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Novel method for mass producing genetically sterile fish from surrogate broodstock via spermatogonial transplantation

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Abstract

A stable system for producing sterile domesticated fish is required to prevent genetic contamination to native populations caused by aquaculture escapees. The objective of this study was to develop a system to mass produce stock for aquaculture that are genetically sterile by surrogate broodstock via spermatogonial transplantation (SGTP). We previously discovered that female medaka carrying mutations on the follicle-stimulating hormone receptor (*fshr*) gene become sterile. In this study, we demonstrated that sterile hybrid recipient females that received spermatogonia isolated from sex-reversed XX males (*fshr* (-/-)) recovered their fertility and produced only donor-derived *fshr* (-) X eggs. Natural mating between these females and *fshr* (-/-) sex-reversed XX males successfully produced large numbers of sterile *fshr* (-/-) female offspring. In conclusion, we established a new strategy for efficient and mass production of sterile fish. This system can be applied to any aquaculture species for which SGTP and methods for producing sterile recipients can be established.

Keywords : Spermatogonial transplantation, germline stem cells, sterile fish production, fshr mutants

Introduction

Global aquaculture production has grown steadily and recently reached 106 million tons, accounting for more than half of the total of aquatic production, including wild capture [1]. In response to growing demand for seafood production, selective breeding technology is already in widespread use for aquaculture species. Indeed, many domesticated fish are now farmed worldwide [2]. However, most aquafarming activities using open sea cages carry a concern about escapees from aquaculture sites then polluting the gene pool of wild populations because the domesticated fish are genetically distinct owing to the modification to incorporate commercially valuable phenotypic characters. The escapees can possibly compete for ecological niche and even hybridize with wild counterparts. Hence, this kind of escapee-induced ecological disruption must be avoided as soon as possible in order to protect wild fish populations [3]. Further, a genetically modified (GM) Atlantic salmon was recently approved as seafood by the US Food and Drug Administration (FDA) and is being sold in the Canadian market [4]. Therefore, it is imperative to quickly establish reliable biological containment system of GM fishes.

Obviously, one of the promising methods to prevent above-mentioned escapee problems is sterilization of the farmed fish. Traditionally, triploidization techniques were developed in several farmed fish species to produce functionally sterile fish by inducing a meiotic disorder. However, triploid fish show lower early survival, and triploidization treatment is not always 100% effective, resulting in production of unwanted fertile diploids [5].

More recent strategies have implemented molecular biology-based techniques to modify germ cells in fish. In an attempt to ablate germ cells, delivery of antisense morpholino oligonucleotide (MO) into fertilized eggs generates germ cell-ablated fish by knocking down (KD) the function of *dead end* (*dnd*), a gene essential for germ cell survival [6-8]. However, a method requiring one-by-one microinjection is not practically applicable for industrial-based seedling production in hatcheries. Genome editing techniques have become a powerful tool for modifying target gene sequences for controlling fertility. Wargelius *et al*. [9] applied the CRISPR-Cas9 system to knockout (KO) the *dnd* gene in salmon, suggesting the possibility of germ cell-free salmon in which biallelic mutations are found in the founder generation. Although similar KO fish can be produced by mating heterogenic males and females, subsequent genotyping is necessary to eliminate the heterogenic mutants and fish lacking the target mutation. From the standpoint of commercial aquaculture

production, Wong and Zohar [10] recently proposed a bath immersion method with a *Vivo*-conjugated MO against *dnd* gene [11] that induced sterility in zebrafish. Nevertheless, the most crucial limitation of these methodologies is that once a perfect sterile fish is generated, these fish will never pass their sterile characteristics to offspring.

Therefore, a new technique is still required in order to establish a strategy for mass producing sterile fish. Here, we designed and established a new strategy for the mass production of genetically sterile fish by the surrogate broodstock system [12] via spermatogonial transplantation (SGTP) [13] using medaka, *Oryzias latipes*, as a model species (Fig. 1). Previously, Murozumi *et al*. [14] generated a follicle-stimulating hormone receptor (*fshr*) KO mutant in medaka: the male *fshr* (-/-) displayed normal testicular development with complete fertility, whereas the female *fshr* (-/-) had sterile ovary in which vitellogenesis was inhibited due to loss of reception to Fsh signal (Fig. 1A).

In other words, if aquaculture fish stocks that are all female *fshr* (-/-) can be produced, they are expected to all be sterile. However, it is theoretically impossible to mate *fshr* (-/-) parents because female *fshr* (-/-) cannot produce any eggs. Since *fshr* is expressed only in ovarian somatic cells and not in germ cells, we expected that transplanting *fshr* (-/-) germ cells to wild type female recipients carrying normal Fshr would cause the recipient ovary to nurse the donor-derived *fshr* (-/-) germ cells and to produce *fshr* (-) eggs. Further, in order to obtain all female offspring, masculinized XX-males could be mated with recipients that received spermatogonia isolated from XX sex-reversed males (Fig. 1B). Other candidate donor cells, oogonia carrying XX sex chromosomes, do not contain as many transplantable germ-line stem cells as spermatogonia [15] and are not suitable for germ cell transplantation in small fish species such as medaka, which has small gonads.

Further, by using sterile hybrids as recipients, the resulting recipients can only produce donor-derived *fshr* (-) eggs but not recipient-derived wild type eggs (Fig. 1B). In this study, sterile hybrid fish [16] were used as recipients, and the recipients that received XX and *fshr* (-/-) spermatogonia were examined to determine whether they only produce the expected donor-derived X eggs with the nonsense mutation on the *fshr* gene (Fig. 1C).

