

1 **Potential and advantages of goldfish as a surrogate host for producing grass carp**
2 **gametes**

3 Huan Ye^{1,#}, Peng Yu^{2,#,*}, Xiao-Jing Yang^{1,#}, Hua-Mei Yue¹, Xiao-Ya Zhou³, Zi-Ling
4 Qu¹, Ling Huang¹, Yan-Ling Hou¹, Hui-Hui Kong¹, Cai-Rong Li¹, Zhi Li¹, Gang Yang¹,
5 Rui Ruan¹, Li Zhou^{2,4}, Jian-Fang Gui^{2,4}, Chuang-Ju Li^{1,*}

6 ¹ Key Laboratory of Freshwater Biodiversity Conservation, Ministry of Agriculture and
7 Rural Affairs, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery
8 Sciences, Wuhan, Hubei 430223, China

9 ² State Key Laboratory of Breeding Biotechnology and Sustainable Aquaculture,
10 Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei 430072,
11 China

12 ³ College of Animal Science and Technology, Anhui Agricultural University, Hefei,
13 Anhui 230036, China

14 ⁴ University of Chinese Academy of Sciences, Beijing 100049, China

15 [#] Authors contributed equally to this work

16 ^{*} Corresponding authors, Email: yupeng@ihb.ac.cn; lcj@yfi.ac.cn



ABSTRACT

Grass carp (*Ctenopharyngodon idellus*), which accounts for approximately 10% of global freshwater aquaculture production, is an important aquaculture species in Asia. Nevertheless, advancements in breeding have been substantially hindered by an extended sexual maturation period of 4–5 years. Germline stem cell transplantation (GSCT) presents a potential technology to accelerate fish breeding by shortening the generation interval through the use of recipients with a shorter sexual maturation period. In this study, smaller-bodied goldfish recipients (*Carassius auratus*) characterized by a shorter generation interval, high fecundity, and scalable year-round gamete output, were employed for the production of grass carp gametes. Donor testicular cells isolated from the testes of 1.5-year-old grass carp were intraperitoneally transplanted into 4 days post-hatching germ cell-depleted goldfish larvae generated by knocking down the *dead end (dnd)* gene. At 6 months post-transplantation (mpt), 11 out of 21 male goldfish recipients successfully produced functional donor-derived grass carp sperm, with an average milt volume of 754 μ L. Unlike sterile zebrafish recipients, which lack ovarian development, sterile goldfish recipients supported the differentiation of donor grass carp spermatogonia stem cells into oogonia and their subsequent development into late-vitellogenic oocytes within the recipient gonadal somatic environment by 10 mpt. These findings identify goldfish as a promising recipient for grass carp GSCT, providing a foundation for the rapid, large-scale production of donor-derived grass carp sperm and supporting future efforts to produce donor-derived grass carp eggs through

38 reproductive regulation.

39 **Keywords:** Grass carp; Germline stem cell transplantation; Goldfish; Surrogacy;
40 Sperm

41 INTRODUCTION

42 According to the Food and Agriculture Organization of the United Nations (FAO),
43 aquatic animal products constitute a vital source of high-quality proteins, comprising
44 15% of the animal protein intake globally, and exceeding 50% in several Asian and
45 African countries (FAO, 2026b). Aquaculture contributes over 50% of the total aquatic
46 animal production, thereby addressing the increasing global demand for aquatic foods.
47 Grass carp (*Ctenopharyngodon idellus*) is an important farmed fish, representing
48 approximately 10% of global freshwater aquaculture output (FAO, 2025; FAO, 2026a;
49 Gui, 2024). Originally native to eastern Asia, grass carp has been extensively
50 introduced and established in various regions worldwide (Xie et al., 2018). The genetic
51 enhancement of grass carp is essential for the sustainable and healthy development of
52 its aquaculture industry. Various breeding technologies, including selective breeding,
53 hybridization, molecular marker-assisted breeding, gynogenesis, and gene editing,
54 have been employed in the genetic improvement of grass carp (Wang et al., 2024).
55 However, breeding progress has been considerably impeded by the long sexual
56 maturation period of grass carp (4–5 years).

57 Germline stem cell transplantation (GSCT) represents a highly effective
58 reproductive biotechnology for generating donor gametes by using surrogate hosts

(Goto & Saito, 2019; Jin et al., 2021; Yoshizaki & Yazawa, 2019). This technique involves the extraction of germline stem cells (GSCs) from donor gonads, followed by their transplantation into recipient hosts. Within the recipient gonadal environment, the colonized donor GSCs proliferate, differentiate, and mature into functional gametes. This process can shorten the breeding cycle by using a closely related surrogate with a smaller body size and shorter generation interval. For instance, through xenogeneic GSCT, small-bodied (~13 g in body weight) grass puffer (*Takifugu alboplumbeus*) recipients have generated functional donor eggs and sperm of the tiger puffer (*T. rubripes*) (Hamasaki et al., 2017). Similarly, surrogate goldfish (*Carassius auratus*) have produced both donor eggs and sperm of common carp (*Cyprinus carpio*) following spermatogonia transplantation (Franěk et al., 2021). Hybrid little tuna surrogates, at merely 8 months of age and approximately 1 kg in body weight, produced donor-derived Pacific bluefin tuna (*Thunnus orientalis*) sperm (Kawamura et al., 2024). Pacific bluefin tuna typically require 3–5 years to reach sexual maturity and can approach 100 kg body weight. More recently, recipient zebrafish (*Danio rerio*) generated donor grass carp sperm (~1 µL) within 3 months following transplantation; however, donor eggs were not obtained (Ye et al., 2026). Therefore, these findings highlight the importance of the recipient for the efficient production of both donor eggs and sperm.

For grass carp, goldfish is considered a suitable surrogate host. Originating from wild crucian carp through approximately 1 800 years of selective breeding in China, goldfish have diversified into over 300 strains and are widely recognized as both a

popular ornamental species and a valuable model for studies in evolutionary genetics and developmental biology (Chen et al., 2020; Li et al., 2025; Yu et al., 2021). Beyond these values, goldfish exhibit remarkable reproductive characteristics that make them ideal for surrogate propagation. Under optimal conditions, goldfish can achieve sexual maturity and spawn approximately 4 months post-fertilization, with the ability to re-spawn every 4–7 days throughout the year, producing up to 10 000 eggs per clutch (Yu et al., 2022). Moreover, goldfish lacking endogenous germ cells due to *dead end* (*dnd*) gene knockdown exhibit gonadal sexual dimorphism (Goto et al., 2012), a trait distinct from that observed in zebrafish (Slanchev et al., 2005). This trait is essential for donor egg production. Collectively, the combination of a short generation time, high fecundity, and the presence of sexually dimorphic gonads despite germ cell deficiency positions goldfish as a promising surrogate for producing grass carp gametes.

In this study, we identified the optimal developmental stage at which goldfish larvae can serve as recipients. We conducted comprehensive cellular, histological, and molecular analyses of germ cell-depleted gonads generated using *dnd* morpholino (MO)-mediated knockdown. The colonization, proliferation, and development of intraperitoneally transplanted grass carp spermatogonia stem cells (SSCs) within the recipient gonads were systematically examined. Lastly, we characterized the donor-derived grass carp sperm produced by recipient goldfish and evaluated its functionality.

MATERIALS AND METHODS

Experimental fish

Goldfish of the lionhead strain were reared in aquaria maintained at temperatures ranging from 22–26°C, under a 14 h light/10 h dark photoperiod (lights on at 0900h, lights off at 2300h) at the National Aquatic Biological Resource Center. The rearing protocols and conditions adhered to those outlined by Yu et al. (2022). Briefly, goldfish larvae with a total length (TL) of less than 2 cm were initially fed live nauplii and subsequently acclimated to a commercial diet (Guangdong Yuequn Ocean Biotechnology Co., Ltd., China). Artificial propagation was conducted using healthy, sexually mature goldfish generally older than 150 days post-hatching (dph), as detailed in prior research (Yu et al., 2022). Grass carp aged 1.5 years (TL, 57.67±1.30 cm; body weight [BW], 1.98±0.12 kg; gonadosomatic index, 0.020±0.0018%) were sourced from a commercial farm in Jiayu County, Xianning City, Hubei Province, and were sacrificed for donor SSC preparation. The fish experiments adhered to the Guidelines for the Care and Use of Laboratory Animals of the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (YFI2024YH04).

Experimental design

The experimental workflow comprised six sequential stages: (1) establishment of germ cell-depleted goldfish recipients through *dnd* knockdown; (2) transplantation of grass carp testicular cells into goldfish larvae; (3) colonization of donor-derived SSCs within recipient gonads; (4) development of donor-derived SSCs in recipient gonads; (5) production of donor-derived sperm by surrogate goldfish recipients; and (6) development of donor-derived oocytes within recipient gonads (Figure 1). Three independent trials were performed for each experiment. The sample size and the

corresponding analytical approaches employed at each stage are indicated within rounded rectangles in Figure 1.

Histological analysis of goldfish larvae

To characterize early goldfish gonadal development, larvae with TLs of 6.13 ± 0.10 mm, 7.02 ± 0.03 mm, and 8.44 ± 0.17 mm were collected at 3, 5, and 7 dph, respectively. The larvae were fixed in Bouin's solution (Phygene, China), followed by dehydration, paraffin embedding, and transverse sectioning at a thickness of $4.50 \mu\text{m}$ using a microtome (Leica Microsystems, Germany). Thereafter, the sections were stained with hematoxylin and eosin (H&E), and imaged using an upright microscope (DM5000; Leica Microsystems, Germany) equipped with a digital camera (DFC365FX; Leica Microsystems, Germany). The total number of primordial germ cells (PGCs) per larva was quantified from serial sections of the entire specimen, and the mean was calculated across five individuals.

Preparation of germ cell-depleted goldfish recipients

The *dnd* gene antisense morpholino (MO) for goldfish (5'-TCCATGCCGCTGTCCACCTGTGATG-3') was designed as described previously (Goto et al., 2012), and synthesized by Gene Tools LLC (Philomath, USA). Endogenous PGCs were depleted by injecting $200 \mu\text{mol/L}$ *dnd*-MO into goldfish embryos at the 1-cell stage. To determine the efficiency of PGC ablation, embryos were co-injected with artificially synthesized green fluorescent protein (GFP)-*zfnanos3* 3' untranslated region (UTR) mRNA at a concentration of $150 \text{ ng}/\mu\text{L}$. Eighteen *dnd* morphants at 50 dph (nine males, nine females; TL, 2.63 ± 0.09 cm; BW, 0.36 ± 0.04 g)

were sampled for histological observation, while 18 *dnd* morphants at 5 months old (8 males, 10 females; TL, 11.61±0.20 cm; BW, 41.13±2.21 g) were used to assess gonadal development and related gene expression.

Total RNA was isolated separately from the gonads of both *dnd* morphants and wild-type (WT) goldfish at 5 months old using a FastPure Tissue/Cell Total RNA Isolation Kit (Vazyme, China). The extracted RNA was subsequently reverse transcribed into complementary DNA (cDNA) using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan) following the manufacturer's protocol. Reverse transcription PCR (RT-PCR) amplification was conducted in a 20 µL reaction volume, comprising 10 µL of 2×Phanta UniFi Master Mix (Vazyme, China), 1 µL of diluted cDNA, 0.4 µL of each primer, and 8.2 µL of DNase-free water. RT-PCR was performed using primers specific to germ cell (*vasa*) and gonadal somatic cell (*foxl2*, *cyp11b*, and *gsdf*) genes (Supplementary Table S1).

The thermal cycling protocol comprised 33 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 10 s. The PCR products were subjected to electrophoresis on a 2.0% agarose gel stained with GoldView (Yeast, China), cloned into a pcDNA3.1(+) vector (Thermo Fisher Scientific, USA), and sequenced by Wuhan Tianyi Huayu Gene Technology Co., Ltd., China.

Immunohistochemistry of donor grass carp testes

Grass carp were initially anesthetized using 2% tricaine methanesulfonate (Sigma-Aldrich, USA), and testes were dissected, cut into small pieces, and fixed in Bouin's solution at 4°C for 20 min. The fixed testicular samples were embedded in paraffin,

sectioned, and immunohistochemically stained, following previously established protocols (Ye et al., 2017). A primary anti-Vasa antibody (1:400, ab209710; Abcam, UK) was used to identify germ-cell types. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, USA). Fluorescence signals were acquired using a confocal microscope (LSM800; Carl Zeiss, Germany).

