A controllable on-off strategy for the reproductive containment of fish

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A major impediment to the commercialization and cultivation of transgenic fish is the potential ecological risks they pose to natural environments: a problem that could be solved by the production of sterile transgenic fish. Here, we have developed an on-off reproductive containment strategy for fish that renders the offspring sterile but leaves their parents fertile. TG1 (Tol2-CMV-GFP-pA-CMV-gal4-pA-Tol2) and TG2 (Tol2-CMV-RFP-pA-5×UAS-as/dnd-pA-Tol2) zebrafish lines were established using a GAL4/UAS system. While the parental lines remained fertile, in the hybrid offspring, GAL4 induced 5×UAS to drive the transcription of antisense dnd, which significantly down-regulated endogenous dnd expression. This disrupted the migration of primordial germ cells (PGCs), led to their apoptosis, and resulted in few or no PGCs migrating to the genital ridge. This process induced sterility or reduced fertility in adult fish. This on-off strategy is a potentially effective means of generating sterile fish for commercialization while retaining fertility in brood stocks, and offers a novel method to mitigate the ecological risks of fish introductions.

Fish are the last wild food available to humans1. However, wild fish stocks are under increasing pressure from overfishing as the global demand for fish increases alongside rapid population growth. Overfishing compromises the long-term sustainability of fisheries resources and results in biodiversity loss, potentially leading to ecosystem collapse2,3. Aquaculture is, therefore, considered to be the only long-term sustainable solution to supply our growing demand for fish4. The introduction of exotic fish species to the environment is an effective method of sourcing fish with valuable traits for aquaculture, but there is an ecological risk associated with it: the introduction of the exotic species may be economically viable but can also cause substantial economic and ecological damage should the species become invasive. Transgenic technologies offer another means of producing new fish varieties that exhibit physiologically and commercially desirable traits for aquaculture, and are an important factor in the sustainable development of future aquaculture industries. Since the first rapid-growth transgenic fish in the world was developed by our group 30 years ago5, many fish breeds with commercially desirable traits (e.g. rapid growth, cold tolerance, enhanced disease resistance) have been generated using transgenic technologies6-8. However, to date, no transgenic fish has been approved for release into a natural environment, or for commercial cultivation as food. The “AquaBounty” transgenic salmon (Oncorhynchus tschawytscha) is perhaps the most notable example of current attempts to commercialize the cultivation of transgenic fish. This variety was declared safe for consumption by the US Food and Drug Administration in 2010, and is nearing approval for commercialization9. The primary impediment to the commercialization of transgenic fish is the concern over their potential ecological risk to natural ecosystems. As a result of their superior viability and competitive ability, the inadvertent release or escape of transgenic fish into natural environments can alter natural community structure. Additionally, transgenic fish may also interbreed with native fish populations resulting in gene introgression to the wild.

A key means of eliminating the potential ecological risk posed by invasive and transgenic fish is fertility control to make individuals infertile10-15. At present, however, there are few cost-effective means of controlling reproduction in fish. Generating fish that are triploid rather than diploid is currently the most common strategy used to develop sterile fish. However, in commercially important species, the ‘triploidization’ rate (achieved via physical or chemical methods) rarely approaches 100%, and varies greatly among species, treatment methods and egg quality16. Although up to 99.8% of the all-female transgenic salmon produced by AquaBounty are triploid and sterile, some researchers are still concerned about the non-zero possibility that fertile escapees will be produced17. Moreover, in some fish species triploidization is known to produce defects such as impaired growth and reduced disease resistance16. For example, compared with diploids, the growth rate of triploid tilapia (Oreochromis
the on-off strategy for controlling reproduction in transgenic fish using the GALA/UAS inducible system and the dnd antisense knock-down technique is shown in Figure 1. The TG1 line expressed the transcriptional activator GAL4, driven by the CMV promoter (Fig. 1a). The TG2 line expressed antisense dnd RNA under the control of 5 × UAS (Fig. 1b). The expression level of the UAS-regulated gene was related to the number of UAS repeat units. We evaluated the effect of a range of UAS repeat units (from 1 to 11) on egfp transgene activation induced by GAL4, and found the expression level of 5 × UAS: egfp to be strongest (Supplementary Fig. 1). Based on this result, the TG2 line was established with a transplant of 5 × UAS: antisense dnd to get the strongest expression of antisense dnd in the hybrids of TG1 and TG2 lines (which are hereafter referred to as the TG3 line; Fig. 1c).