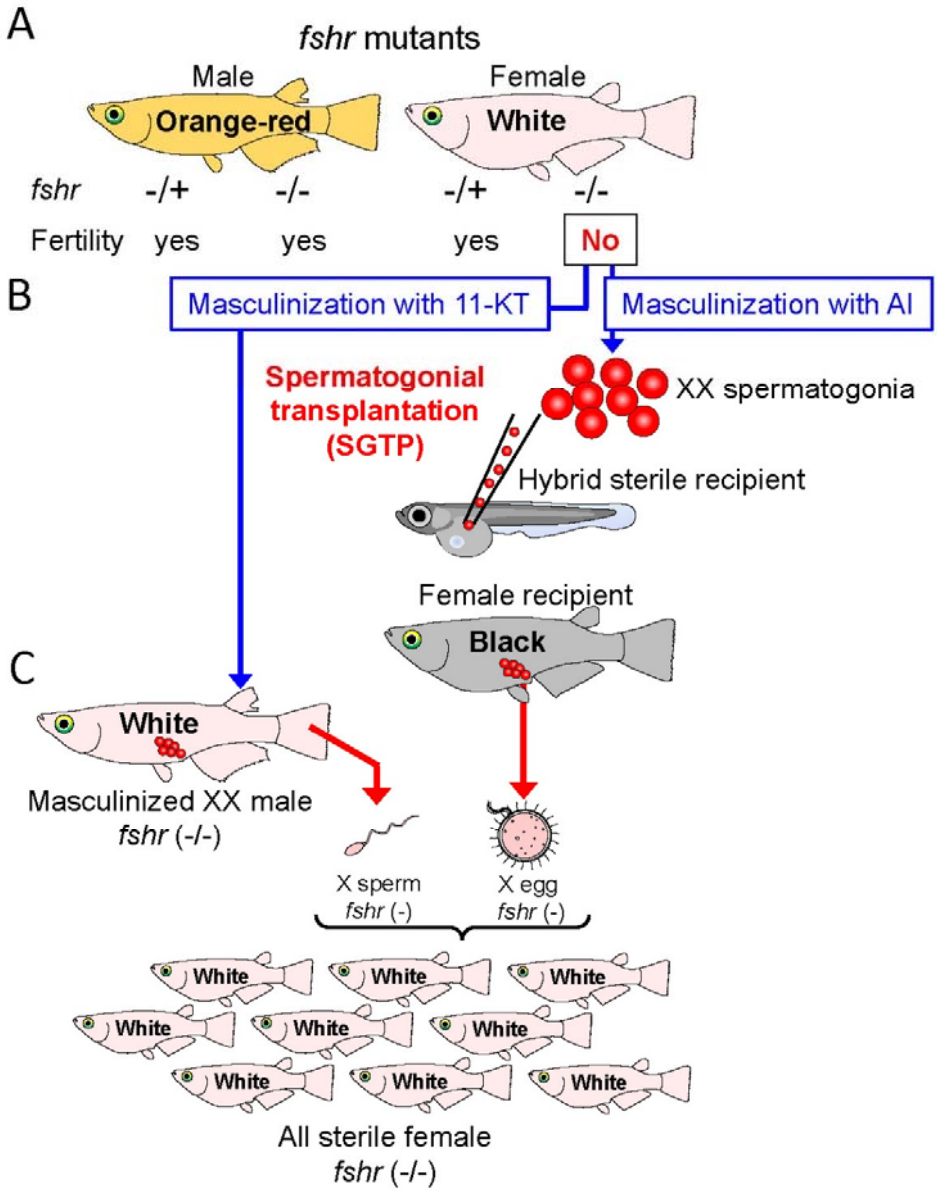

Fig. 1

Fig. 1. Schematic representation of the production strategy of all sterile offspring by surrogate broodstock. A) Among all follicle stimulation hormone receptor (*fshr*) mutants, only the homozygotic (-/-) female mutants are sterile [14]. Since the *fshr*-mutant strain was established on the genetic background of the Hd-rR strain, females display white body color [20]. *fshr* (-/-) females with white body color were selected and used as donor fish for spermatogonia transplantation (SGTP). B) The female *fshr* (-/-) mutants were masculinized by aromatase inhibitor (AI) treatment. The obtained testicular germ cells containing XX spermatogonia (red cells) were transplanted into the peritoneal cavity of hybrid sterile recipients. Since the hybrid sterile recipients exhibited abnormalities in meiotic division of their endogenous germ cells [25, 26], the donor-derived germ cells can dominate and differentiate into functional gametes depending on the recipient sex. Meanwhile, the XX male *fshr* (-/-) mutants were generated by treatment with 11-ketotestoasterone (11-KT) to be used for the subsequent mating tests. C) The masculinized XX male *fshr* (-/-) mutants only produced X sperm possessing the *fshr* mutation. By mating this masculinized XX male with female recipients that possess donor-derived XX *fshr* (-/-) germ cells, all resulting F1 offspring are XX female *fshr* (-/-) mutants, which are all sterile.

Materials and Methods

Fish

olvas-gfp transgenic medaka [17] was kindly provided by Dr. Minoru Tanaka (Nagoya University, Japan) and used as a donor. In addition, *fshr*-mutant fish obtained from a medaka TILLING library [14] used as a donor possessed a nonsense mutation (R631X) resulting in a truncation of the BXXBB motif for G protein activation and a carboxyl terminus (amino acids 632-687). For hybrid recipient preparation, wild type *Oryzias latipes* were purchased from a local supplier (Tochigi, Japan), while *Oryzias curvinotus* (Hong Kong, RS269) were kindly provided by Dr. Sakaizumi of Niigata University (Niigata, Japan) via The National BioResource Project-Medaka (NBRP-Medaka) [18]. The fish used in the present study were reared in dechlorinated water at 25 ± 1°C under a light-dark cycle (LD 14:10) at the Tokyo University of Marine Science and Technology (TUMSAT, Shinagawa, Japan). All rearing methods for both adult fish and larvae were performed with the experimental protocols specialized for medaka [19]. All experiments were carried out in accordance with the guide for the care and use of laboratory animals from TUMSAT.

Preparation of masculinized XX males from *fshr* (-/-) mutants as donors

For donor preparation, the embryos of *fshr* (-/-) mutants were obtained by natural mating between the female *fshr* (+/-) and male *fshr* (-/-) mutants. To produce masculinized XX males from *fshr* (-/-) mutants, hatched larvae were fed dry feed containing an aromatase inhibitor (AI, namely Letrozol, Tokyo Chemical Industry, Tokyo, Japan) at 200 μg/g diet for one month. After one month feeding of AI, fish were fed brine shrimp. Since the *fshr*-mutant strain was established on the genetic background of the Hd-rR strain, the female mutant X^rX^r has a white body color, and the mutant male X^rY^R has an orange-red body color [20]. Hence, we were able to screen the *fshr* mutants to find the masculinized XX males by checking first for white body color and secondly, male characteristics, namely the shape of the dorsal and anal fins. Then, genotypes of each masculinized XX male of *fshr* mutants were analyzed by the Surveyor mutation detection assay and genomic PCR detection of the *dmy* gene as detailed below. For mating tests, the masculinized XX male *fshr* (-/-) mutants were generated by treatment with an androgen as reported [19]. In brief, fertilized eggs were incubated in embryo culture medium containing 11-ketotestosterone (11-KT, Cosmo Bio, Tokyo, Japan) at 50-250 ng/ml until hatching. The medium was changed daily to maintain an optimal concentration of 11-KT.

Preparation of the sterile hybrids to be used as recipients

Through natural mating, we prepared F_1 hybrids from reciprocal crosses between *O. latipes* and *O. curvinotus* that exhibit abnormalities in gametogenesis—the males are sterile but the females rarely lay a very few diploid eggs [16]. The collected embryos were reared in a 9 cm Petri dish filled with tap water at 26°C until hatching under a light-dark cycle (LD 14:10) and subsequently used for the SGTP.

