited in oocyte. After fertilization, the message in the oocyte was alloted into PGCs but not somatic cells because of the 3’UTR of nanos3 that allows the mRNA be stable in PGCS but be degraded in somatic cells. In other words, only the PGC cells in the embryos derived from female transgenic zebrafish can express the fusion protein of NsfB-mCherry. Therefore, once the embryos are incubated with MTZ, the PGC cells in the embryos derived from the female transgenic zebrafish will be killed no matter the embryos themselves are transgenic or not. Conforming to the above hypothesis, our results demonstrated that early MTZ treatment completely eliminated PGCs in the embryos derived from the female transgenic zebrafish. With development, no germ cells were detected in the gonads of the zebrafish developed from the MTZ-treated embryos (Fig. 3), and only cavity-shaped structures derived from somatic cells were observed (Fig. 4), resulting in all male-like sterile adults without reproductive function (Figs. 4 and 5). As an anti-anaerobe and protozoaicide, prolonged treatment with high-dose MTZ may induce adverse effects. Therefore, the duration and dose of MTZ treatment are the key parameters for targeted conditional elimination. Previously, Curado et al. utilized 10 mmol/L MTZ to treat transgenic NTR juvenile zebrafish under the control of different promoters and found that cell ablation occurred in the heart, pancreas, and liver at 24 h, accompanied by functional defects. White et al. adopted 5 mmol/L MTZ to treat transgenic animals and conditionally removed the ovariay. Hsu et al. treated transgenic zebrafish using 5 mmol/L MTZ for 14 d and demonstrated that the testis was completely or partially destroyed. In this study, we demonstrated that a dose of 10 mmol/L Mtz for 48 h from 1-cell stage exerted no significant adverse effects on zebrashembyros and all the embryos treated with Mtz developed into male-like sterile adult. The results revealed that short-term MTZ intervention with 10 mM is sufficient to effectively eliminate germ cells at early development. However, 5 mM is not optimal amount to kill all the germ cells in zebrafish embryos because Dai et al. administered 5 mM MTZ to treat transgenic zebrafish Tg(dnd:NTER-EGFP + 3’UTR) for 20 days from 18 dpf and found that all MTZ-treated transgenic fish exclusively developed into males with subfertilities.

In aquaculture, males of multiple species, such as tilapia (Oreochromis niloticus) and yellow catfish (Tachysurus Fulvidraco), outperform females in terms of breeding and growth. For example, if male and female tilapia are raised together, they reach sexually mature at the age of 3–5 months and they then mate ad libitum to produce offspring. The adults derived from the offspring are in small body size, far less than the market size. Therefore, production of all-male fish may substantially improve aquaculture yield of the defined fish species. In this study, we found no germ cells were detected in the gonads of MTZ-treated zebrafish at 20 dpf. In addition, no PGCs-derived germ cells except somatic cell-derived supporting structures were observed in the gonads of MTZ-treated transgenic zebrafish. Interestingly, the MTZ-treated adult zebrafish retained the morphology and behavioral characteristics of males, but failed to produce sperm despite chasing and attempting to mate with females for spawning. The results demonstrate that zebrafish with conditional elimination of PGCs at early development grow up as sterile male-like fish. Therefore, the NTR/MTZ system established to conditionally eliminate PGCs in this study provides an alternative novel strategy for producing all-male fish, which is probably a practical direction for fishery industrialization. Provided that the female transgenic fish is a heterozygote for the transgene, only half of their offspring will be transgenic and the other half will be non-trangenic but their germ cells were all killed when they are incubated with MTZ. Therefore, one can obtain non-trangenic all male-like fish in aquaculture using this developed technique system.
Transplantation of fish germ cells offers a novel strategy for rapid breeding and seed conservation, but this strategy is unsuitable for industrialized application due to low transplantation efficiency (which may be enhanced depending on the donor fish cells and the quality of the recipient fish). Currently, multiple recipient fish species lack a sterile line. Conventionally physical and chemical approaches have been employed, but they have either failed to completely eliminate the germ cells or resulted in substantial variability of outcome, which greatly increases the workload for subsequent screening. Furthermore, these non-targeted methods can alter other tissues. Thus, the experimental endpoint cannot be controlled and reproducibility is poor. In this study, the germ cells of adult zebrafish developed from the MTZ-treated embryos were completely eliminated, which can await the germ cell chimeras between the donor and recipient fish and facilitate subsequent screening. Alternatively, the somatic cell-derived supporting structures remain in intact, thereby creating a favorable environment for the development and differentiation of donor cells and enhancing transplantation efficiency. Thus, induced elimination of germ cells as demonstrated in this study may facilitate the systematic development of appropriate recipient fish for the transplantation of allogenic germ cells.