Donor testicular cell transplantation

Following dissection under sterile conditions, donor grass carp testes were carefully trimmed using fine scissors and tweezers to remove the peritoneum, adipose tissue, and any blood clots. Single-cell suspensions of testicular cells were prepared using enzymatic digestion according to previously described methods with some modifications (Ye et al., 2017). Specifically, the minced testicular fragments were subjected to protease digestion at 30°C for 1.5–2.0 h. The resultant cell suspensions were sequentially filtered through 100- and 40-µm cell strainers to eliminate any undigested tissue remnants. Before transplantation, the testicular cells were labeled with the fluorescent cell tracker dye PKH26 (Sigma-Aldrich, USA) to enable in vivo tracking. A 100-nL suspension containing approximately 100 000 donor cells was injected into the peritoneal cavity near the genital ridge region of goldfish at 4 dph. Following transplantation, the larvae were transferred to fresh water and maintained under standard rearing conditions.

Colonization and development of donor-derived cells in goldfish recipients

At 21 days post-transplantation (dpt), both the transplanted and control goldfish were fixed using Tissue-Tek Ufix (Sakura Finetech, Japan) for 1 min on ice. Subsequently,

the fish were dissected under a stereomicroscope to examine the colonization of donor grass carp SSCs through fluorescence observation (DM5000B; Leica Microsystems, Germany). To further assess the proliferation, differentiation, and development of transplanted SSCs within recipient gonads, histological analyses were conducted on gonads from WT, *dnd* morphants, and transplanted goldfish at 3 months post-transplantation (mpt, TL, 7.32±0.19 cm; BW, 17.74±0.98 g), 6 mpt (TL, 11.34±0.29 cm; BW, 41.69±1.64 g), and 10 mpt (TL, 14.45±0.34 cm; BW, 106.20±7.15 g). The same gonadal samples were analyzed using RT-PCR with grass carp-specific *vasa* primers. Detailed methods are provided in the subsequent section.

PCR detection of donor-derived germ cells in recipients

Total RNA was extracted from the gonads of WT goldfish, *dnd* morphants, and transplanted goldfish at 3, 6, and 10 mpt. These RNA samples were subsequently reverse-transcribed into cDNA as detailed in the “Preparation of germ cell-depleted goldfish recipients” section. Utilizing the *vasa* cDNA sequences from grass carp and goldfish, species-specific primers were designed *Civasa*-RNA-F/R for grass carp and *Cavasa*-RNA-F/R for goldfish. The *β-actin* cDNA sequences of both grass carp and goldfish were referenced to generate the common primer (*β-actin*-F/R) as the positive control (Supplementary Table S1). The PCR reaction mixture and thermal cycling conditions were consistent with those outlined in the “Preparation of germ cell-depleted goldfish recipients” section. The resulting PCR products were resolved on 2.5% agarose gels, cloned, and sequenced.

RNA sequencing and analysis

212 The quality of gonadal RNA from WT and *dnd*-MO goldfish, WT grass carp, and
213 goldfish that successfully underwent GSCT with exclusively donor-derived grass carp
214 germ cells was assessed. RNA from testes and ovaries at 6 and 10 mpt, respectively,
215 was used to construct cDNA libraries through a poly(A) enrichment technique,
216 followed by sequencing on a NovaSeq 6000 platform (Illumina, USA). Approximately
217 6 and 10 G of clean data, with more than 94% of bases having Phred quality scores ≥ 30
218 (Q30), were generated for grass carp and goldfish, respectively. The genomes of grass
219 carp (GCF_019924925.1) and goldfish (GCA_019720715.2) were downloaded from
220 the National Center for Biotechnology Information (NCBI) database
221 (<https://www.ncbi.nlm.nih.gov/>) and integrated into a joint reference genome. To
222 prevent conflicts arising from homologous chromosomes, species-specific prefixes
223 (“CI_” for grass carp and “CA_” for goldfish) were appended. A consolidated genome
224 index was then constructed using STAR software (v.2.5.2b). Paired-end clean reads of
225 150 bp (PE150) were aligned to the joint reference genome using STAR. To enhance
226 the specificity and reliability of the alignment results, secondary and supplementary
227 alignments were removed, and only reads with a mapping quality (MAPQ) score ≥ 30
228 that aligned uniquely to the consolidated reference genome were retained. Following
229 the rigorous filtering procedures outlined above, high-confidence, unique alignment
230 results were obtained for subsequent coverage analysis. To assess coverage patterns at
231 the chromosomal scale, the combined reference genome was segmented into sliding
232 windows of a fixed size (default: 10 kb). Utilizing the BEDtools software suite
233 (v.2.27.1), we computed the coverage fraction for each genomic window. This metric

is defined as the ratio of the number of bases covered by at least one sequencing read to the total length of the window. Subsequently, we determined the average coverage fraction per window and the base-weighted overall coverage fraction for each chromosome. These calculations were performed to assess the overall coverage levels across chromosomes in these two species for different samples. To minimize the impact of assembly fragments and small scaffolds on statistical outcomes, only chromosomes longer than 10 Mb were included in the analysis. Chromosomes were categorized and organized according to species prefixes. Using the chromosome-level coverage statistics, we constructed a matrix correlating sample information with chromosome coverage, and subsequently generated a heatmap to visualize the results.

PCR detection of donor DNA in the recipient sperm

To determine whether the sperm produced by the transplanted recipients originated from recipient goldfish or donor grass carp, genomic DNA (gDNA) was extracted from sperm samples from transplanted goldfish, WT grass carp, and WT goldfish using a TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (Takara, Japan). Species-specific primers for grass carp (*Civasa*-DNA-F/R) and goldfish (*Cavasa*-DNA-F/R), targeting the *vasa* gDNA sequences, were designed to confirm the presence of donor-derived grass carp sperm in the transplanted goldfish recipients (Supplementary Table S1). The PCR procedures and conditions were identical to those described in the “Preparation of germ cell–depleted goldfish recipients” section.

Scanning electron microscopy of surrogate-derived spermatozoa

Sperm samples were obtained from the transplanted goldfish, WT goldfish, and WT

grass carp for analysis via scanning electron microscopy (SEM). Fresh sperm samples from three individuals within each group were fixed in 2.5% glutaraldehyde overnight at 4°C, followed by post-fixation in 1% osmium tetroxide (Servicebio, China). The samples were dehydrated through a graded series of ethanol and subsequently subjected to critical-point drying utilizing a Critical Point Dryer (K850; Quorum Technologies Ltd., UK). Thereafter, the samples were sputter-coated with platinum using an Ion Sputtering Apparatus (MC1000; Hitachi High-Tech Corporation, Japan). Spermatozoa ultrastructure was assessed using a scanning electron microscope (SU8100; Hitachi High-Tech Corporation, Japan). To compare the morphological differences in spermatozoa among the three groups, 30 spermatozoa from each individual were randomly selected, and their head areas were subsequently determined using ImageJ software (v.1.53c).

Genome resequencing and analysis

The quality of sperm DNA from WT goldfish, WT grass carp, and successfully transplanted goldfish was assessed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA). DNA libraries were constructed using an MGIEasy FS DNA Library Prep Set (BGI, China) and subjected to paired-end sequencing with 150 base pairs on a DNBSEQ-T7 platform (MGI Tech Co., Ltd., China). Approximately 10 and 18 G clean data, with a quality score (Q30) exceeding 95%, were generated for grass carp and goldfish, respectively. To facilitate a comparative analysis of read alignment and genomic coverage between the two species, a consolidated reference genome was developed as detailed in the “RNA sequencing and analysis” section, and whole-

genome coverage analysis was performed. The Burrows–Wheeler Aligner maximal exact match (BWA-MEM) algorithm was employed to align the resequencing reads from each sample to the consolidated reference genome, and the resulting Binary Alignment/Map (BAM) files were subsequently sorted and indexed. To enhance the reliability of the alignment results, only primary alignments with a MAPQ score ≥ 30 were retained, excluding secondary and supplementary alignments, for further statistical analysis. BEDtools (v.2.27.1) was used to calculate the coverage breadth, defined as the proportion of bases covered by reads on each chromosome. A heatmap was generated to visually depict the differences in coverage among various samples on the consolidated genome.

Sperm motility analysis and in vitro fertilization of surrogate-derived sperm

To induce spermiation in both transplanted and WT goldfish, a high-efficiency reproduction reagent (Shanghai Biological Science Co., Ltd., China) was administered via intraperitoneal injection at a dosage of 200 U/kg. Approximately 8–10 h post-hormone injection, milt was collected from the urogenital papilla and maintained on ice until fertilization. Subsequently, sperm kinetic parameters, including the percentage of progressive motility (PMOT, %), curvilinear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), and average path velocity (VAP, $\mu\text{m/s}$), were evaluated using a computer-assisted sperm analysis (CASA) system (CEROS II; Hamilton Thorne, USA). The concentration and volume of the milt were also quantified. Ovulation in grass carp was induced by administering luteinizing hormone–releasing hormone A2 (Ningbo Sansheng Pharmaceutical Co., Ltd., China) at a dosage of 8 $\mu\text{g/kg}$. Eggs were retrieved

10–12 h post-hormone injection by gently applying pressure to the abdomen of females. *In vitro* fertilization was conducted using the following parental combinations: GSCT sperm with WT grass carp eggs, WT grass carp sperm with WT grass carp eggs, WT goldfish sperm with WT goldfish eggs, and WT goldfish sperm with WT grass carp eggs. Sperm and eggs from three individuals within each group were utilized, and the fertilization rate was subsequently calculated. Following hatching, five individuals from each experimental group were randomly selected and pooled for DNA extraction. Subsequent genetic analysis was conducted using PCR with species-specific *vasa* primers, as detailed in the “PCR detection of donor DNA in the recipient sperm” section. Larvae were imaged using a stereomicroscope (V16; Carl Zeiss, Germany) at 1 and 21 dph.

Statistical analysis

The data are presented as the mean±standard error (SE). The *Shapiro–Wilk* test indicated that the distribution of the head area of spermatozoa from transplanted goldfish, WT goldfish, and WT grass carp did not follow a normal distribution. Consequently, the *Kruskal–Wallis* test, followed by *Dunn’s* multiple comparisons test, was used to evaluate the statistical significance of differences in the head area of spermatozoa among these groups. For PMOT, VCL, VSL, and VAP, the normality and homogeneity of variances were confirmed using *Shapiro–Wilk* and *Bartlett’s* tests. Subsequently, a one-way analysis of variance (ANOVA), followed by *Tukey’s* multiple comparison test, was conducted to evaluate statistical significance. Differences were deemed significant at $P<0.001$. Statistical analyses were performed, and graphs were

generated, using GraphPad Prism 9 (GraphPad Software, USA). Phylogenetic distances between donor and recipient species were estimated using TimeTree (<http://www.timetree.org/>).

RESULTS

Early gonadal development of goldfish

To determine the optimal timing for conducting GSCT in recipient goldfish larvae, we examined endogenous PGC migration and genital ridge formation by analyzing serial sections of whole larvae. In larvae ranging from 6.13 mm (3 dph) to 7.02 mm (5 dph), PGCs characterized by large, round nuclei were observed to settle in the dorsal region of the peritoneal cavity, where the genital ridge is expected to form (Figure 2A–F). At both stages, the PGCs had not yet been encapsulated by gonadal somatic cells. The average numbers of PGCs per individual were 21.00 ± 2.39 and 31.00 ± 2.97 (Supplementary Figure S1), respectively. In larvae measuring 8.44 mm (7 dph), the genital ridges were distinctly formed, and a single layer of gonadal somatic cells enveloped PGCs (Figure 2G–I). At this stage, the number of PGCs increased to 46.40 ± 3.22 (Supplementary Figure S1). These findings suggest that PGC migration in goldfish is completed by 5 dph.

Morphological and molecular characteristics of germ cell-depleted gonads following *dnd* knockdown

To evaluate the efficacy of germ cell depletion induced by the MO targeting *dnd* (*dnd*-MO), both *dnd*-MO and GFP-*zfnanos3* 3' UTR mRNA were injected into embryos at

the 1-cell stage. GFP-labeled PGCs were present in $62.28 \pm 0.85\%$ of embryos injected only with GFP-*zfnanos3* 3' UTR mRNA (Figure 3A; Table 1). In contrast, embryos co-injected with *dnd*-MO and GFP-*zfnanos3* 3' UTR mRNA exhibited an almost complete absence of GFP-labeled PGCs ($94.00 \pm 0.89\%$; Figure 3B; Table 1), indicating that the administered dose of *dnd*-MO effectively induced PGC depletion in goldfish. A comprehensive histological analysis of gonads at 50 dph was conducted to further investigate germ cell ablation in morphants. In WT fish, all examined ovaries (9/9) exhibited an ovarian cavity and primary oocytes at the perinuclear stage (Figure 3C), while the testes (9/9) contained dispersed spermatogonia (Figure 3D). In contrast, the gonads of morphants appeared to differentiate into two distinct structures resembling ovaries (Figure 3E) and testes (Figure 3F), respectively. Notably, nearly all morphants (17/18) lacked germ cells.