Spermatogonia transplantation (SGTP)

Intraperitoneal transplantation of medaka testicular cells was carried out based on the methodology established [21]. In detail, testicular cell suspensions were prepared from 2-month-old homozygous *olvas-gfp* (20 mm total length, TL) or from 3-month-old masculinized XX male *fshr* (-/-) mutant (28 mm TL). Freshly isolated testis were minced and incubated with 0.5 ml of Leiboviz's L-15 medium (pH 7.8) with 10% FBS containing 2 mg/ml collagenase H (Roche, Mannheim, Germany), 1.7 mg/ml dispase II (Godo-Shuzo, Tokyo, Japan) and 450 Units/ml DNase I (Sigma-Aldrich, Tokyo, Japan) for 1 hour at 26°C. During the incubation, gentle pipetting 50 times was conducted every 30 min to aid cell dispersion. The cell suspension was centrifuged at 100 × *g* for 5 min, washed once with L-15/10% FBS medium, filtered through a 42-μm pore size nylon screen to eliminate undissociated cell clumps and then re-suspended in 20 μl of L-15/10% FBS medium. An aliquot of approximately 60 nl of cell suspension, containing ~15,000 cells, was intraperitoneally transplanted into newly hatched fry. For tracking donor cells after SGTP, dissociated testicular cells were stained with fluorescent dye PKH26 (Sigma-Aldrich) as described previously [22].

Surveyor mutation detection assay for the mutated *fshr* genome

In order to distinguish the genotype of *fshr* mutants (e.g., *fshr* (-/-), (+/-) or (+/+)), Surveyor mutation detection was performed. The single-nucleotide nonsense mutation on the *fshr* exon 14 (R631X) [14] was detected using the IDT Surveyor Mutation Detection Kit for Standard Gel Electrophoresis (Integrated DNA Technologies, Coralville, IA) as described elsewhere [23]. First, genomic DNA was extracted from a clipped dorsal fin using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Then, the genomic DNA fragment containing the target mutation (described above) was amplified by PCR with a primer set producing an amplicon of 396 bp (Fw-*fshr*,

ATCTACTTGACCTACCGCAAGC and Rv-*fshr*, ACTGAGAGTATTGGCCAAGGAG). PCR was performed with Ex Taq (TaKaRa Bio, Shiga, Japan) at 95°C for 3 min, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, followed by a final elongation step of 72°C for 3 min. Second, for DNA duplex formation, the PCR product was hybridized with itself or with PCR products amplified from wild type genomic DNA according to the protocol in the Surveyor Kits manual. Third, hetero/homoduplex DNA samples were incubated with Surveyor Enhancer S and Surveyor nuclease S at 42°C for 60 min to specifically cleave the heteroduplex region. Fourth, duplex DNA treated with Surveyor nuclease was analyzed on a 2% TBE agarose gel. For example, heteroduplex amplicons were formed by hybridizing the *fshr* (-/-) amplicon with the wild type amplicon or by hybridizing the *fshr* (+/-) amplicon with itself. This heteroduplex amplicon should be cleaved into two DNA fragments (150 and 246 bp). Meanwhile, homoduplex amplicons were formed by hybridizing the *fshr* (-/-) amplicon with itself or by hybridizing the wild type amplicon with itself. These homoduplexes should not be cleaved and remain as intact amplicons (396 bps).

Genotyping for the *dmy* gene

The genetic sex of each fish was confirmed by detecting *dmy* located on the Y chromosome [24] (NCBI accession number; AY129241.1). The specific primer set for *PG17/dmy* was redesigned and produced an amplicon of 988 bp (Fw-DMY, CCTGAAGTGGTGGTGAAGAATGAAG and Rv-DMY, ACGGTACCTGGTACTGCTGGTAGTTG). A specific primer set for *Actb* (NCBI accession number; NC_019866.1) was used as an internal control (Fw-actb, ACTACCTCATGAAGATCCTG and Rv-actb, TTGCTGATCCACATCTGCTG; amplicon length of 671 bp). The PCR was performed with Ex Taq (TaKaRa Bio) and a thermal cycle with 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C (65°C for *actb*), and 90 s (30 s for *actb*) at 72°C, followed by a final elongation step of 72°C for 3 min. PCR products were electrophoresed on 0.7% TBE agarose gel (2% gel for *actb*).

Histology

Gonads were fixed with Bouin's solution at 4°C for 12 hours, embedded in paraffin wax and then sliced into 5-μm-thick sections. Paraffin sections were stained with hematoxylin and eosin (HE) for morphological observation of gonad development.

Data Availability

Strains used are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures and tables.

Results

Sterile hybrid recipients can produce functional gametes from donor-derived germ cells

Because triploid recipient production in medaka has unreliable success and a risk for the production of diploid individuals, we first chose hybrid recipients for SGTP as alternatives to triploids in this study. To confirm the potential of hybrid recipients as surrogate broodstock, we examined whether their gonads can support gametogenesis of donor-derived germ cells after SGTP. For hybrid recipients, we chose hybrids from reciprocal crosses between *Oryzias latipes* and *Oryzias curvinotus* that exhibit abnormalities in gametogenesis of endogenous germ cells [16, 25, 26]. For donor germ cells, we used testicular cells isolated from *olvas-gfp* transgenic medaka (Figs. 2A, B). These cells were transplanted into the peritoneal cavity of hybrid recipients (Fig. 2C). Ten days after SGTP, the colonized donor germ cells were easily identified by GFP fluorescence inside the immature gonad of the hybrid recipient, *O. latipes* ♀ × *O. curvinotus* ♂ (Fig. 2D). At 3 months after the SGTP, the female recipients (*O. latipes* ♀ × *O. curvinotus* ♂) matured and naturally spawned eggs, while no females holding eggs were found in control recipients without SGTP (Table 1). In particular for recipients with SGTP, spawning females had gonads filled with GFP-positive ovarian germ cells (Figs. 2E, e). In contrast, the ovaries of females in the control group remained immature (Fig. 2F), even though the ovaries of wild type females at same age matured with fully vitellogenic oocytes (Fig. 2f).

The sex ratio of hybrid recipients was biased to female in both combinations of reciprocal crosses between *O. latipes* and *O. curvinotus* (Table 1). In addition, SGTP did not affect the sex ratio of hybrid recipients. As a result, we were not able to produce male recipients in enough numbers to find any colonized GFP-positive germ cells in their testes. Therefore, spawning females with SGTP were mated with wild type males to confirm whether the donor-derived eggs were functional or not, and normal numbers of fertilized eggs were obtained by natural mating (Fig. 2G). These eggs were all GFP-positive (Figs. 2H, h) and the developed larvae possessed GFP-positive germ cells in the presumptive gonadal area (Figs. 2I, i). Since no difference was observed in the efficiency of SGTP for female recipients from reciprocal crosses between *O. latipes* and *O. curvinotus* (Table 1), we chose hybrid recipients (*O. latipes* ♀ × *O. curvinotus* ♂) for the rest of this study.