**Experimental Methods**

**Ethics statement.** Experimental protocols using zebrafish as a research subject in this study were approved by the Aquatic Animal Research Committee at Pearl River Fishery Research Institute, China. All methods were performed in accordance with the relevant guidelines and regulations.

**Generation of transgenic zebrafish.** A Tol2-nanos3-nfsB-mCherry-nano3-3′UTR vector (Fig. 1A) was constructed using traditional molecular recombination. In the expression construct of Tol2, the CDS of nfsB-mCherry, encoding a fusion protein of NfsB nitroreductase fuzed with mCherry reporter, was inserted between the promoter of zebrafish nano3 and its 3′ UTR in order for the transcription of the fused gene to be controlled by zebrafish nano3 promoter and the transcript to be only stably present in PGCs.

A transgenic line was established by microinjecting the transgenic construct plus transposase mRNA into zebrafish fertilized eggs as described previously. F0 zebrafish were bred to sexual maturity, and the females selected and mated. PCR was run in 95°C 30 s, followed by 40 cycles of 95°C 5 s, 60°C 34 s, and 72°C 15 s. Each amplification was repeated 3 times. The curve was plotted with X axis representing log of copy number and Y denoting the average of Ct.

**Transgene copy number determination.** Quantitative PCR was performed using the equipment of ABI 7500. To determine the copy number of transgene in the genome of transgenic zebrafish, two standard curves (log of copy number vs amplification cycle threshold) were set up by the real time PCR using the pair of primers of ACGAACGACCAACTAACTCT and TTAGACAAGCTCCCTTGGC (actb), and CATCCGACCCACTTCCCA and CCACTCTGAC AGACATCGT (nfsb), respectively. The standard template of actb DNA sample was the genome DNA isolated from the caudal fin of wild type zebrafish using the traditional genomic DNA isolation method. In contrast, the standard template of nfsb DNA sample was the cloning vector of the transgene. The copy number of target template was calculated using the equations of DNA concentration (ng/μL) × 10^6 × 1 μL × 10^-3 × 6.02 × 10^21/(6593 × 650) (for transgene nfsb) or DNA concentration (ng/μL) × 10^-6 × 1 μL × 10^-3 × 6.02 × 10^23/(1.7 × 10^9 × 650) (for actb). The PCR reaction was performed in a 20 μL volume including 10 μL of SYBR Premix Ex Taq II (2×), 0.8 μL of 10 μM forward primer, 0.8 μL of 10 μM reverse primer, 0.4 μL of Rox Reference Dye II (50×), 6 μL of ddH2O, and 2 μL of standard DNA template. The PCR was run in 95°C 30 s, followed by 40 cycles of 95°C 5 s, 60°C 34 s, and 72°C 15 s. Each amplification was repeated 3 times. The curve was plotted with X axis representing log of copy number and Y denoting the average of Ct.

To determine the copy number of the transgene in the transgenic zebrafish genome, genomic DNA was isolated from the caudal fin of the transgenic zebrafish as described above and then used as the template of the quantitative PCR amplifying the transgene and the endogenous actb gene. The copy number of transgene was finally calculated from the standard curve by comparing it with actb that are two copies in the zebrafish genome.

**MTZ treatment.** Female zebrafish with positive phenotype (mCherry fluorescence) and genotype (nfsb) were mated with wild type male and the fertilized eggs collected. Metronidazole (MTZ) was dissolved in DMSO and adjusted to 10 mM with water. Sixty mCherry-positive fertilized eggs were obtained and treated with 10 mM MTZ in a 500 mL beaker at 28 ± 1°C under darkness. After 24 h, the medium was exchanged with fresh MTZ. After another 24 h, the solution was exchanged for egg water and incubation was continued. Growth and development status were monitored regularly hereafter. All treatments were conducted in triplicate.

**Effect of MTZ treatment on PGC morphology.** After 24, 36, 48, 60 and 72 h of MTZ treatment, four embryos per group were randomly selected and PGC morphology and distribution assessed under fluorescence stereoscopy.