In 5-month-old WT goldfish, the differentiation between ovaries and testes was evident based on their external morphology (Figure 3G, I). At this developmental stage, the ovaries contained a substantial number of fully grown oocytes (Figure 3H), while the testes comprised spermatogonia, primary and secondary spermatocytes, and spermatids within each seminal lobule (Figure 3J). In contrast, in *dnd* morphants, type I gonads exhibited an ovarian-like structure with an ovarian cavity but were devoid of oocytes (9/10; Figure 3K, L). Type II gonads, on the other hand, consisted of seminiferous lobule-like structures lacking germ cells (8/8; Figure 3M, N). Moreover, *vasa* expression was detected in the gonads of WT goldfish but not in the gonads of *dnd* morphants (Figure 3O). The ovarian granulosa cell marker gene *foxl2* was

predominantly expressed in the ovaries of WT goldfish and in type I gonads (Figure 3O). In contrast, the expression of the testicular Leydig cell gene *cyp11b* was primarily observed in the testes of WT goldfish and in type II gonads (Figure 3O). The gonadal somatic cell gene *gsdf* was expressed in both gonads of WT goldfish and *dnd* morphants (Figure 3O). Overall, the ablation of germ cells did not influence sex differentiation in goldfish.

Transplantation of grass carp testicular cells into goldfish recipients

Vasa-positive signals were specifically observed in the germ cells of donor grass carp testes. Moreover, no distinct testicular lobules were observed, and isolated and paired spermatogonia, presumed to be SSCs, were dispersed throughout the testes (Figure 4A). Approximately 100 000 donor grass carp testicular cells, labeled with PKH26 (Figure 4B, C), were intraperitoneally transplanted into 4 dph goldfish larvae (Figure 4D). PKH26-labeled cells were subsequently detected in the peritoneal cavity of recipients using fluorescence microscopy (Figure 4E), whereas no fluorescence was observed in non-transplanted larvae. The survival rates at 21 dpt were $82.96 \pm 0.89\%$ for the transplanted group and $92.63 \pm 1.56\%$ for the non-transplanted group (Table 2).

Incorporation and differentiation of donor SSCs in gonads of germ cell-depleted goldfish recipients

At 21 dpt, fluorescence microscopy detected PKH26-labeled donor testicular cells in the gonads of transplanted goldfish (Figure 5A, B), whereas no red fluorescence was detected in the gonads of control fish (Figure 5C, D). The colonization rate of donor SSCs in germ cell-depleted recipients was 75.00% (18/24). At 3 mpt, WT ovaries were

replete with primary growth oocytes (Figure 5E), while WT testes comprised different types of germ cells, including spermatogonia, primary and secondary spermatocytes, and spermatids (Figure 5F). Conversely, irrespective of sex, the gonads of *dnd* morphants were devoid of germ cells and instead displayed structures analogous to ovarian cavities and seminiferous lobules (Figure 5G, H). In the GSCT group, gametogenesis was observed in 11 out of 15 transplanted goldfish (Table 2), including 7 females and 4 males. Compared with WT goldfish gonads, transplanted gonads exhibited slower development, with ovaries containing only early perinuclear oocytes and testes only comprising spermatogonia (Figure 5I, J). Moreover, based on differences in the *vasa* gene sequence between donor grass carp and recipient goldfish, species-specific primers were designed to distinguish donor- and recipient-derived germ cells (Supplementary Figure S2; Supplementary Figure S3A). RT-PCR using these species-specific primers revealed that, in 10 of the 15 transplanted goldfish, germ cells originated exclusively from transplanted grass carp SSCs, with no detectable endogenous goldfish germ cells (Figure 5K). Notably, only one transplanted goldfish (TP-14) contained germ cells from both the donor grass carp and the recipient goldfish.

Gametogenesis of colonized grass carp SSCs in recipient goldfish

In WT goldfish, light yellow oocytes were observed within the ovaries (Figure 6A), whereas milk-white testes were identified at 6 mpt (Figure 6C). Subsequent histological analysis revealed that WT ovaries contained a substantial number of fully grown oocytes (Figure 6B), whereas WT testes predominantly comprised spermatids (Figure 6D). In contrast, the gonads of both female and male *dnd* morphants were thin and

translucent (Figure 6E, G). No germ cells were observed; however, ovarian cavity-like and seminiferous lobule-like structures were present in the female and male gonads, respectively (Figure 6E–H). In successful GSCT goldfish, ovarian development progressed only to the cortical alveolus stage at 6 mpt. Even at 10 mpt, the ovaries of GSCT goldfish exhibited a similar appearance to those of WT goldfish (Figure 6I), although most oocytes were at the late vitellogenic stage (Figure 6J). In contrast, both the morphological and histological characteristics of testes in successful GSCT goldfish were comparable to those of WT testes (Figure 6K, L). The presence of donor grass carp germ cells in recipient goldfish gonads was further confirmed by donor *vasa* gene expression (Supplementary Figure S4). Donor-specific *vasa* amplicons were detected in 16 out of 23 (69.57%) recipient ovaries and 11 out of 21 (52.38%) recipient testes. Notably, only one GSCT goldfish (TP-7) contained both donor and recipient *vasa* amplicons.

To further characterize the gonadal transcriptomic profiles of successful GSCT in goldfish, RNA sequencing was conducted on grass carp *vasa*-positive gonads from GSCT goldfish, as well as on WT gonads from both goldfish and grass carp. The resulting transcripts were aligned to a consolidated reference genome comprising both goldfish and grass carp sequences for RNA-seq alignment and coverage analysis (Figure 6M). In WT grass carp ovaries, the average base-weighted coverage fractions were 0.628 for the grass carp genome and 0.004 for the goldfish genome. Correspondingly, testes exhibited average base-weighted coverage fractions of 0.820 for the grass carp genome and 0.005 for the goldfish genome. In contrast, *dnd* morphant

ovaries exhibited average base-weighted coverage fractions of 0.008 and 0.612 for the grass carp and goldfish genomes, respectively, while their testes showed values of 0.008 and 0.567, respectively. Within the GSCT group, the average base-weighted coverage fractions for grass carp *vasa*-positive ovaries and testes in goldfish were 0.603 and 0.773, respectively, for the grass carp genome, and 0.278 and 0.420, respectively, for the goldfish genome (Supplementary Table S2). These results suggest that the GSCT gonads were chimeric and contained transcripts derived from both grass carp and goldfish.

Production of grass carp sperm in recipient goldfish

Following hormonal induction, semen was successfully collected from 11 of 21 transplanted male goldfish. Subsequently, species-specific *vasa* DNA fragments were amplified from gDNA (Supplementary Figure S2; Supplementary Figure S3B). Of the 11 semen samples, 10 exclusively contained gDNA from the donor grass carp, while only one sample, from transplanted goldfish TP-Sp2, contained gDNA from both donor grass carp and recipient goldfish (Figure 7A). To further confirm the production of donor grass carp sperm by recipient goldfish, SEM was used to compare the ultrastructure of spermatozoa from WT grass carp, WT goldfish, and donor-derived grass carp spermatozoa produced by recipient goldfish (GSCT spermatozoa). The head area of spermatozoa from WT grass carp ($1.98 \pm 0.01 \mu\text{m}^2$) was significantly smaller than that of WT goldfish ($2.66 \pm 0.01 \mu\text{m}^2$). However, GSCT spermatozoa exhibited a head area ($2.03 \pm 0.01 \mu\text{m}^2$) similar to that of WT grass carp spermatozoa, suggesting that recipient goldfish successfully produced donor grass carp sperm (Figure 7B–H).

Moreover, the sperm concentration and milt volume of successfully transplanted germ cell-free recipients were $(5.64 \pm 0.47) \times 10^9$ spermatozoa/mL and 754.40 ± 65.18 μ L, respectively; both were lower than those of WT goldfish (Figure 7I). Finally, genetic consistency among goldfish, grass carp, and GSCT sperm was assessed using whole-genome resequencing (Figure 7J; Supplementary Table S3). The genomic sequences obtained from these samples were mapped to the consolidated reference genome containing both goldfish and grass carp sequences. The analysis revealed that the GSCT sperm genome was consistent with that of grass carp but differed substantially from that of goldfish.

To evaluate the functionality of surrogate grass carp sperm produced by recipient goldfish, these sperm were utilized to fertilize eggs from both WT grass carp and goldfish. CASA indicated no significant differences in sperm progressive motility and kinetics (VCL, VSL, and VAP) among spermatozoa from goldfish, grass carp, and GSCT sperm (Figure 8A). The offspring produced from GSCT sperm and grass carp eggs exhibited normal development (Figure 8B, C). The fertilization and hatching rates were $51.36 \pm 6.93\%$ and $67.93 \pm 3.89\%$, respectively, which were comparable to those achieved with WT grass carp sperm and WT eggs ($53.04 \pm 4.53\%$ and $72.65 \pm 1.62\%$). Furthermore, these offspring could be reared without any detectable abnormalities (Figure 8F, G). In contrast, goldfish sperm fertilized grass carp eggs at a low rate of $5.38 \pm 0.66\%$, and all resulting hybrid embryos exhibited malformations and died within 4 days post-fertilization (dpf; Figure 8E). PCR analysis further demonstrated that larvae produced from GSCT sperm only contained grass carp gDNA (Figure 8I).

DISCUSSION

In this study, functional donor grass carp sperm was successfully generated by sterile goldfish recipients through xenogeneic GSCT in a notably short timeframe. Grass carp typically require 5 years to reach sexual maturity and achieve a body weight of approximately 10–20 kg (Li et al., 2023; Lin et al., 2022). In contrast, goldfish hosts produced donor-derived grass carp sperm at approximately 6 months of age, with an average body weight of 40.48 g. Although the use of surrogate goldfish extends the time required to obtain donor grass carp sperm by 3 months relative to that required by zebrafish recipients (Ye et al., 2026), the resulting semen volume is considerably larger (754 μL vs. 1 μL), enhancing practicality for breeding purposes. Furthermore, employing goldfish to produce grass carp sperm substantially reduces the spatial and financial demands of maintaining grass carp broodstock. Consequently, this study offers a viable approach to expedite grass carp genetic breeding.

The selection of an appropriate larval-stage recipient for GSCT is crucial for the successful incorporation of donor-derived GSCs into the recipient's genital ridge. Prior research, including studies on *Nibeia mitsukurii* (Takeuchi et al., 2009), *Rhodeus ocellatus ocellatus* (Octavera & Yoshizaki, 2019), and *Plecoglossus altivelis* (Ichida et al., 2025), has demonstrated that recipients lose the capacity to guide donor GSCs to the genital ridge when endogenous PGCs become fully encased by gonadal somatic cells. Histological analyses of goldfish larvae indicated that PGCs migrate and settle at the genital ridge between 3–5 dph, whereas by 7 dph, PGCs are enveloped by a single

layer of gonadal somatic cells. These findings suggest that goldfish larvae at 3–5 dph are optimal candidates for use as recipients. Franěk et al. (2021) reported that host goldfish produced donor common carp gametes when transplantation was conducted at 6 dpf. The present study also observed high transplantation efficiency when GSCT was performed before 5 dph: 75% of recipients were identified as chimeras at 21 dpt, and 50% of male recipients produced donor-derived grass carp sperm.