Table 1. Spermatogonia transplantation with sterile hybrid recipients of medaka.

Exp.*	SGTP†	Hybrid type of recipients‡	Donors§	Injected cells¶	No. of recipients#	Sex ratio of recipients (F:M)	No. of spawning females‖
No. 1	TP	*O. latipes* ♀ × *O. curvinotus* ♂	XY male *olvas-gfp*	1.2×10^4	19	14:5	7 (50%)
	Non-TP		-	-	27	24:3	0
No. 2	TP	*O. curvinotus* ♀ × *O. latipes* ♂	XY male *olvas-gfp*	1.2×10^4	20	15:5	7 (47%)
	Non-TP		-	-	18	12:6	0
No. 3	TP	*O. curvinotus* ♀ × *O. latipes* ♂	XX male *fshr* (-/-)	9×10^3	38	26:12	5 (19%)
	Non-TP		-	-	23	17:6	0**
No. 4	TP	*O. latipes* ♀ × *O. curvinotus* ♂	XX male *fshr* (-/-)	3×10^3	28	25:3	10 (40%)
	Non-TP		-	-	18	14:4	0**
No. 5	TP		XX male *fshr* (-/-)	3×10^3	19	17:2	9 (53%)
	Non-TP		-	-	16	15:1	0

*Five SGTP experiments (No. 1-5) were conducted with two donor strains (No. 1-2 with *olvas-gfp* transgenics and No. 3-5 with *fshr* mutants).

†TP; the recipients that underwent SGTP, Non-TP; the recipients without SGTP (controls).

‡Each hybrid recipient was prepared by natural mating of reciprocal crosses between *O. latipes* and *O. curvinotus.*

§Donor testicular cell suspension was prepared from the testis of XY male *olvas-gfp* or the masculinized XX male *fshr* (-/-) fish.

¶Total number of injected testicular cells per recipient.

#Number of recipient embryos allocated to TP or non-TP groups.

‖Number of female recipients that naturally spawned eggs. The spawning females produced at least 10 eggs per day and repeated spawning almost every day. Double asterisks (**) in non-TP groups indicate that few females rarely spawned very few eggs (2-4 eggs/each). Efficiencies for successful production of the spawning females are bracketed (100×spawning females/female recipients that underwent SGTP (%)).

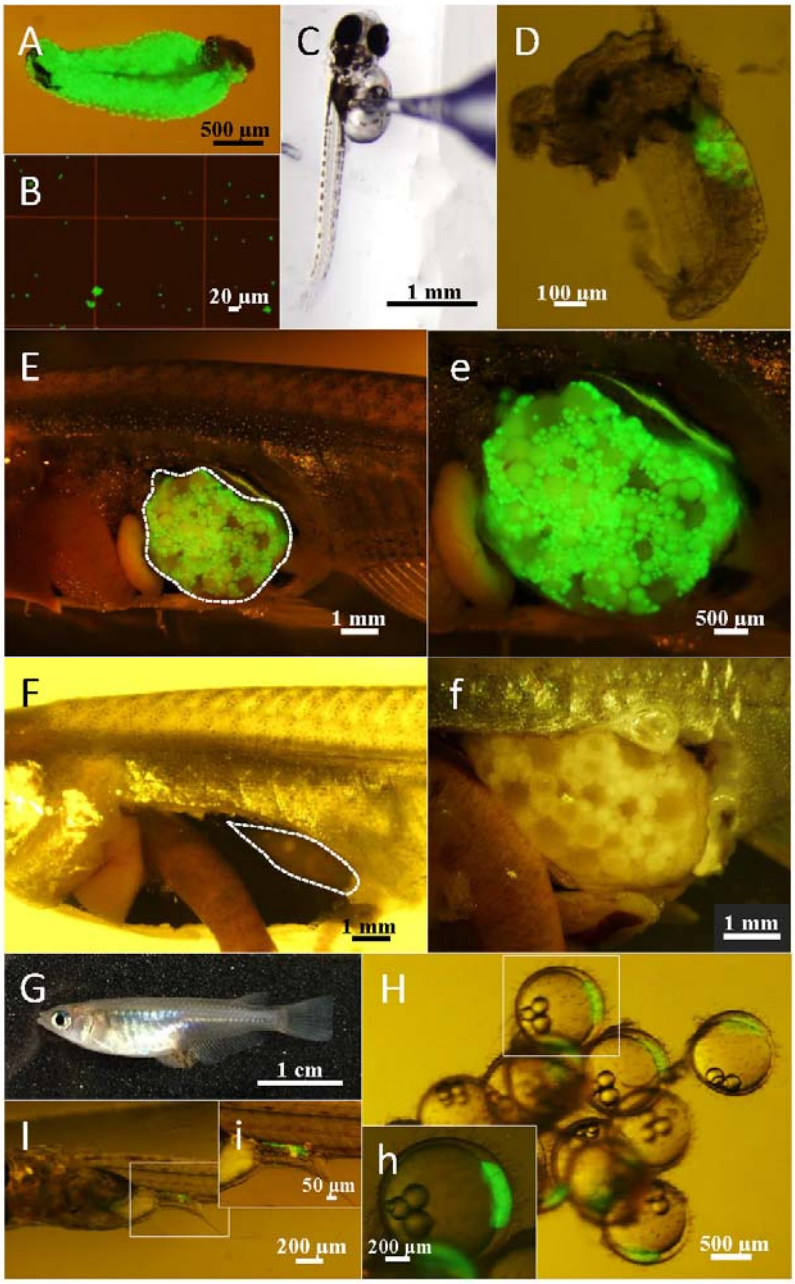

Fig. 2

Fig. 2. The hybrid sterile recipients supported the gametogenesis of donor-derived spermatogonia taken from *olvas-gfp* medaka. A) A donor testis excised from an *olvas-gfp* male at 2 months old had a total length of 20 mm. B) Testicular cell suspension after enzymatic dissociation. C) Intraperitoneal spermatogonia transplantation (SGTP) by glass needle into a hybrid recipient at hatching stage. D) Isolated gonad from the recipient that underwent SGTP at 20 days post-injection (dpi). Colonized and proliferated donor-derived germ cells are labeled by a green fluorescence protein (GFP). E) The donor-derived germ cells labeled with GFP developed as ovarian germ cells (e.g., oocytes) in a female recipient at 4 months old with a magnified image of the ovary (e'). F, f) Without SGTP, a female recipient showed sterile ovary at 4 months old (F, negative control), while a wild type female (*O. latipes*) showed mature ovary at 4 months old (f). G) A spawned female hybrid recipient that underwent SGTP. H) Spawned eggs obtained from the hybrid recipient by mating with a wild type male. All fertilized eggs show GFP fluorescence in the blastodisc with a magnified image of fertilized egg (h). I) A hatched larvae at 10 days post-fertilization (dpf) shows characteristics identical to the *olvas-gfp* donor fish with a magnified image of the presumptive gonadal region (i).