During the development of wild-type (WT) embryos, the expression level of endogenous dnd, nanos1 and triRd7 mRNA was initially high then decreased rapidly (Fig. 2a–c). There were no significant differences in the endogenous dnd mRNA level between the TG3 and the WT lines at the stages of 1-cell, 1k-cell and oblong (Fig. 2a). Meanwhile, dnd mRNA level in the TG3 line was markedly lower than that of the WT line at the stages of 50%-epiboly and 3-somite (p < 0.01; Fig. 2a). The dnd gene encodes an RNA binding protein that can bind to the 3′ untranslated regions of nanos1 and triRd7 mRNAs to protect them from miR-430-mediated repression. Hence, the expression level of nanos1 and triRd7 mRNAs can mirror the level of the DND protein. Our study showed that the patterns of nanos1 and triRd7 expression change were similar to those of dnd during early development, both in the TG3 line and in the WT embryos (Fig. 2b, c). The nanos1 and triRd7 mRNA levels were normal at the 1-cell, 1k-cell and the oblong stage in the TG3 line, but were significantly lower than those of the WT line at the 50%-epiboly stage (p < 0.05) and at the 3-somite stage (p < 0.01; Fig. 2b, c). These results suggest that the endogenous dnd expression could be significantly repressed by GAL4/UAS induced antisense dnd in the TG3 line during early embryogenesis.

PGC migration was impaired and the number of PGCs was reduced in TG3 embryos. The dnd gene is essential for the migration and survival of PGCs in zebrasfish. We labeled PGCs with antisense vasa probes to detect the migration and number of PGCs during the embryogenesis. At 4.3 hours post-fertilization (hpf), when compared with the normal distribution of PGCs seen in the majority of WT embryos 87% (141/166), about 72% (94/130) of TG3 embryos showed unusual distribution of some PGCs, with 20 hpf and 24 hpf, the number of PGCs which had migrated near the genital ridge varied among TG3 embryos. Moreover, when compared to the WT embryos the number of PGCs in TG3 embryos had visibly reduced in 79% (109/138) of the TG3 embryos at 20 hpf and 67% (127/185) of the TG3 embryos at 24 hpf. Furthermore, there were almost no PGCs remaining in about 14.8% (23/155) of the TG3 embryos at 24 hpf (Fig. 2e).

In order to label the PGCs using green fluorescent protein (GFP) in living embryos, TG4-construction was undertaken by replacing the GFP element of TG1-construction using a red fluorescent protein (RFP) element (Fig. 3a). Then a TG5 line was derived by crossing the TG2 and TG4 lines. To label the PGCs, the 5 end-capped mRNA encoding GFP along with nanos1 3′UTR was then injected into the TG5 line and the WT embryos at the 1-cell stage. At 10 hpf under a fluorescence stereoscope, the PGCs could be observed to converge into two clusters that were distributed symmetrically on both sides of the dorso axis in 83% (332/400) of the WT embryos and 32% (128/395) of the TG5 embryos, and were distributed far from the dorso axis in the remaining embryos. At 13 hpf, the PGCs lay close to the dorso axis and strongly arrayed in 86% (344/400) of the WT embryos and 36% (132/369) of the TG5 embryos, but lay far from the dorso axis.
axis in the remaining embryos (Fig. 3b). Meanwhile, at 20 hpf and 24 hpf there was a variable number of PGCs among the TG5 embryos, but a constant number of PGCs among the WT embryos. The number of PGCs visibly decreased in 80% (279/347) of the TG5 embryos at 20 hpf and 62% (169/273) of the TG5 embryos at 24 hpf (Fig. 3b). No PGCs were detected in 17% (47/273) of the TG5 embryos at 24 hpf (Fig. 3c). We randomly selected 10 embryos with PGCs labeled by GFP from the TG5 and the WT lines at 24 hpf, and observed that the GFP fluorescence intensity of the PGCs within the WT embryos was four times that of the PGCs within the TG5 embryos (Fig. 3d).

To confirm whether the PGCs underwent apoptosis, 5'end-capped mRNA encoding GFP followed by nanos1 3'UTR was injected into the TG5 and the WT embryos at the 1-cell stage. The embryos were then analyzed for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL assay). The results showed that part of the PGCs underwent apoptosis in 13.4% (19/142) of the TG5 embryos at 20 hpf and 16% (20/124) of the TG5 embryos at 24 hpf (Fig. 3e).