Donor-derived egg production is particularly desirable through GSCT because established cryopreservation methods for fish eggs and embryos are lacking (Qin et al., 2025). Two key factors are crucial for egg production via GSCT. First, the gonadal sex differentiation of the recipient should occur independently of the presence of PGCs, allowing germ cell-depleted gonads to exhibit sexual dimorphism. Consistent with previous findings (Goto et al., 2012), knocking down the *dnd* gene does not affect ovarian somatic cell differentiation in goldfish. Second, the genetic distance between donor and recipient species is a significant determinant. Current intergeneric GSCT studies have yielded two outcomes regarding donor gamete production (Goto & Saito, 2019; Yoshizaki & Yazawa, 2019). When the phylogenetic distance between donor and recipient species is within 28.0 million years ago (Ma), both donor eggs and sperm can be produced via the surrogate host. This phenomenon has been demonstrated in the Salmonidae family using *Salmo salar* and *Oncorhynchus mykiss* (28.0 Ma) as donor and recipient, respectively (Hattori et al., 2019); in the Cyprinidae family using *Cyprinus carpio* and *Carassius auratus* (25.8 Ma) (Franěk et al., 2021); and in the Acheilognathinae subfamily using *Rhodeus ocellatus ocellatus* and *Tanakia lanceolata*

(26.7 Ma) (Octavera et al., 2023). However, when the divergence time between donor and recipient species exceeds 51.0 Ma, even within the same family, only donor sperm can be generated by the recipient. For instance, functional donor-derived yellowtail (*Seriola quinqueradiata*) sperm were produced by recipient jack mackerel (*Trachurus japonicus*), but no donor-derived primary oocytes were observed in the recipient gonad (Morita et al., 2015). In the Cyprinidae family, recipient zebrafish generated only donor-derived sperm from goldfish (Saito et al., 2008), Chinese rare minnow (*Gobiocypris rarus*) (Zhang et al., 2022), and grass carp (Ye et al., 2026), even when male zebrafish were treated with estradiol, which induces ovary formation and subsequent egg production in WT zebrafish (Saito et al., 2008). In the current study, the 90-million-year evolutionary divergence between goldfish and grass carp did not affect the development of donor SSCs within ovaries of the recipient. A comprehensive genomic analysis revealed two distinct subgenomes in goldfish, which diverged from their common ancestor with grass carp approximately 32.8–36.8 Ma (Chen et al., 2020). This relatively close genetic relationship between goldfish and grass carp may enhance the potential for successful development of donor SSCs within the ovaries of recipients. Nonetheless, the distinct characteristics of the eggs—demersal in goldfish and semi-buoyant in grass carp—may contribute to the maturation failure of grass carp eggs in goldfish, potentially because of differences in the nutritional requirements of these egg types. Therefore, further studies should explore nutritional supplementation and reproductive regulation to promote the maturation of donor-derived grass carp eggs within recipients. Production of functional donor-derived grass carp eggs would

substantially broaden the utility of this surrogate system.

The incomplete depletion of endogenous PGCs using *dnd*-MO in approximately 6% of goldfish may lead to hybrids in GSCT offspring. Residual PGCs in recipients may limit the effectiveness of knockdown-based sterilization because manual microinjection can result in interindividual variability in the volume of reagent delivered. A similar phenomenon has been observed in other fish species (Octavera & Yoshizaki, 2019; Tao et al., 2022). In light of the potential for hybridization between grass carp and goldfish, previous research (female grass carp×male common carp [2n=100]) and our current findings (female grass carp×male goldfish [2n=100]) indicate that progeny from the cross between female grass carp and male cyprinids with 100 chromosomes exhibit developmental abnormalities and ultimately fail to survive post-hatching (Stanley, 1976; Wu et al., 1981; Ye et al., 1989). In the reciprocal cross between female common carp and male grass carp (Stanley, 1976; Wu et al., 1981), a small percentage of eggs (3.45%) successfully developed into hatched larvae; however, the survival rates were markedly reduced, reaching only 0.035% by the age of 3 months. Given that lionhead goldfish, used as recipients in the present study, produce an average of $8\,900 \pm 3\,300$ eggs per spawning event (Yu et al., 2022), even assuming a similar survival rate, only approximately 3 viable offspring would be expected from a single spawning event. Consequently, although the occurrence of viable hybrid offspring cannot be entirely ruled out in fertile female recipient goldfish, the extremely low survival potential of such hybrids implies that the risk of substantial unintended hybrid contribution is likely limited. Nonetheless, whether the reciprocal cross between female goldfish and male

grass carp exhibits similar developmental outcomes remains to be experimentally determined.

When donor spermatozoa are produced by surrogate hosts, their morphology more closely resembles that of the donor species than that of the host species. For example, the flagellum length of spermatozoa from surrogate goldfish was comparable to that of donor common carp, but not to that of recipient goldfish (Franěk et al., 2021). Similar findings were observed in inter-family GSCT involving Japanese flounder (*Paralichthys olivaceus*) and turbot (*Scophthalmus maximus*) as donor and recipient, respectively (Zhou et al., 2021). Conversely, recipient zebrafish reportedly influence the morphology of surrogate Chinese rare minnow spermatozoa (Zhang et al., 2022). A different outcome was observed when recipient zebrafish produced donor grass carp spermatozoa (Ye et al., 2026). In the present study, when recipient goldfish produced grass carp sperm, the morphological characteristics of surrogate grass carp spermatozoa were indistinguishable from those of donor grass carp. These findings suggest that the morphology of surrogate-derived spermatozoa is predominantly germ cell–autonomous.

In conclusion, we successfully established an intergeneric GSCT system using germ cell–depleted goldfish as recipients, enabling the production of large quantities of functional donor-derived grass carp sperm. Notably, donor-derived SSCs differentiated into oogonia and subsequently developed into late-vitellogenic oocytes within the recipient gonadal somatic environment. Effective measures, such as nutritional and reproductive regulation, should be implemented to facilitate the maturation of surrogate oocytes. Overall, this technology holds great promise for significantly shortening the

584 generation interval in grass carp and other economically important cyprinid species
585 with prolonged sexual maturation periods.

586 **DATA AVAILABILITY**

587 The raw RNA sequencing and whole-genome resequencing data have been deposited
588 in the Genome Sequence Archive at the China National Center for Bioinformation
589 (<https://ngdc.cncb.ac.cn/gsa/>) under accession numbers CRA045597 and CRA045596,
590 respectively.

591 **SUPPLEMENTARY DATA**

592 Supplementary data to this article can be found online.

593 **COMPETING INTERESTS**

594 The authors declare that they have no competing interests.

595 **AUTHORS' CONTRIBUTIONS**

596 H.Y.: Writing–original draft, Investigation, Methodology, Funding acquisition, Data
597 curation, Conceptualization. P.Y.: Investigation, Resources, Writing–original draft,
598 Conceptualization. X.J.Y.: Investigation, Validation, Formal analysis. H.M.Y.:
599 Validation, Writing–original draft. X.Y.Z.: Investigation, Data curation. Z.L.Q.:
600 Validation, Writing–original draft. L.H.: Validation, Data curation. Y.L.H.: Validation,
601 Methodology. H.H.K.: Data curation. C.R.L.: Data curation. Z.L.: Investigation. G.Y.:

Software, Investigation. R.R.: Investigation, Validation. L.Z.: Writing–review & editing, Methodology. J.F.G.: Writing–review & editing, Conceptualization. C.J.L.: Writing–review & editing, Funding acquisition, Supervision, Conceptualization. All authors read and approved the final version of the manuscript.

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REFERENCES

- Chen D, Zhang Q, Tang W, et al. 2020. The evolutionary origin and domestication history of goldfish (*Carassius auratus*). *Proceedings of the National Academy of Sciences of the United States of America*, **117**(47): 29775–29785.
- FAO. 2026a. Cultured Aquatic Species Information Programme-*Ctenopharyngodon idellus*. *Ctenopharyngodon idella* (Valenciennes 1844). Text by Weimin, M.. In: Fisheries and Aquaculture [online]. Updated 2004–12–15 [Cited Sunday, August 9th 2026], http://www.fao.org/fishery/culturedspecies/Ctenopharyngodon_idellus/en
- FAO. 2025. Fishery and Aquaculture Statistics – Yearbook 2023. FAO Yearbook of Fishery and Aquaculture Statistics. Rome. <https://doi.org/10.4060/cd6788en>
- FAO. 2026b. The State of World Fisheries and Aquaculture 2026 – Blue Transformation: turning vision into impact. Rome. <https://doi.org/10.4060/cd8357en>

622 Franěk R, Kašpar V, Shah MA, et al. 2021. Production of common carp donor-derived
 623 offspring from goldfish surrogate broodstock. *Aquaculture*, **534**: 736252.
 624 Goto R, Saito T. 2019. A state-of-the-art review of surrogate propagation in fish.
 625 *Theriogenology*, **133**: 216–227.
 626 Goto R, Saito T, Takeda T, et al. 2012. Germ cells are not the primary factor for sexual
 627 fate determination in goldfish. *Developmental Biology*, **370**(1): 98–109.
 628 Gui JF. 2024. Chinese wisdom and modern innovation of aquaculture. *Water Biology*
 629 *and Security*, **3**(3): 100271.
 630 Hamasaki M, Takeuchi Y, Yazawa R, et al. 2017. Production of tiger puffer *Takifugu*
 631 *rubripes* offspring from triploid grass puffer *Takifugu niphobles* parents. *Marine*
 632 *Biotechnology*, **19**: 579–591.
 633 Hattori RS, Yoshinaga TT, Katayama N, et al. 2019. Surrogate production of *Salmo*
 634 *salar* oocytes and sperm in triploid *Oncorhynchus mykiss* by germ cell transplantation
 635 technology. *Aquaculture*, **506**: 238–245.
 636 Ichida K, Suzuki K, Amano Y, et al. 2025. Production of ayu (*Plecoglossus altivelis*)
 637 offspring using cryopreserved spermatogonia from surrogate broodstock. *Aquaculture*,
 638 **609**: 742948.
 639 Jin YH, Robledo D, Hickey JM, et al. 2021. Surrogate broodstock to enhance
 640 biotechnology research and applications in aquaculture. *Biotechnology Advances*, **49**:
 641 107756.
 642 Kawamura W, Yazawa R, Takeuchi Y, et al. 2024. Bluefin tuna sperm production is
 643 hastened by surrogacy in small Euthynnus. *Nature Communications*, **15**(1): 8128.

644 Li LL, Xu QL, Yi WJ, et al. 2025. Arachidonic acid metabolism down-regulation-
 645 mediated tumor necrosis factor signaling contributes to cutaneous fibrosis and skull
 646 hyperplasia in goldfish hoods. *Research*, **8**: 0786.

647 Li L, Balto G, Xu X, et al. 2023. The feeding ecology of grass carp: A review. *Reviews*
 648 *in Aquaculture*, **15**(4): 1335–1354.

649 Lin S, Milardi M, Gao Y, et al. 2022. Sustainable management of non-native grass carp
 650 as a protein source, weed-control agent and sport fish. *Aquaculture Research*, **53**(17):
 651 5809–5824.

652 Morita T, Morishima K, Miwa M, et al. 2015. Functional sperm of the yellowtail
 653 (*Seriola quinqueradiata*) were produced in the small-bodied surrogate, jack mackerel
 654 (*Trachurus japonicus*). *Marine Biotechnology*, **17**(5): 644–654.

655 Octavera A, Yamakawa K, Yoshizaki G. 2023. The volume and shape of bitterling eggs
 656 are more strongly influenced by germ cell autonomy than by the surrounding somatic
 657 cells. *Fish Physiology and Biochemistry*, **49**(5): 967–981.

658 Octavera A, Yoshizaki G. 2019. Production of donor-derived offspring by allogeneic
 659 transplantation of spermatogonia in Chinese rosy bitterling. *Biology of Reproduction*,
 660 **100**(4): 1108–1117.

661 Qin W, Pan J, Xiao J, et al. 2025. Current progress of the long-term preservation for
 662 fish embryos, female reproductive cells, and tissues. *Reviews in Aquaculture*, **17**(4):
 663 e70057.

664 Saito T, Goto-Kazeto R, Arai K, et al. 2008. Xenogenesis in teleost fish through
 665 generation of germ-line chimeras by single primordial germ cell transplantation.

666 *Biology of Reproduction*, **78**(1): 159–166.

667 Slanchev K, Stebler J, De La Cueva-Mendez G, et al. 2005. Development without germ
 668 cells: The role of the germ line in zebrafish sex differentiation. *Proceedings of the*
 669 *National Academy of Sciences of the United States of America*, **102**(11): 4074–4079.

670 Stanley JG. 1976. Production of hybrid, androgenetic, and gynogenetic grass carp and
 671 carp. *Transactions of the American Fisheries Society*, **105**(1): 10–16.

672 Takeuchi Y, Higuchi K, Yatabe T, et al. 2009. Development of spermatogonial cell
 673 transplantation in Nibe croaker, *Nibea mitsukurii* (Perciformes, Sciaenidae). *Biology of*
 674 *Reproduction*, **81**(6): 1055–1063.

675 Tao B, Liao X, Chen L, et al. 2022. Germ cells are not essential for sexual dimorphism
 676 of gonads in common carp, *C. carpio* L. *Aquaculture*, **547**: 737501.