Masculinization of *fshr* (-/-) mutants using aromatase inhibitor treatment

To obtain XX spermatogonia with the homozygous mutation on the *fshr* gene, we performed masculinization with aromatase inhibitor (AI) treatment to *fshr* (-/-) mutants. Genetic sex was easily distinguished by body color (Figs. 3A-C) with XY males and XX females exhibiting orange-red and white body colors, respectively. After AI treatment, out of 40 offspring produced by mating between the female *fshr* (+/-) and male *fshr* (-/-) mutants, we identified three genetic females (Figs. 3C, no. 2-4 in Fig. 3E) that showed secondary sex characteristics of males (Figs. 3c, c'). These characters were not observed in normal XX females (Figs. 3D, d) but were observed in normal XY males (Figs. 3b, b'). Then, three masculinized XX males (no. 2-4) and one normal XY male (no. 1) were subjected to the Surveyor mutation detection assay to find the *fshr* (-/-) mutants. The Surveyor assay identified two fish that were *fshr* (-/-) mutants (no. 3 and 4 in Fig. 3E). In parallel, genotyping for the *dmy* gene confirmed that these *fshr* (-/-) mutants were genetically XX males (Fig. 3F). The testis at 2 months old (Fig. 3G) containing spermatogonia (Figs. 3I, i) were excised and used for enzymatic dissociation for further SGTP. At the same age, normal XX females possessed ovaries with developing oocytes (Fig. 3H). Donor testicular cells showed red fluorescence by PKH26, identifying them as dissociated testicular cells after SGTP (Fig. 3J). Testicular cells prepared from a single donor fish were sufficient for injections into approximately 40 recipients (Exp. 3-5 in Table 1).

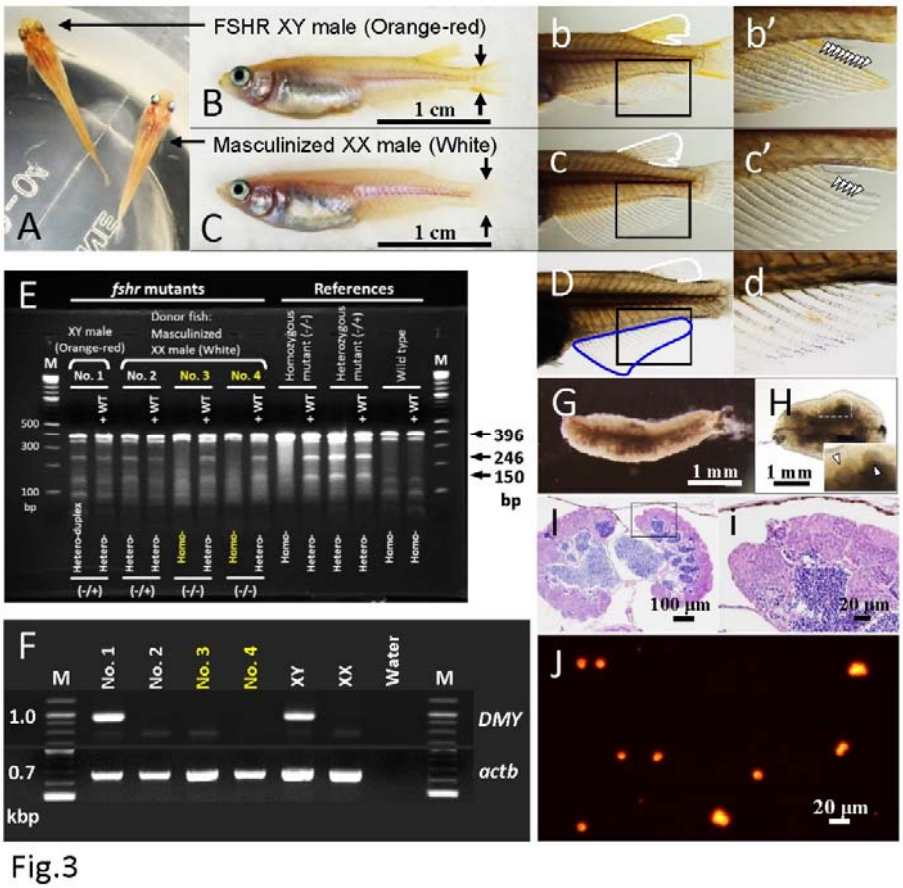

Fig.3

Fig. 3. Preparation of XX spermatogonia from *fshr* (-/-) mutants as donor cells. A) As donor fish, *fshr* (-/-) mutants have the genetic background of the Hd-rR strain [14]. This congenic strain has X^rY^R male resulting in an orange-red body color, and X^rX^r female resulting in a white body color [24]. Remarkably, orange-red lines are observed on the base of caudal fin of X^rY^R (arrows in B), while no color is seen in X^rX^r (arrows in c). Hence, genetic sex can be distinguished by body color. B) An XY male *fshr* (-/-) mutant with orange-red body color and its secondary characteristics of dorsal fin with a saw-toothed distal edge (outlined in white in b, c) and anal fin with small papillar processes on the posterior area (arrowheads in b', c'). C) A masculinized XX male *fshr* (-/-) mutant with white body color and its secondary characteristics

of the dorsal fin (c) and anal fin (c') displaying as male. D, d) In contrast, the female shows secondary characteristics on the dorsal fin with a smooth distal edge (outlined in white in D) and triangle shaped-anal fin without papillar process (outlined by blue in D and d). E) Surveyor mutation assay for detection of the mutated *fshr* allele. The amplicons of the mutated *fshr* genome appeared at 396 bp. CEL I nuclease recognizes and cleaves the hetero-duplex DNA in which the mutated nucleotide is positioned in the *fshr* genome. Homozygous mutant (-/-) and wild type DNA formed homo-duplex DNA resulting in no cleavages, while heterozygous mutant (+/-) DNA and homozygous mutant (-/-) DNA mixed with wild type DNA formed hetero-duplex DNA with cleavages that produced two short fragments (246 and 150 bp) as show in the references (right side). In the donor samples (*fshr* mutants, left side), the masculinized XX male *fshr* mutants (No. 2-4) were screened by the Surveyor assay and two fish (No. 3 and 4) were identified as the *fshr* (-/-) mutants to be used as donors for SGTP. M represents molecular weight marker (100-bp DNA ladder). Fragment sizes, in base pairs, are indicated on the right. F) Genotyping of the *dmy* gene identified the masculinized XX males from *fshr* (-/-) mutants with homo-duplex DNA as donors. *Actb* was used as an internal control for PCR amplification. G) An isolated testis from the masculinized XX male *fshr* (-/-) mutant at 3 months old had a total length of 28 mm. H) A typical ovary with growing oocytes (arrowheads in inset) isolated from non-treated 3 months old medaka. I) A cross section of the donor testis from masculinized *fshr* (-/-) mutant stained with hematoxylin and eosin (HE) and with a magnified image of the area enclosed in the rectangle in i. J) Dissociated testicular cells prepared from a masculinized XX male *fshr* (-/-) mutant are labeled with PKH26 prior to SGTP.