TG3 adult fish were sterile or exhibited limited reproductive capacity. The majority of juvenile zebrafish reach sexually mature within three to four months\textsuperscript{39}. In this study, we evaluated reproductive capacity at 4.5 months of age as at this age all the WT fish were able to be naturally inseminated. In this experiment, the fertilization rate of WT fish was 89.27%. While all the TG3 males chased the WT females, 30.8% (53/172) failed to fertilize the eggs. About a third (31%; 15/48) of the TG3 females did not spawn after stimulus by the WT males. For convenience, fish that did not reproduce are termed TG3-1, while those that did reproduce are termed TG3-2. Although TG3-2 individuals could reproduce, the fertilization rate of TG3-2 males was significantly lower than that of the WT males (Table 1). Likewise, the relative fecundity of TG3-2 females was significantly lower than that of the WT females (Table 1).

TG3-2 individuals, only a portion of the lobular cavities lacked mature sperm. In the ovaries of WT females, most oocytes were at stage III. However, in the ovaries of TG3-1 females, the majority of oocytes were at stage I or II, with only a few oocytes having developed to stage III. In ovaries of TG3-2 females, meanwhile, most oocytes were at stage III, but were loosely aligned and fewer in number (Fig. 4a). The results of the TUNEL analysis revealed that apoptosis occurred more frequently in the gonads of the TG3-1 and TG3-2 fish than in the WT fish, regardless of sex. The cells undergoing apoptosis were not evenly
distributed, and the oocytes undergoing apoptosis were always at stage I or stage II (Fig. 4b).

The hybrid offspring exhibited methylation of the UAS and the mosaic expression of the UAS-regulated gene. The reproductive capacity of TG3 adults varied from complete sterility (TG3-1) to partial reproductive capacity (TG3-2). To understand the cause of this variation, we established the TG6 transgenic line, in which 5' UAS directly regulated the expression of egfp (Fig. 5a). Mosaic expression of egfp was observed in the embryos of the hybrid offspring of the TG4 and the TG6 lines, and varied among individuals (Fig. 5b). Mosaic expression of egfp was also observed in the testes and the ovaries of the hybrids, again with variation among individuals (Fig. 5c, d). We detected little dnd mRNA level in the gonads of TG3 adults (TG3-1 p < 0.01; TG3-2 p < 0.05), and significantly lower dnd mRNA level in the gonads of TG3-1 individuals than in TG3-2 individuals (p < 0.05; Fig. 5e, f). The gal4 mRNA level did not differ significantly between the TG3-1 and the TG3-2 adults (Fig. 5g, h). For the UAS repeat (CGGAGTAC-TGTCCTCGAG) contained two CpG sites, which were the targets of methylation. Hence, we examined the methylation status of 5' UAS and the minimal promoter E1b (5' UAS-E1b). The results showed that the 5' UAS-E1b sequence was susceptible to methylation in 77.5% of the TG3-1 males, 81.2% of the TG3-1 females, 88.3% of the TG3-2 male, and 90.8% of the TG3-2 females (Fig. 5i).

Discussion

The potential ecological risks posed by transgenic fish are a key impediment to their commercial use and development. Controlling the fertility of transgenic fish offers a solution to this problem. However, to date, a method of transferring the ‘infertility’ trait of the transgenic fish to its offspring while maintaining a self-propagating genetic line has not been established. The galactose regulated upstream promoter element (GAL4) is a transcriptional activator in yeast that contains not only the DNA-binding domain but also the transcriptional activation domain. The upstream active sequence (UAS) is a type of regulatory sequence like a eukaryotic enhancer that occurs in yeast. GAL4 can specifically recognize UAS and drive the transcription of genes following UAS: a system that has strong specificity and easily controlled. In our study, TG1 and TG2 transgenic lines were generated using this GAL4/UAS system with dnd as the target gene. We have shown that the expression of dnd mRNA was significantly suppressed, resulting in disrupted migration of the PGCs and their apoptosis in the hybrid embryos. This process led either to sterility (TG3-1) or to poor reproductive ability (TG3-2) in adults. Our novel strategy (which ensures that the parental transgenic generation remains fertile while their hybrid offspring become sterile) can effectively maintain the valuable traits of transgenic fish while avoiding issues of ecological risk via the production of sterile offspring.