677 Wang Y, Liu W, Li Z, et al. 2024. Improvement and application of genetic resources
 678 of grass carp (*Ctenopharyngodon idella*). *Reproduction and Breeding*, **4**(3): 126–133.

679 Wu W, Lin L, Xu D. 1981. A tetraploid hybrid species-female *Cyprinus carpio* × male
 680 *Ctenopharyngodon idellus*. *Acta Hydrobiologica Sinica*, **7**(3): 433–436. [in Chinese]

681 Xie C, Li J, Li D, et al. 2018. Grass carp: the fish that feeds half of China. In:
 682 *Aquaculture in China*. Hoboken: Wiley-Blackwell, 93–115.

683 Ye D, Hao Y, Zhu J, et al. 2026. Ultra-fast generation of all-female grass carp via
 684 transplantation of female germline stem cells into zebrafish. *Science China Life*
 685 *Sciences*, DOI: 10.1007/s11427-026-3272-2

686 Ye H, Li CJ, Yue HM, et al. 2017. Establishment of intraperitoneal germ cell
 687 transplantation for critically endangered Chinese sturgeon *Acipenser sinensis*.

688 *Theriogenology*, **94**: 37–47.

689 Ye Y, Wu Q, Chen R. 1989. Studies on cytology of crosses between grass carp and
 690 carp-asynchronization between nucleus and cytoplasm in distant hybridization of fishes.
 691 *Acta Hydrobiologica Sinica*, **13**(3): 234–239. [in Chinese]

692 Yoshizaki G, Yazawa R. 2019. Application of surrogate broodstock technology in
 693 aquaculture. *Fisheries Science*, **85**(3): 429–437.

694 Yu P, Wang Y, Li Z, et al. 2022. Causal gene identification and desirable trait recreation
 695 in goldfish. *Science China Life Sciences*, **65**(12): 2341–2353.

696 Yu P, Wang Y, Yang WT, et al. 2021. Upregulation of the PPAR signaling pathway
 697 and accumulation of lipids are related to the morphological and structural
 698 transformation of the dragon-eye goldfish eye. *Science China Life Sciences*, **64**(7):
 699 1031–1049.

700 Zhang F, Hao Y, Li X, et al. 2022. Surrogate production of genome-edited sperm from
 701 a different subfamily by spermatogonial stem cell transplantation. *Science China Life*
 702 *Sciences*, **65**: 969–987.

703 Zhou L, Wang X, Liu Q, et al. 2021. Successful spermatogonial stem cells
 704 transplantation within pleuronectiformes: first breakthrough at inter-family level in
 705 marine fish. *International Journal of Biological Sciences*, **17**(15): 4426–4441.

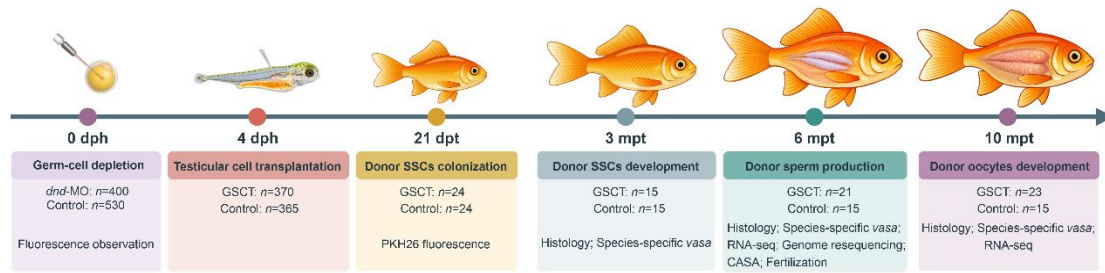


Figure 1 Schematic diagram of the experimental procedure

GSCT, germline stem cell transplantation; dph, days post-hatching; dpt, days post-transplantation; mpt, months post-transplantation; SSCs, spermatogonia stem cells; *dnd*-MO, morpholino targeting *dead end*; RT-PCR, reverse transcription PCR; RNA-seq, RNA sequencing; CASA, computer-assisted sperm analysis; *n*, sample size.

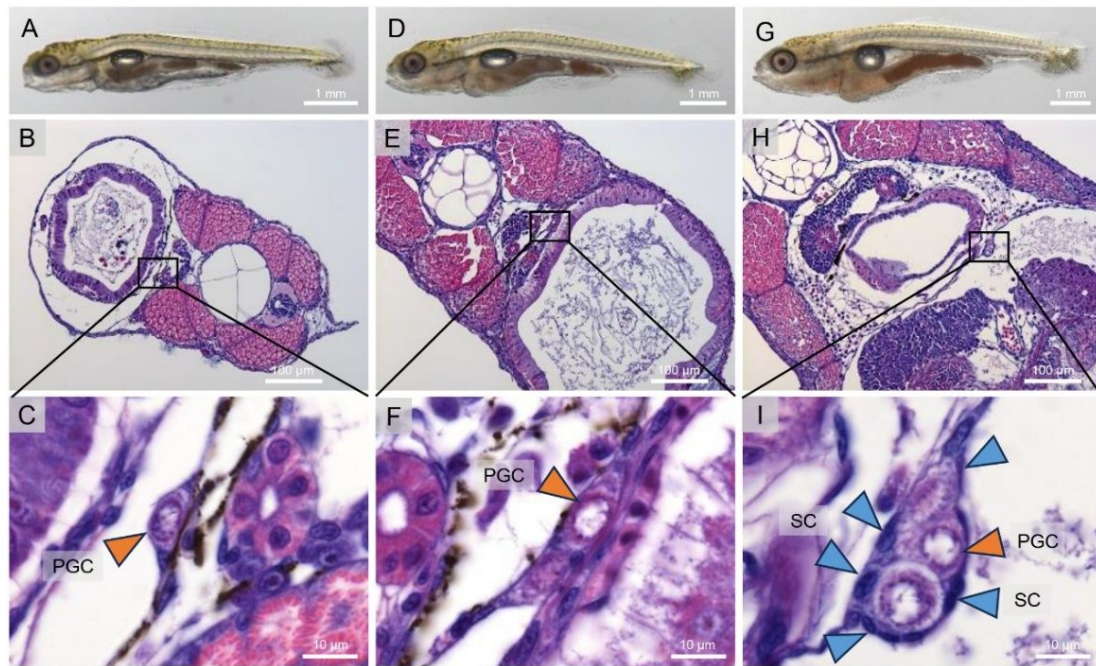


Figure 2 Early gonadal development of recipient goldfish larvae

A–C: Goldfish larvae appearance (A) and H&E-stained transverse sections (B) at 3 dph (TL 6.13 mm). A magnified view of the boxed region in B is shown in (C). D–F: Goldfish larvae appearance (D) and H&E-stained transverse sections (E) at 5 dph (TL 7.02 mm). A magnified view of the boxed region in E is shown in (F). G–I: Goldfish larvae appearance (G) and H&E-stained transverse sections (H) at 7 dph (TL 8.44 mm). A magnified view of the boxed region in H is shown in (I). PGC, primordial germ cell; SC, gonadal somatic cell; H&E, hematoxylin and eosin; TL, total length; dph, days post-hatching.

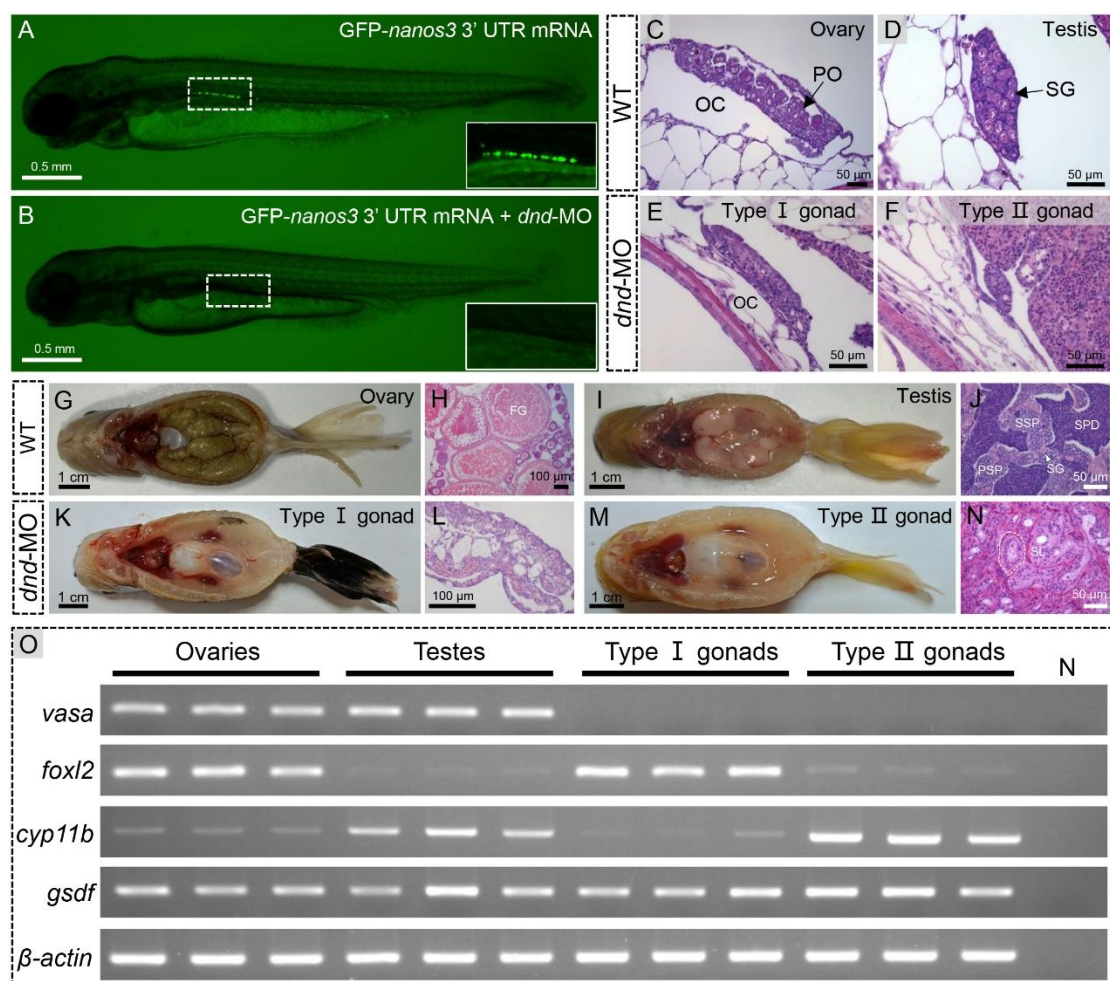


Figure 3 Morphological and molecular characteristics of goldfish gonads following *dnd* knockdown using morpholino (MO)

A, B: Fluorescence images of goldfish larvae (1 dph) with GFP-labeled PGCs. GFP-*nanos3* 3' UTR mRNA was injected alone (A) or together with *dnd*-MO (B) into one-cell stage goldfish embryos to label PGCs and evaluate *dnd*-MO induced germ cell depletion. C–F: Histological structures of wild-type (WT) and *dnd*-MO goldfish gonads at 50 dph. H&E-stained images of the gonads of WT females (C), WT males (D), *dnd*-MO females (E), and *dnd*-MO males (F). G–N: Morphological and histological analyses of WT and *dnd*-MO gonads (5-month-old). External morphology and corresponding H&E-stained sections of ovaries (G, H) and testes (I, J) in WT goldfish, and gonads of *dnd*-MO females (K, L) and males (M, N). O: Gene expression analysis of germ cell marker *vasa*, ovarian granulosa cell gene *foxl2*, testicular Leydig cell gene *cyp11b*, and supporting cell gene *gsdf* in WT and *dnd*-MO goldfish gonads. β -actin was used as an internal control. N, negative control. PO, primary oocytes; OC, ovarian cavity; SG, spermatogonia; FG, fully grown oocytes; PSP, primary spermatocytes; SSP, secondary spermatocytes; SPD, spermatids; SL, seminiferous lobule; H&E, hematoxylin and eosin; GFP, green fluorescent protein; PGCs, primordial germ cells; UTR, untranslated region; dph, days post-hatching.