Surrogate broodstock produced all *fshr* (-/-) females in the F1 generation

In the 3 months after SGTP, several recipient females that received XX spermatogonia with the homozygous mutation on the *fshr* gene started to spawn eggs and were used for a mating test with masculinized XX males of *fshr* (-/-) mutants (Fig. 1C, Exp. 4 in Table 2). Fertilized eggs were obtained from several hybrid female recipients that underwent SGTP (Fig. 4A). The spawning female recipients had a developed ovary that contained vitellogenic oocytes (SGTP; Figs. 4B, b, D), while female recipients that did not undergo SGTP did not show gonadal development (Non-SGTP; Figs. 4C, E, e) even though the fish showed typical dorsal fin shape as female (Fig. 4c).

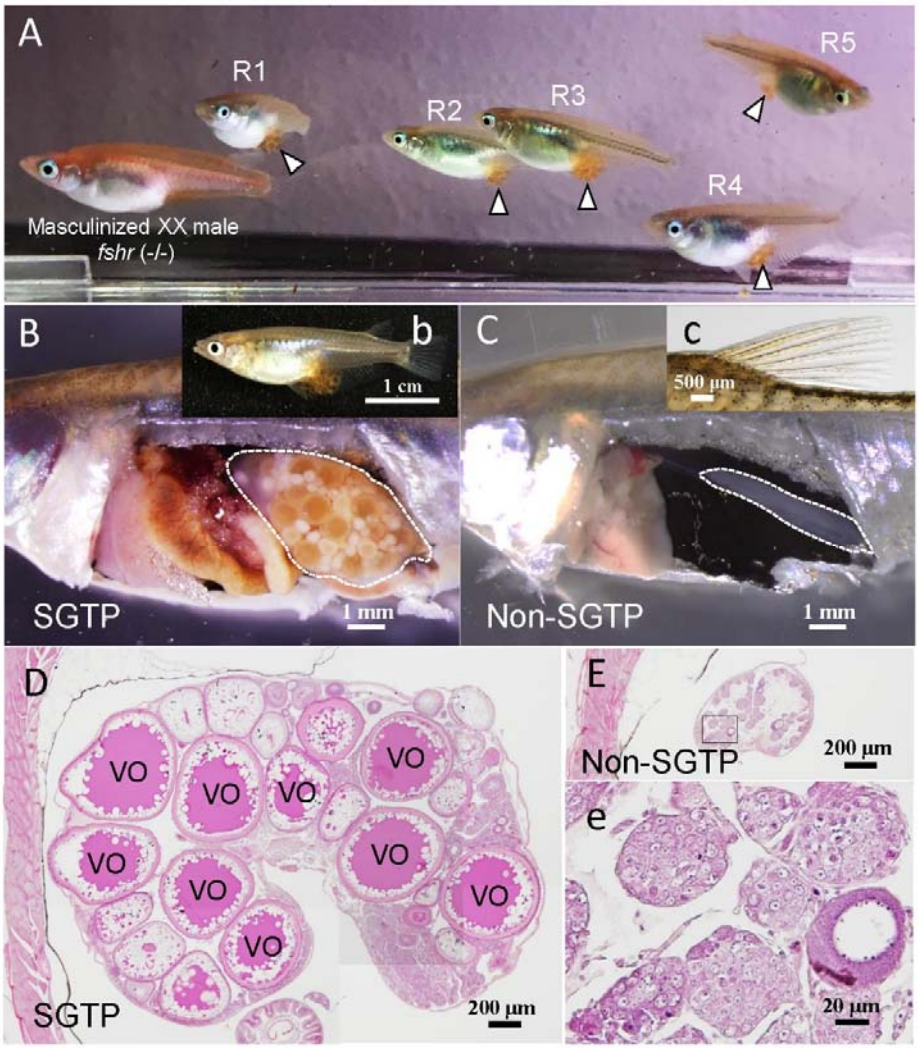

Fig. 4

Fig. 4. Surrogate broodstock that underwent SGTP with XX spermatogonia from *fshr* (-/-) mutants produced fertilized eggs by natural mating. A) Mating test between the mature female recipients (arrow heads, n=5) and the masculinized XX male *fshr* (-/-) mutants. B, C) Microscopic observations of ovaries by opening the abdominal cavity of the fish with SGTP (B, b) and without SGTP (C). Dashed line outlines the edge of the ovaries (B, C). C) The dorsal fin of the recipient without SGTP shows female secondary characteristics (c). D, E) Histological observations for the ovaries from fish with SGTP (D) and without SGTP (E) with a magnified image of the area enclosed in the rectangle in e.

Table 2. Mating test for female recipients.

Exp.*	No. of spawning females[†]	No. of mature males[‡] (XX male *fshr* (-/-))	Mating period[§]	No. of fertilized eggs[¶]	No. of triploid eggs[#]	No. of adults[‖]
No. 4	5	3	27 days	920	3	644

*Two mating tests were conducted for each hybrid recipient type from reciprocal crosses as described in Table 1.
[†]Number of spawning females subjected to the mating test in a fish tank.
[‡]Number of the mature males (i.e., masculinized XX male *fshr* (-/-) prepared by 11KT treatment) subjected to the mating test in a fish tank.
[§]Tests were performed every day within a period of time by natural mating.
[¶]Total egg number obtained during the mating test.
[#]Total number of triploid eggs obtained during the experimental period. The eggs showed relatively bigger diameter and hatching larvae displayed deep-black melanophores, which is a phenotype of the recipient.
[‖]Total number of adult fish obtained by mating tests.

Throughout the mating test, five hybrid female recipients (*O. latipes* ♀ × *O. curvinotus* ♂) produced more than 900 donor-derived fertilized eggs during a month (Exp. 4 in Table 2), and their offspring showed normal development and reached adult size at 3 to 4 months old (Fig. 5A). As a representative sample, we assessed the first batch of their offspring consisting of 20 adult fish (Figs. 5A-C) produced by hybrid female recipients (recipients; left panel in Fig. 5A, *O. latipes* ♀ × *O. curvinotus* ♂ from Exp. 4 in Table 2,). All 20 fish had white body color (offspring; right panel in Fig. 5A). The Surveyor assay revealed that the 20 fish were all *fshr* (-/-) mutants (Fig. 5B). In addition, genotyping of the *dmy* gene confirmed that the 20 fish were all XX females (Fig. 5C). The 20 fish showed retarded oogenesis (Figs. 5D, d) as observed in the female *fshr* (-/-) mutants (Figs. 5F, f). On the other hand, control female *fshr* (+/-) mutants at same age had mature ovaries containing many vitellogenic oocytes (Figs. 5E, e).