The fact that both sterile individuals (TG3-1) and individuals with poor reproductive ability (TG3-2) appeared in the TG3 line is likely associated with the methylation of UAS sequence. Genes of interest driven by 5' UAS in the Gal4/UAS system showed strong expression in stable transgenic zebrafish lines. However, repetitive sequences attract methylation. Li and colleagues reported the methylation of 5' UAS in a human cell culture system. Moreover, Engineer and colleagues found transcriptional silencing of genes.
driven by 5′ UAS in transgenic Arabidopsis, and they suggested that the silencing is due to methylation, possibly at the level of 5′ UAS. In zebrafish, transcriptional repression was correlated with the CpG methylation of 14′ UAS, and single insertions containing 4′ UAS were found to be far less susceptible to methylation than insertions containing 14′ UAS. In our study, we detected the methylation of 5′ UAS in TG3 transgenic zebrafish according to a reported method. Methylation of the 5′ UAS-E1b sequence appeared in the gonads of the TG3 line, and there was significantly less methylation of the 5′ UAS-E1b sequence in the TG3-1 individuals than in the TG3-2 individuals. Accordingly, the level of endogenous dnd mRNA was significantly lower in the gonads of the TG3-1 line compared with the TG3-2 line. Moreover, the gal4 gene driven by the CMV promoter was found to be effectively expressed in the gonads of TG3 transgenic fish and the level of gal4 mRNA did not differ between the TG3-1 and TG3-2 lines; further, GFP or RFP under the control of the CMV promoter was strongly expressed in the next generation (F5) of these transgenic lines (see Supplementary Fig. 2a). These findings indicate that the CMV promoter is active in transgenic zebrafish. The mosaic expression of the egfp gene regu-

**Table 1 | Body weight, gonad somatic index (GSI), fertilization and relative fecundity of WT, TG3-1, and TG3-2 fish.**

|          | Weight (g) | GSI       | Fertilization rate (%) | Relative fecundity |
|----------|------------|-----------|------------------------|--------------------|
| WT (♂, n = 10) | 0.2886 ± 0.067 | 1.13 ± 0.13 | 89.27 ± 5.00          | --                 |
| TG3-1 (♂, n = 10) | 0.2681 ± 0.03 | 0.41 ± 0.04** | 0                      | --                 |
| TG3-2 (♂, n = 10) | 0.2729 ± 0.09 | 0.65 ± 0.17** | 42.37 ± 19.97**       | --                 |
| WT (♀, n = 10) | 0.3674 ± 0.07 | 10.04 ± 2.74 | 89.27 ± 5.00          | 2519 ± 225         |
| TG3-1 (♀, n = 10) | 0.3661 ± 0.065 | 5.43 ± 0.43** | 80.01 ± 12.34         | 1035 ± 441**       |
| TG3-2 (♀, n = 10) | 0.3734 ± 0.07 | 7.38 ± 1.16** | 80.01 ± 12.34         | 1035 ± 441**       |

*Figure 3 | Validation of the migration, number and apoptosis of PGCs in the TG5 and the WT embryos. (a) Schematic of the expression gene cassette of the plasmids used to establish the TG4 line. (b) A part of PGCs labeled by GFP (red arrows) migrated abnormally or lagged behind in the TG5 embryos at 10 hpf and 13 hpf, compared with the WT embryos. (c) The number of PGCs decreased in the TG5 embryos at 20 hpf and 24 hpf compared with the WT embryos. The red arrow indicates the location of PGCs. (d) WT embryos contained approximately four times the number of PGCs as the TG5 embryos (detected by GFP fluorescence intensity of PGCs at 24 hpf (n = 10). (e) Apoptosis of some PGCs was detected (red arrows) within some of the TG5 embryos. (Scale bars: 100 μm).**
levels, and the methylation level of the promoter is also known to differ between different cells from the same tissue. This suggests that the patterns of methylation in zebrafish might be similar to mammals. Germ cells from different individuals or from the same individual might exhibit different levels of UAS methylation leading to different levels of mosaic expression of antisense dnd RNA. These differences could generate variation in the numbers of germ cells undergoing apoptosis in the gonads of the TG3 individuals, resulting in TG3 embryos that develop into adults with different reproductive capacities. To our knowledge, this is the first time methylation of 5 × UAS has been identified in zebrafish, so these findings may prove useful for research on the regulation of gene expression with the Gal4/UAS system in zebrafish.