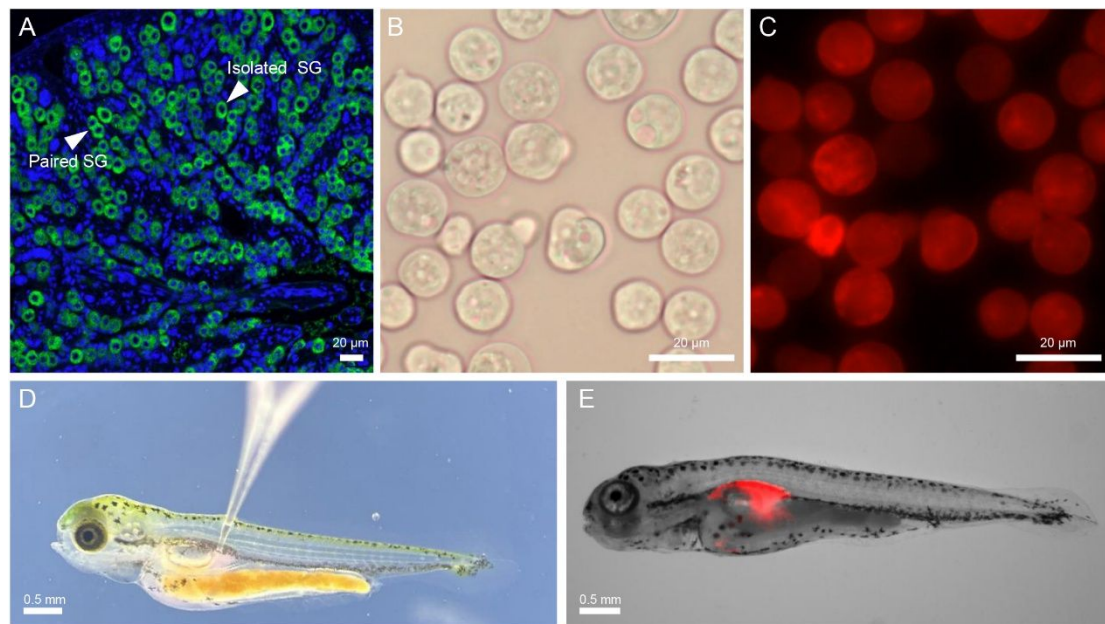


Figure 4 Intraperitoneal transplantation of grass carp testicular cells into goldfish recipient larvae

A: Immunofluorescence staining for Vasa in grass carp testis. B, C: PKH26-labeled grass carp testicular cells in bright-field (B) and fluorescent (C) views. D: Intraperitoneal transplantation of PKH26-labeled testicular cells into 4 dph goldfish larvae. E: Fluorescent image of recipient goldfish showing PKH26-labeled donor cells. SG, spermatogonia; dph, days post-hatching.

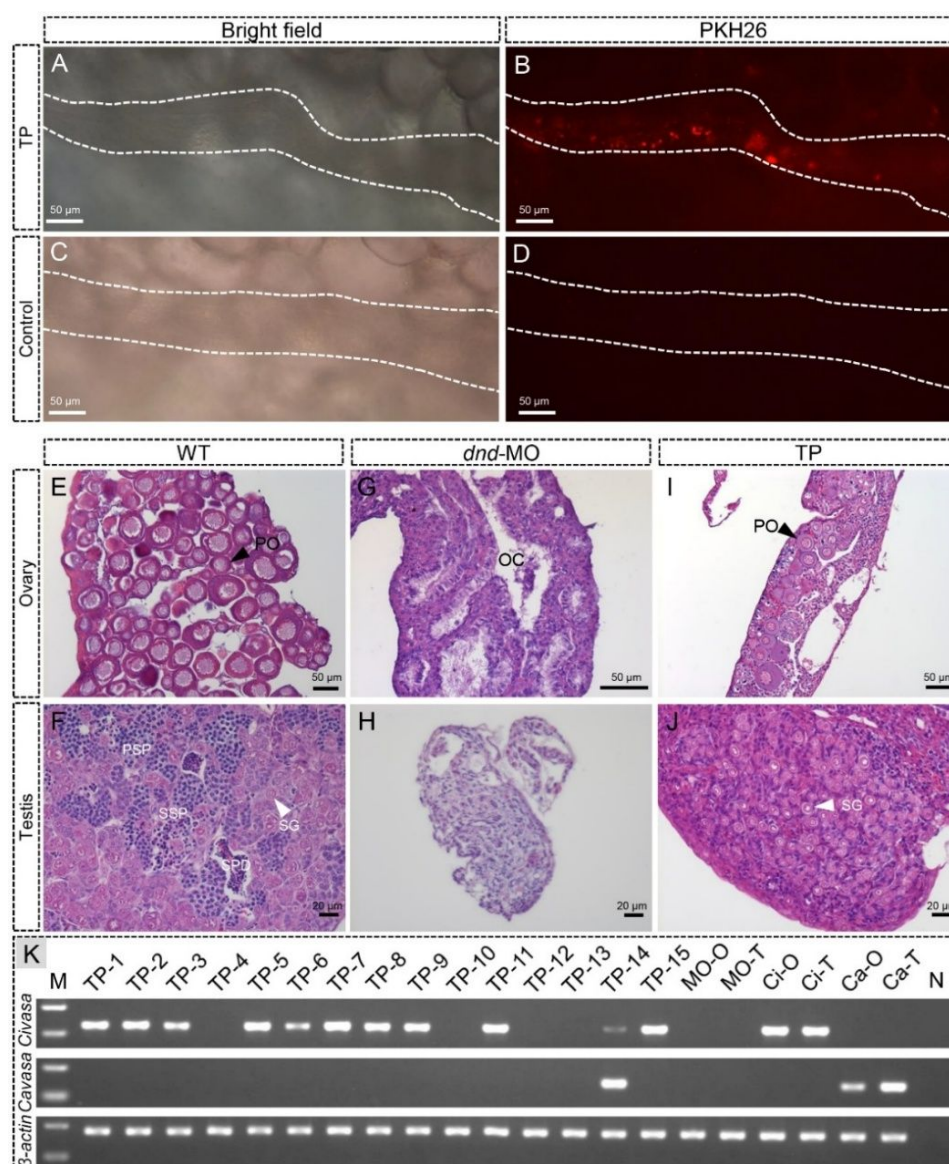


Figure 5 Colonization and differentiation of donor-derived spermatogonia stem cells (SSCs) in gonads of germ cell-depleted goldfish recipients

A–D: Incorporation of PKH26-labeled donor SSCs in germ cell-depleted recipients and control fish at 21 dpt. Bright-field (A, C) and fluorescent (B, D) views of PKH26-labeled donor cells in the gonads of recipients and control goldfish. E–J: Histological structures of wild-type (WT), *dnd*-MO, and successfully transplanted goldfish gonads at 3 mpt. H&E-stained images of the gonads of WT females (E) and males (F), *dnd*-MO females (G) and males (H), and transplanted goldfish (TP) females (I) and males (J). K: Reverse transcription PCR (RT-PCR) assay of grass carp *vasa* (*Civasa*) and goldfish *vasa* (*Cavasa*) expression in TP gonads, *dnd*-MO ovaries (MO-O) and testes (MO-T), grass carp ovaries (Ci-O) and testes (Ci-T), and goldfish ovaries (Ca-O) and testes (Ca-T). β -actin was used as an internal control. N, negative control. PO, primary oocytes; OC, ovarian cavity; SG, spermatogonia; PSP, primary spermatocytes; SSP, secondary spermatocytes; SPD, spermatids; H&E, hematoxylin and eosin; MO, morpholino; dpt, days post-transplantation; mpt, months post-transplantation.

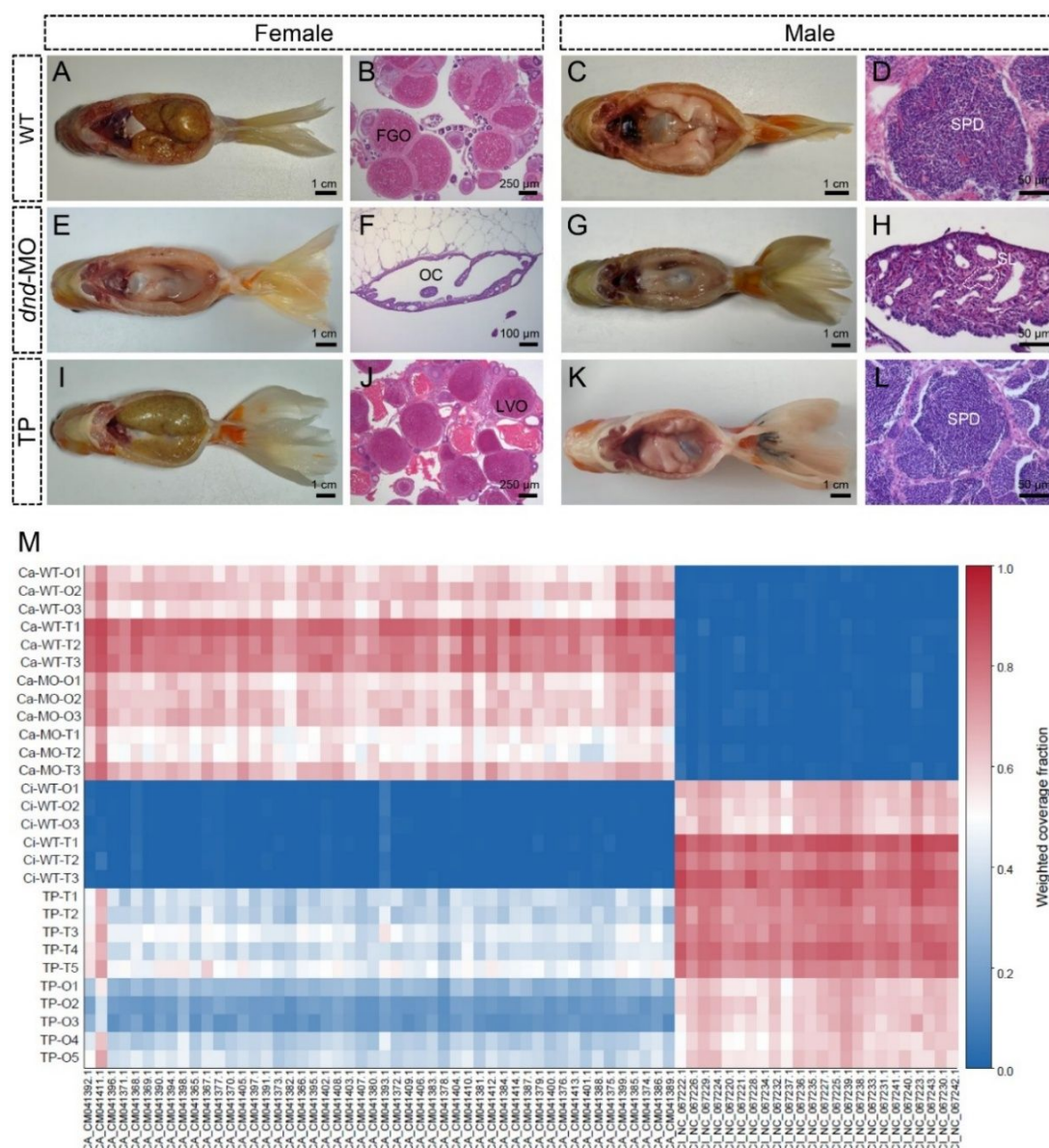


Figure 6 Gametogenesis of colonized grass carp spermatogonia stem cells (SSCs) in recipient goldfish

A–L: Morphology of adult wild-type (WT), *dnd*-MO, and successfully transplanted (TP) goldfish gonads. The external morphology and H&E-stained sections of gonads from WT females (A, B) and males (C, D) at 6 months post-transplantation (mpt), *dnd*-MO females (E, F) and males (G, H) at 6 mpt, and TP females (I, J) at 10 mpt and males (K, L) at 6 mpt, respectively. M: Heatmap showing the weighted coverage fraction of RNA-seq reads of grass carp *vasa*-positive gonads in transplanted goldfish. Weighted coverage fractions were calculated against the consolidated grass carp–goldfish genome for RNA sequencing reads from WT goldfish, *dnd*-MO goldfish, WT grass carp, and grass carp *vasa*-positive gonads from TP gonads. Blue represents low coverage and red represents high coverage; values range from 0 to 1. FGO, fully grown oocytes; SPD, spermatids; OC, ovarian cavity; SL, seminiferous lobules; LVO, late-vitellogenic oocytes; H&E, hematoxylin and eosin; MO, morpholino.