**Effect of MTZ treatment on gross appearance of zebrafish.** Zebrafish body and color changes were observed at each time point and compared between the MTZ treatment and control groups.
Effect of MTZ treatment on gonadal tissues of 20-dpf zebrafish. Ten zebrafish were randomly selected after 20 dpf (days post fertilization) of MTZ treatment and fixed in 4% paraformaldehyde solution overnight at 4 °C. The juveniles were then dehydrated in gradient ethanol, made translucent by xylene treatment, embedded by paraffin, sectioned at 3 μm longitudinally along the body axis or transversely, stained with hematoxylin and eosin (H&E), and finally mounted. The histology of gonadal tissues was compared between MTZ-treated and control groups under light microscopy.

Assessment and validation of reproductive capability. Ten of 100-dpf zebrafish developed from the MTZ-treated embryos were randomly selected and mated with wild-type female zebrafish, and another 10 transgenic male zebrafish were chosen as normal controls. Spawning and reproduction status were compared between the two groups.

Gross observation of gonadal tissues of adult zebrafish. Five of 100-dpf zebrafish developed from the MTZ-treated embryos or control males were randomly chosen and sacrificed under anesthesia using 0.2 mg/ml Tricaine. The size and morphology of the zebrafish gonad were compared between MTZ-treated and control transgenic males. Differences in morphology and positions of liver, intestine, kidney, and other organs were also examined to evaluate extraneous effects of MTZ.

Comparison of gonadal histology in adult zebrafish. Ten of 100-dpf zebrafish developed from the MTZ-treated embryos and control males were randomly chosen, fixed, decalcified for 2 weeks, transverse sectioned, and stained with H&E. The gonadal structure and germ cell morphology were compared.

RT-PCR. Gonads were isolated from the zebrafish developed from MTZ-treated embryos or control embryos without MTZ treatment at 20 dpf and 100 dpf under the stereoscope. Samples were homogenized with a mortar and pestle in liquid nitrogen. Total RNA was then extracted with TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. The quality of the extracted RNA was determined by agarose gel electrophoresis. cDNA was reverse transcribed using the TAKARA PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA, Japan). cDNA was stored at −20 °C for gene expression analysis.

PCR reactions were performed in 25 μl volume containing 9.5 μl dH2O, 1 μl cDNA template, 1 μl (10 μM) of each primer, 12.5 μl 2 × Taq PCR MasterMix (TianGen, China). The sequences of the forward and reverse primers used to detect gene expressions were CCAATATGGATGACTGGGAG and GTCATTTTCCATGAGCTACC (for vasa), TCTAGATGGGTGTTGTGATCT and ACGGTGCAACTGTTCTTCCCTCAG (for zwi), AGTTCCACACGGTTTCGTGATTG and ATCCGTGGGATTAATCGTGACG (for sox9a), CCCAGCATGGTGAACTCTTACG (for actb), and CGTGATGCAATATGGACAGT (for fox12). The PCR was run in 95 °C 5 min, followed by 35 cycles of 95 °C 30 s, 60 °C 30 s and 72 °C 30 s, and finally extended for 5 min at 72 °C. The PCR products were separated by 1.0% native agarose gel electrophoresis.

In situ hybridization. Gonads were isolated from the zebrafish developed from MTZ-treated embryos or control embryos without MTZ treatment at 20 dpf and 100 dpf under the stereoscope. They were fixed in 4% paraformaldehyde solution overnight at 4 °C, dehydrated and embedded as described above, then sectioned at 7 μm transversely. Some sections contained with H&E and others for hybridizations. A 1223 bp vasa cDNA fragment was inserted into a PGEM-Tvector for the synthesis of antisense RNA probes under the drive of T7 promoter by using the digoxigenin RNA Labeling Kit (Roche). The RNA probes were treated with RNase-free TURBO DNase and purified with SigmaSpinTM Sequencing Reaction Clean-Up (Sigma). The sections were digested with proteinase K (10 μg/ml) for 10 min and hybridized with the probes at 65 °C for 14 hours. Chemical in situ hybridization was conducted by developing the signals with BCIP/NBT substrates on sections and post fixed in 50% glycerin. The results was photographed under microscope using digital CCD camera.

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Author Contributions
K.L., Q.Z. and L.J. conceived and designed the experiments; L.Z., Y.F., F.W., C.L., and X.D. did experiments; K.L., Q.Z., L.Z., and Y.F. analyzed the data and wrote the paper.

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