PGCs in zebrafish are located at the blastoderm layer and close to the yolk syncytial layer (YSL) and start to migrate at the dome embryonic stage. By the shield stage, PGCs have migrated close to the dorso axis at which point they begin to cluster. By the 8-somite stage, the PGCs have gathered into two clusters distributed symmetrically on both sides of the dorso axis. In our study, some PGCs remained far from the YSL in the majority of the TG3 embryos at 4.3 hpf, which suggested that dnd expression had been inhibited by the antisense dnd RNA. Continuous transcription of the antisense dnd disrupted the migration of the majority of PGCs at 10 hpf and 13 hpf. We then observed that the PGCs underwent apoptosis at 20 hpf and 24 hpf, resulting in few or no PGCs migrating to the genital ridge. Similar results have been reported to occur when dnd-MO was injected into zebrafish embryos, resulting in a reduction or even disappearance of PGCs in the embryo stage and males that were sterile or exhibited very low reproductive capacity. In general, fish fry containing no or few PGCs develop into males. For example, when the number of germ cells was reduced in zivi mutants, the fish developed into sterile males. In addition, transplantation of single PGC into embryos with no PGCs created male adults with a unilateral gonad. However, in our study, it was interesting to note that the TG3 embryos developed into sterile and low reproductive adults. In zebrafish, dnd is a maternal gene and its expression can also be detected in the gonads of adult fish (see Fig. 2a, Fig. 5e, f). In MO-injected embryos, this inhibitory effect is strongest upon injection with MO and grows gradually weaker due to the degradation of MO, dnd mRNA level decreases rapidly after fertilization (see Fig. 2a). Thus almost no PGCs migrate to the genital ridge and all the fry develop into males. However, in our study, transcription of the gene (regulated by UAS) became gradually stronger when induced by GAL4 (see Supplementary Fig. 2b), which could explain the lower levels of dnd mRNA at the 50%-epiboly and 3-somite stages, but not at the 1-cell, 1k-cell and oblong stages. Additionally, the mosaic expression of the UAS-regulated gene appeared in our research, another factor that could result in the successful migration of some PGCs to the genital ridge. Indeed, the presence of some PGCs is essential for female differentiation in zebrafish, and explains why some females appeared in the TG3 line. Our results also suggest that dnd might play an important role in the survival of germ cells in adult zebrafish.

The mechanism by which the number of germ cells affects sex differentiation remains unknown in zebrafish. Based on the histological features of their gonads, zebrafish are thought to be a bony fish that exhibits a protogynous hermaphrodite at the larval stage. ‘Juvenile ovaries’ are formed in all fries at 10–14 days post-fertilization (dpf), and oocyte apoptosis, which marks the transformation of ‘juvenile ovaries’ into testes occurs in some individuals at 21 dpf. In zivi mutant zebrafish, the number of germ cells has been reported to decrease rapidly or disappear before 21 dpf, such that all fry develop into sterile males. In zebrafish, the mutation of fns1 causes apoptosis of the oocytes that are undergoing meiosis and leads to female-to-male sex reversal. Wong et al. (2011) transplanted ovarian germ cells into the 5 × UAS occurred not only in the larvae but also in the gonads of adults and was variable among individuals. The mosaic expression of UAS-regulated genes has also been reported by other researchers, and similarly attributed to the methylation of the UAS. The mosaic expression of the UAS-regulated gene is even known to occur in stable transgenic lines. Different tissues and different cells from the same tissue have different methylation levels, and the methylation level of the promoter is also known to vary during an organism’s development. In mammals, DNA methylation levels differ among individuals and even in different cells. In humans, meanwhile, almost every sperm cell has its own unique pattern of methylation. We surmised that the patterns of methylation in zebrafish might be similar to mammals.
cells (N = 12 ± 4.7) obtained from three-month-old adults into two-week-old sterile larva and reported that the larva developed into both males and females, of which a few males had low fertility levels while all the females were sterile. In our study, some sterile and low reproductive female adults also appeared in the TG3 line. According to the above lines of evidence, we hypothesized that when the reduction of germ cells occurs before sex differentiation, more individuals differentiate into males. When the reduction of germ cells occurs after sex differentiation, then if the germ cells are absent female-to-male sex reversal transpires. However, the presence of just a few germ cells in the ovaries was evidently enough to maintain the female characteristics and not trigger female-to-male sex reversal. The reasons for this might be that the process of de-differentiation (and re-differentiation) in specialized cells of the gonad tissues (such as sertoli cells in testis and granulosa cells in ovarian) is difficult.