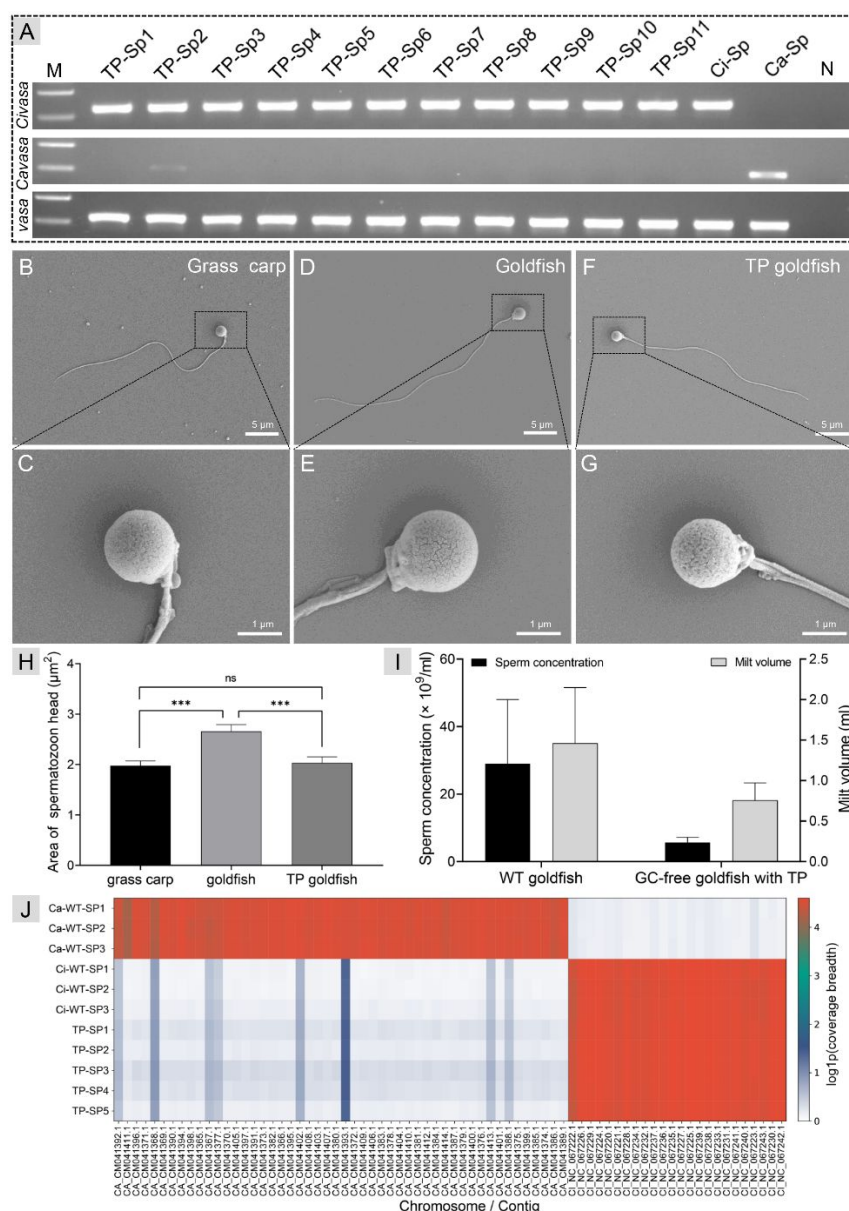


Figure 7 Transplanted goldfish produce donor-derived grass carp sperm

A: Polymerase chain reaction (PCR) detection of *vasa* genomic DNA fragments in sperm from transplanted goldfish using grass carp-specific (*Civasa*), goldfish-specific (*Cavasa*) and common (*vasa*) primers. TP-Sp1–11 represent sperm from 11 successfully transplanted males; Ci-Sp represents grass carp sperm; Ca-Sp represents goldfish sperm. N, negative control. B–G: Scanning electron microscopy images of spermatozoa from grass carp, goldfish, and transplanted goldfish. Magnified images of the sperm heads in B, D, and F are shown in C, E, and G, respectively. H: Head area of spermatozoa comparison among grass carp, goldfish, and transplanted (TP) goldfish. I: Sperm concentration and milt volume in wild-type (WT) goldfish ($n=5$) and TP goldfish ($n=11$). J: Heatmap showing whole-genome sequencing coverage of sperm from WT goldfish (Ca-WT-SP), WT grass carp (Ci-WT-SP), and GSCT goldfish (TP-SP). The horizontal axis represents the chromosomes of the consolidated genome, whereas the vertical axis represents the samples; the colors indicate the log1p (coverage breadth). ns, not significant; ***: $P<0.001$.

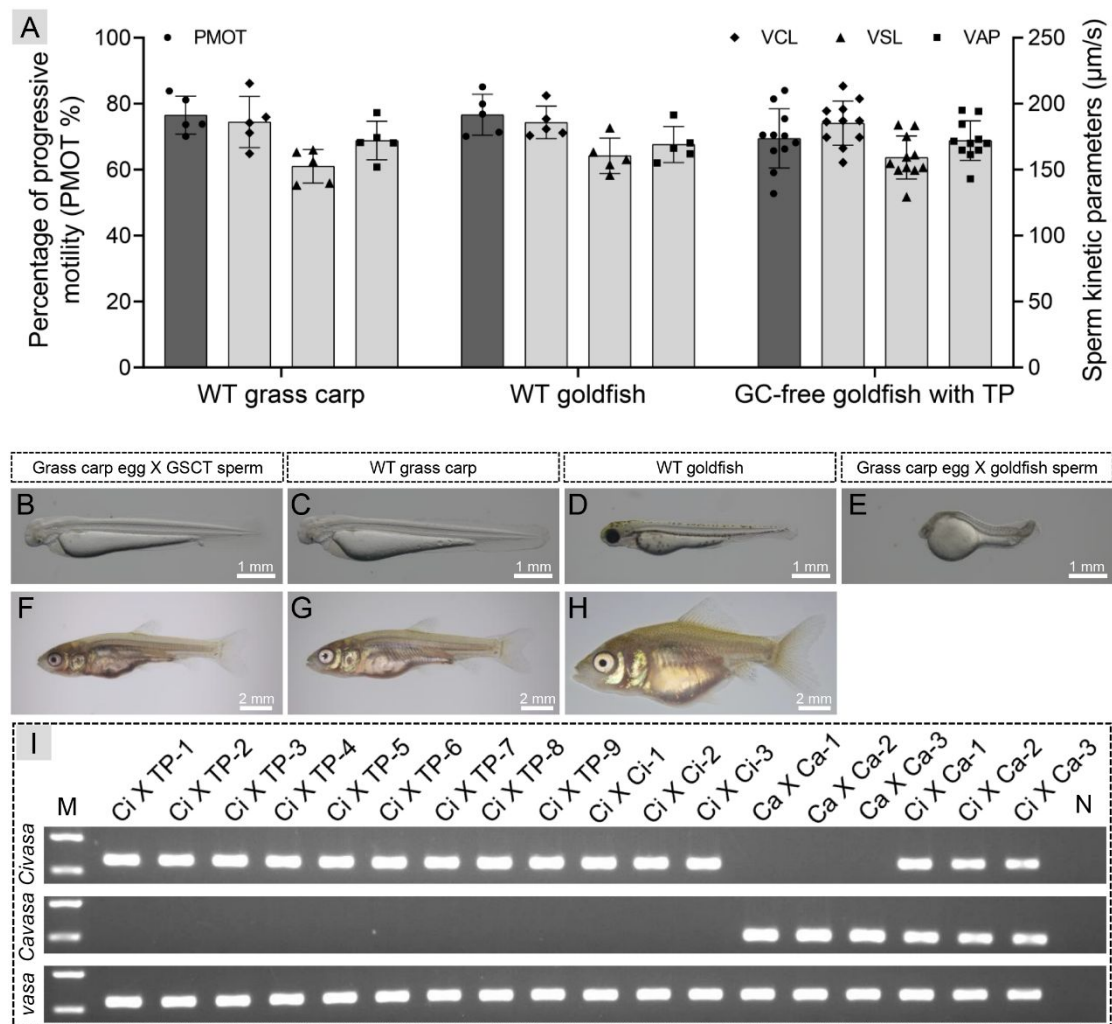


Figure 8 Functional analysis of grass carp sperm produced by surrogate goldfish

A: Progressive motility (PMOT) and sperm kinetic parameters of wild-type (WT) grass carp, goldfish, and GSCT sperm. Parameters include VCL (curvilinear velocity), VSL (straight-line velocity), and VAP (average path velocity). **B–H:** In vitro fertilization of grass carp eggs using GSCT or goldfish sperm. Larvae at 1 and 21 dph obtained from fertilization using GSCT sperm and WT grass carp eggs (**B**, **F**). WT grass carp (**C**, **G**) controls and WT goldfish (**D**, **H**) controls. Larvae at 1 dph obtained from fertilization using goldfish sperm and grass carp eggs (**E**). **I:** Polymerase chain reaction (PCR) detection of *vasa* genomic DNA fragments in larvae from GSCT sperm fertilized with WT grass carp eggs using grass carp-specific (*Civasa*), goldfish-specific (*Cavasa*), and common (*vasa*) primers. Ci X TP-1–9 represent larvae produced by fertilizing WT grass carp eggs with sperm from GSCT males from three parental pairs; Ci X Ci-1–3 represent WT grass carp offspring from three parental pairs; Ca X Ca-1–3 represent WT goldfish from three parental pairs; and Ci X Ca-1–3 represent larvae produced by fertilizing WT grass carp eggs with WT goldfish sperm from three parental pairs. N, negative control; GSCT, germline stem cell transplantation; dph, days post-hatching.

813 **Table 1 Depletion of primordial germ cells (PGCs) by a *dnd*-targeting morpholino**
814 **in goldfish (*Carassius auratus*)**

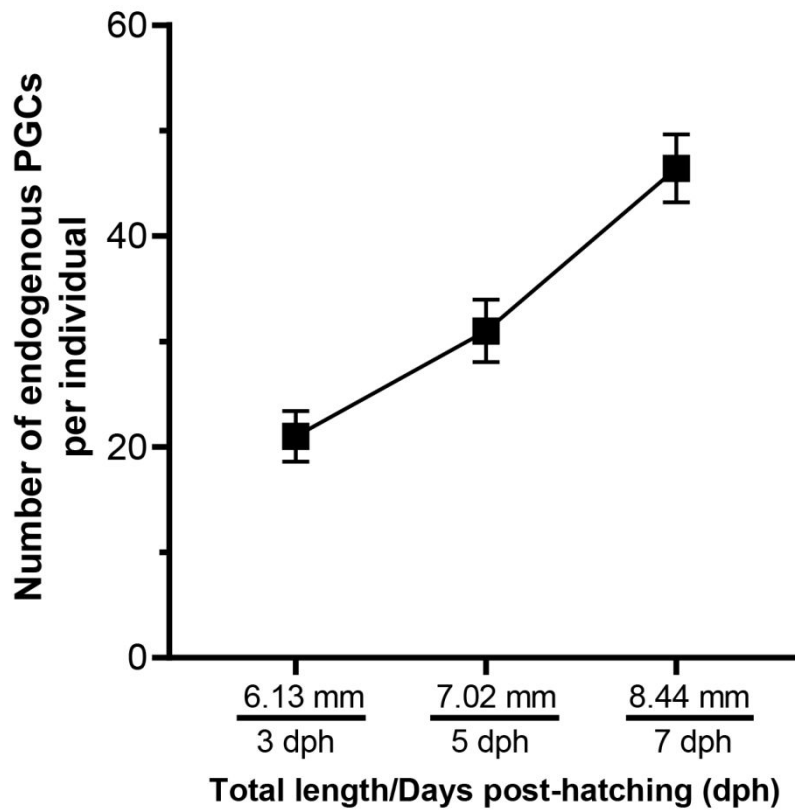
Experimental group	No. of embryos injected	Hatched embryos (%)	Larvae with labeled PGCs/examined (%)
GFP- <i>nanos3</i> 3' UTR mRNA	145	85 (58.62%)	17/28 (60.71%)
	101	62 (61.39%)	21/33 (63.64%)
	112	74 (66.07%)	20/32 (62.50%)
GFP- <i>nanos3</i> 3' UTR mRNA and <i>dnd</i> -MO	129	72 (55.81%)	1/23 (4.35%)
	116	61 (52.59%)	2/27 (7.41%)
	155	83 (53.55%)	2/32 (6.25%)
	189	146 (77.25%)	0/26 (0%)
Control	165	121 (73.33%)	0/25 (0%)
	176	142 (80.68%)	0/31 (0%)

815 Values are shown as counts and percentages. PGCs, primordial germ cells; GFP, green
816 fluorescent protein; UTR, untranslated region; *dnd*-MO, morpholino targeting *dead end*.
817 Each row represents one experimental trial.