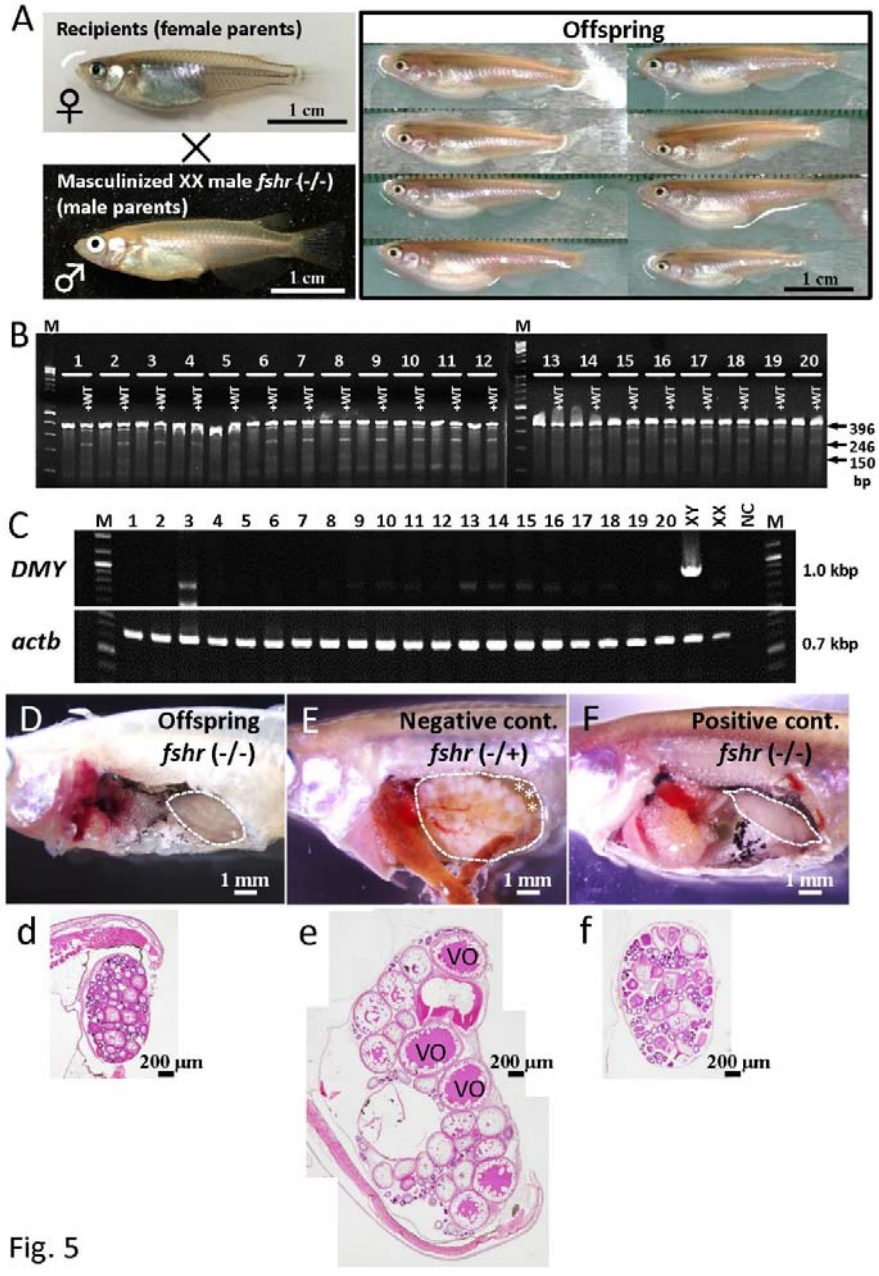

Fig. 5

Fig. 5. Analyses of offspring obtained by mating between recipient females and masculinized XX male *fshr* (-/-) mutants. A) Randomly selected offspring obtained by mating recipients that received XX spermatogonia from *fshr* (-/-) mutants and masculinized XX male *fshr* (-/-) mutants. B) Surveyor mutation detection assay for *fshr* mutation in the offspring genome (n=20). C) Genotyping of the *dmy* gene for offspring (n=20). D, E, F) Microscopic observations of ovaries and histological observation in typical *fshr* (-/-) offspring obtained by mating (D, d), heterogenic *fshr* (+/-) mutant as a

negative control (E, e) and a homogenic *fshr* (-/-) mutant as a positive control (F, f) that indicates exemplary gonad development. All fish were sampled as adults at 4.5 months old. Dashed line outlines the edge of the ovaries. Asterisks indicate vitellogenic oocytes. VO, vitellogenic oocytes.

Discussion

In order to allay concerns about genetic pollution caused by escapees from fish farms, we here developed a new strategy for the mass production of genetically sterile fish by the surrogate broodstock using SGTP [13]. To produce the genetically sterile fish population, we carried out SGTP using *fshr*-mutant donors and sterile hybrid recipients. The hybrid female recipients that underwent SGTP recovered fertility and produced only donor-derived eggs with the *fshr* mutation and successfully produced large numbers of sterile offspring in matings with sex-reversed XX males that also produced *fshr* (-) sperm (Fig. 6).

This surrogate broodstock system has various notable advantages for the efficient and mass production of sterile fish. First, as long as surrogate females producing *fshr*-mutant eggs and sex-reversed and *fshr*-mutated males are produced, these parental fish produce genetically sterile offspring by natural mating. In other words, when this technique is applied to aquaculture species, sterile fish stocks are produced through natural mating, or artificial insemination when needed, with these specially prepared parental stocks. Although the preparation of the parental stocks requires some biotechnological techniques (e.g., gene KO, sterilization of recipients, SG transplantation), these steps could be performed in laboratories that routinely perform them. Second, these parental fish are expected to continuously produce sterile offspring for their entire lifetimes. In this study, we also confirmed that the three female recipients that received *fshr* (-/-) spermatogonia were able to produce functional eggs throughout the entire experimental period. Importantly, at least eggs spawned by one female examined developed into sterile fish with 100% efficiency. Third, the sterile offspring developed with a normal survival rate since no chemical or physical treatments were conducted on the eggs or embryos, as is the case for triploid production [27], chemical sterilization such as with the use of alkylating drugs [28], or delivery of morpholino oligonucleotides [29]. Therefore, this surrogate broodstock system is well-suite for sterile seedling production at commercial scales. Particularly, it is expected that this technique is suitable for sterile fish production in marine fish species that generally exhibit high mortality during the early embryonic stage. Fourth, the resulting offspring produced by the method developed in this study were not genetically modified, making it possible to apply this technique directly for seafood production because *fshr*-mutant donor fish were generated by targeting-induced local lesions in genome (TILLING) [30], which is a well-established technique of breeding agricultural plants [31]. Taking these advantages into consideration, it is conceivable that this surrogate broodstock system can be a silver bullet for industrial-based mass production of sterile fish. In this study, due to the biased sex ratio of the recipient hybrid medaka, we mated the recipient females with sex-reversed XX males in order to produce all female offspring that are sterile. However, in some of the aquaculture species, it is known that sterile fish produced by triploidization [5] or *dnd* knock down [8, 32] show a 1:1 sex ratio, making it possible to use these sterilized fish as both male and female recipients for SGTP, which is simpler than procedures presented in this study.