Our research has shown that antisense dnd RNA regulated by 5 × UAS can inhibit the expression of endogenous dnd. We observed that this process was affected by the methylation of UAS and resulted in partial sterility of the adults. However, we expect that the use of gene editing tools such as TALEN or CRISPR/Cas9 to refine the strategy implemented in this study (namely, to induce sterility in the offspring of two viable transgenic parental lines) will ensure that all hybrid offspring are sterile. This is because these tools enforce complete knockout of the dnd gene, allowing for the establishment of the reproductive switch in fish.

The novel reproductive containment strategy we have developed in this study will be of great use in controlling the reproduction capacity of introduced fish. The introduction of exotic species is a traditional way of obtaining fish with valuable traits for aquaculture, but this process can cause extensive economic and environmental harm. For example, it is estimated that up to 5.4 billion dollars are lost annually in USA due to the negative impacts of exotic fish. Another notable example is the extent of environmental damage caused by the introduction of the Asian carp in the USA. Recently, Thresher et al. (2014) reported a successful means of controlling invasive fish by reducing the effective population size of females. The controllable on-off strategy for the reproductive containment of fish that we have developed provides a novel means of controlling the ecological and economic risks inherent to fish introductions.
TUNEL cell death assay of the gonadal tissues. Gonadal tissues were dissected from 4.5 month-old TG3 fish and WT fish and embedded in Optimal Cutting Temperature (O.C.T. SAKURA). The samples were sectioned at a width of 7 μm using a freezing microtome. The TUNEL cell death assay was performed using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instructions. Images were observed under a fluorescence (Olympus MVX10) and captured using a digital camera (Nikon, MS-SCM) controlled by ACT-2U software (Nikon) after hematoxylin re-dying.

DNA bisulfite sequencing. Gonadal tissues were dissected from 4.5 month-old TG3 adults (TG3-1 males and females, TG3-2 males and females). Each sample contained six gonads of TG3 fish. The methylated DNA was extracted using a genomic DNA kit (Tiangen). The 5′-XAS-Elb sequence was amplified using the primers 5′-TTTTTTTTATAGTTTGTGTGTTG-3′ and 5′-TAAAAATTTCATCAATCAACATTCC-3′. The PCR products were cloned into a PMD-18T vector (TAKARA) and ten clones from each sample were sequenced. The percentage of methylation of all sites (CpG n = 12) was calculated in each sample.

Statistical analyses and image acquisition. T-tests were used to assess the significance of differences between the TG3 line and WT line in the following metrics: quantitative PCR data, body weight, GSI, fertilization rate and fecundity; t-tests were also used to assess the significance of differences between the TG5 line and WT line in fluorescence intensity and between TG3-1 and TG3-2 in quantitative PCR data, body weight, GSI and bisulfate. Images were taken and observation of the embryos and slices was performed using a fluorescence (Olympus MVX10) and a confocal microscope LSM 710 (Karl Zeiss). The brightness and contrast of the images were processed using Adobe Photoshop (Adobe Systems, USA).
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Acknowledgments

We greatly appreciate Mrs. Ming Li for raising the zebrafish. This work was supported financially by the “863” High Technology Project [grant number 2011AA100404], the National Natural Science Foundation [grant number 31325026] and the Key Research Program of the Chinese Academy of Sciences [grant numbers XDA08010106, KSCX2-EW-N-004, 2011FBZ19].

Author contributions

W.H., Z.Z., Y.Z. and I.D. conceived and designed research. Y.Z. and X.C. contributed to the construction of DNA constructs. H.X. conducted HE staining. Y.Z. and W.H. performed experiments, prepared figures 1–5, modified methods and analyzed data. D.L. contributed to the sorting of images. Y.Z., W.H. and J.C. wrote the manuscript. All authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhang, Y. et al. A controllable on-off strategy for the reproductive containment of fish. Sci. Rep. 5, 7614; DOI:10.1038/srep07614 (2015).

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