In terms of the commercial use of sterile fish production, microinjection technique has enabled the delivery of various molecules for the ablation of germ cells from fish embryos of aquaculture species [8, 9]. However, this technique has limitations in that the processing quantity and control of quality are dependent on handling skill. To overcome these bottlenecks, Wong and Zohar [10] recently developed a bath immersion method with a *Vivo*-conjugated MO [11] against zebrafish *dead end* (*dnd*-MO-*Vivo*) that effectively causes miss-migration of primordial germ cells during the embryonic stage to induce sterility. This approach is very practical in commercial aquaculture since the immersion treatment can be used to process large numbers of fish eggs. However, the downside of this technique is that a scaling up of bath immersion method with a *Vivo*-MO is cost-prohibitive for seedling production in aquaculture. In addition, the immersion method (e.g., optimal temperature, time and concentration) for efficient delivery must be validated separately for each fish species.

To generate sterile fish, most previous studies have focused on the ablation of germ cells by knocking down a gene that is essential for germ cell survival or normal development [6-8]. Furthermore, the CRISPR-Cas9 system also made it possible to generate germ cell-free salmon [9]. In either case, however, these sterile phenotypes resulting from germ cell ablation are not inheritable to the next generation. In contrast, we approached this challenge from the somatic cells by knocking out *fshr*, an essential gene for oogenesis, which results in the complete developmental arrest of oocytes due to a lacking of Fsh signal [14]. Importantly, in this surrogate broodstock system, the hybrid recipients could fully support oogenesis of the Fshr-deficient germ cells throughout their reproductive period. Therefore, we concluded that as long as functional Fshr is produced in gonadal supporting cells, it is not required in germ cells. Further, the reason for sterility of hybrid medaka used in this study has been proven to be germ cells and not somatic cells or from the endocrine system since hybrid recipients recovered fertility simply by transplanting foreign germ cells.

When applying this technique to aquaculture species, the possibility of sex reversal of F1 offspring should be considered since it can be induced by various environmental factors, such as endocrine disruptors [33] or extreme rearing conditions or stressors [34]. Although only the females of the *fshr*-mutant medaka used in this study showed sterile phenotype, it has been reported that *fshr*-mutant XX-medaka sometime shows spontaneous masculinization [14]. Therefore,

seeking other mutant donors that are sterile in both sexes will be an important task in the future, and such a donor will make it possible to establish a more reliable system for the mass production of sterile fish. Since hybrids are not always sterile, recipients should be sterilized by either *dnd* KD [8] or KO [9], triploidization [27, 35, 36], or hybridization [37].

Assuredly, this system can be utilized for aquaculture production of GM aquaculture fishes. Recently, growth hormone (GH)-overexpression transgenic salmon [38] was approved for human consumption by the FDA and has started to be sold in Canada [4]. Combination with the mass production technique of sterile fish developed in this study would make it possible to establish a reliable biological containment system of GM aquaculture fishes. Since SGTP has already been shown to be applicable to various groups of fishes [21, 27, 36, 39-42], as long as genetically sterile donors are obtained by mutation of somatic genes (but not germ cell genes), this technique can be applied to various aquaculture-target species. In addition to solving problems with escapees, sterile fish production would contribute to suppress various negative side effects in aquaculture production caused by reproductive maturation [43], such as reduction of growth, fillet quality, and disease tolerance. Moreover, sterilization of valuable fish strains that are produced by selective breeding programs and that were time- and labor-intensive to produce can protect the interests of seedling producer. Thus, we believe that the use of genetically sterile fishes is a very attractive option for the future of aquaculture.

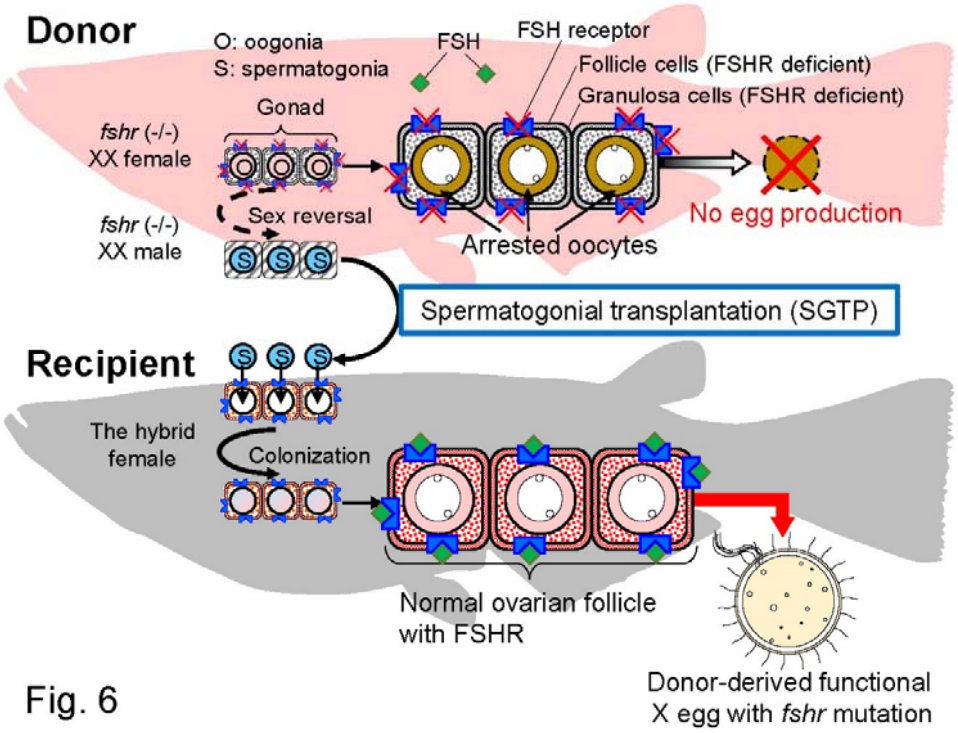

Fig. 6. Schematic representation of the surrogate broodstock system with transplantation of spermatogonia (SGTP) carrying the *fshr* mutation for mass production of fish stock that are genetically sterile.

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wrote the paper; K.N., G.Y., T.K., and Y.Y. contributed critical discussion on all analysis of data.
Competing interests: There are no competing interests.

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