818 **Table 2 Survival and colonization rates of donor grass carp spermatogonia stem cells transplanted into goldfish recipients**

Trials	Initial larvae (<i>n</i>)	Survivors at 21 dpt (%)	Colonized/examined at 21 dpt (%)	Gametogenesis/examined at 3 mpt (%)	Males producing grass carp sperm/examined (%)	Females with grass carp late-vitellogenic oocytes/examined (%)
1	126	104 (82.54%)	6/8 (75.00%)	4/5 (80.00%)	3/6 (50.00%)	4/6 (66.67%)
2	120	98 (81.67%)	5/8 (62.50%)	4/5 (80.00%)	6/10 (60.00%)	3/6 (50.00%)
3	124	105 (84.68%)	7/8 (87.50%)	3/5 (60.00%)	2/5 (40.00%)	9/11 (81.82%)
Con 1	142	135 (95.07%)	0/8 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Con 2	116	108 (93.10%)	0/8 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Con 3	107	96 (89.72%)	0/8 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)

819 dpt, days post-transplantation; mpt, months post-transplantation; Con, non-transplanted control. Each row represents one trial.



820

821 **Supplementary Figure S1 Number of primordial germ cells (PGCs) in goldfish**

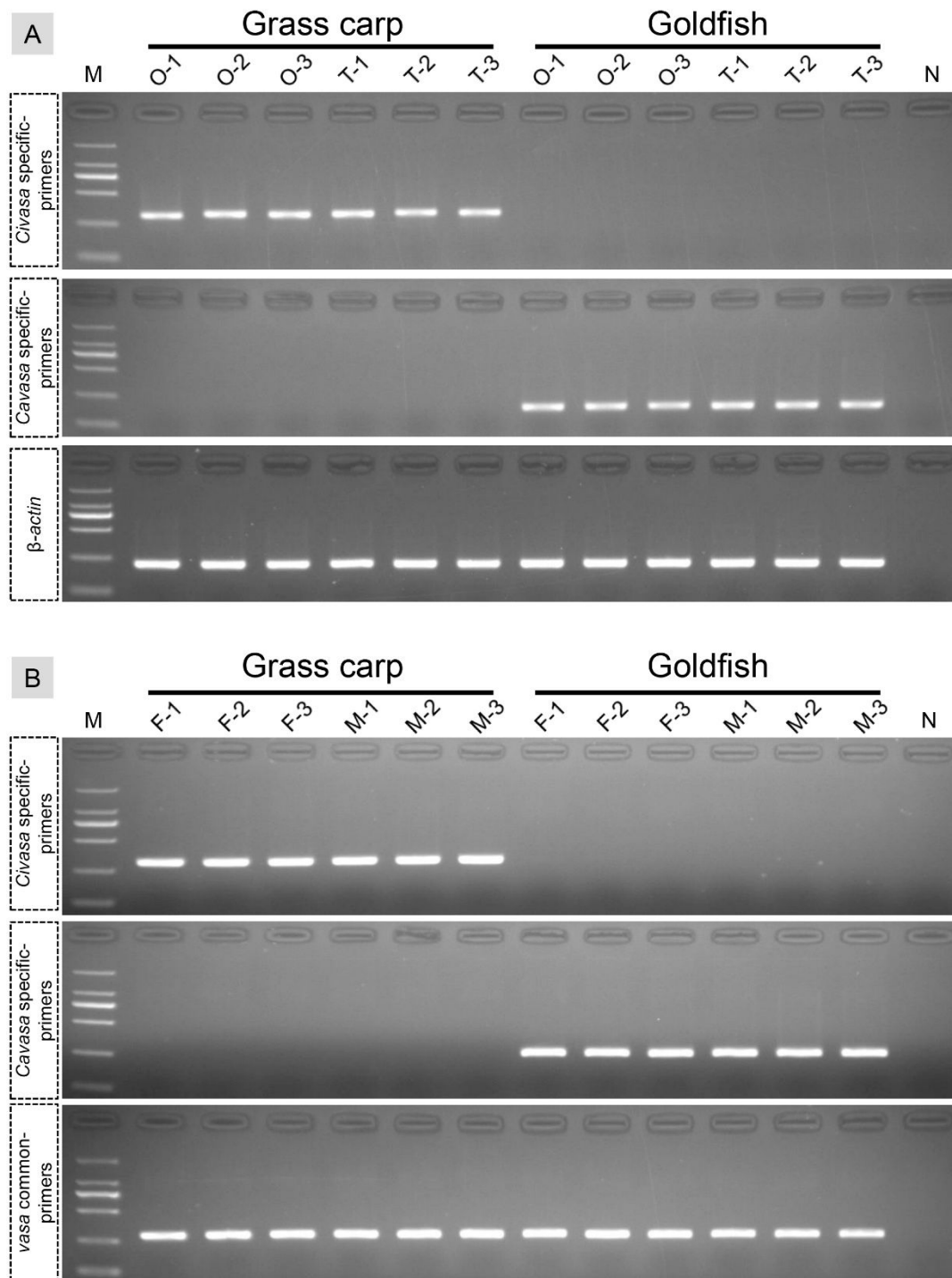
822 **larvae at the indicated stages**

823 PGCs, primordial germ cells; dph, days post-hatching.

824

826 **grass carp**

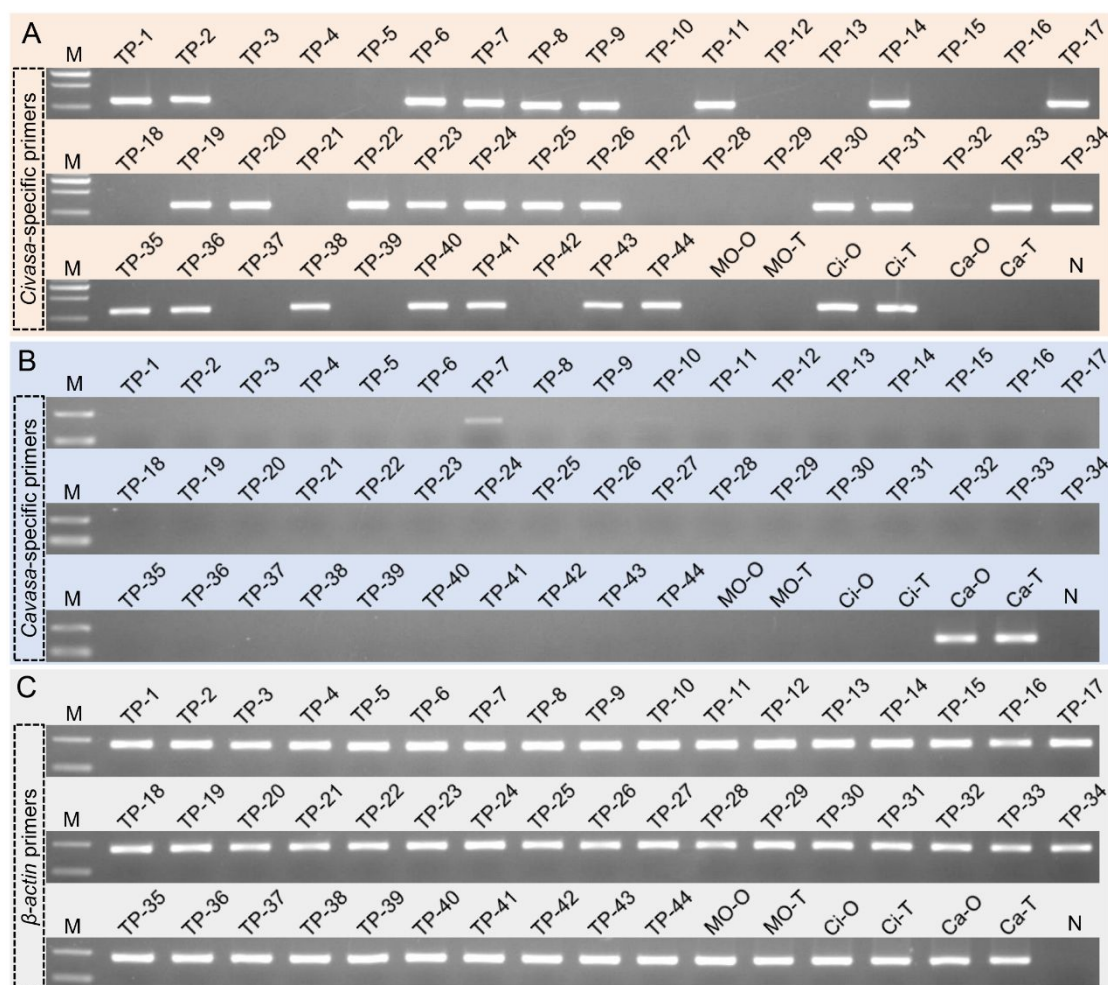
831



Supplementary Figure S3 Species-specific analysis of *vasa* cDNA and genomic

DNA fragments in grass carp and goldfish

A: PCR detection of *vasa* cDNA fragments using grass carp-specific (*Civasa*) and goldfish-specific (*Cavasa*) primers in ovaries (O-1–3) and testes (T-1–3) of grass carp and goldfish. β -actin was used as an internal control. B: Polymerase chain reaction (PCR) detection of *vasa* genomic DNA fragments using species-specific and common primers in females (F-1–3) and males (M-1–3) of grass carp and goldfish. M, DNA marker DL-2000; N, negative control.



Supplementary Figure S4 RT-PCR analysis of grass carp *vasa* (*Civasa*) and goldfish *vasa* (*Cavasa*) gene expression in transplanted goldfish gonads

A: Reverse transcription PCR (RT-PCR) detection of *vasa* cDNA fragments using grass carp-specific (*Civasa*) primers in transplanted (TP) gonads, germ cell-depleted gonads (*dnd*-MO ovary, MO-O; *dnd*-MO testis, MO-T), and positive control gonads (grass carp ovary, Ci-O; grass carp testis, Ci-T; goldfish ovary, Ca-O; goldfish testis, Ca-T). B: RT-PCR detection of *vasa* cDNA fragments using goldfish-specific (*Cavasa*) primers in TP gonads, germ cell-deficient gonads, and positive control gonads. C: β -actin was used as an internal control. M, DNA marker; N, negative control.

851 **Supplementary Table S1 Primer sequences used for polymerase chain reaction**
852 **(PCR)**

853 **Supplementary Table S2 Weighted coverage fractions of RNA sequencing reads**
854 **from wild type (WT) goldfish, *dnd*-MO goldfish, WT grass carp, and successfully**
855 **transplanted recipient gonads with grass carp *vasa* expression on the consolidated**
856 **reference genomes of grass carp and goldfish**

857 **Supplementary Table S3 A comprehensive whole-genome coverage analysis of**
858 **genome resequencing data from wild type (WT) goldfish, WT grass carp, and**
859 **successfully transplanted (TP) sperm**

题目：金鱼借腹生殖产生草鱼配子的潜力与优势

叶欢^{1, #}, 余鹏^{2, #, *}, 杨晓静^{1, #}, 岳华梅¹, 周晓雅³, 屈紫玲¹, 黄玲¹, 侯彦岭¹, 孔辉辉¹, 李彩荣¹, 李志¹, 杨刚¹, 阮瑞¹, 周莉^{2, 4}, 桂建芳^{2, 4}, 李创举^{1, *}

¹ 中国水产科学研究院长江水产研究所, 农业农村部淡水生物多样性保护重点实验室, 中国湖北武汉 430223

² 中国科学院水生生物研究所, 水产品种质创制与高效养殖全国重点实验室, 中国湖北武汉 430072

³ 安徽农业大学动物科技学院, 中国安徽合肥 230036

⁴ 中国科学院大学, 中国北京 100049

共同第一作者

* 共同通讯作者, 电子邮箱: yupeng@ihb.ac.cn; lcj@yfi.ac.cn

摘要

草鱼 (*Ctenopharyngodon idellus*) 是亚洲重要的养殖鱼类, 其产量约占全球淡水养殖总产量的 10%。然而, 草鱼初次性成熟周期长达 4–5 年, 严重制约了其育种进程。生殖干细胞移植技术利用性成熟周期较短的受体产生供体配子, 从而缩短世代间隔时间, 为加快鱼类育种提供新的途径。本研究选用体型较小、世代间隔较短、繁殖力高且可全年规模化繁殖的金鱼 (*Carassius auratus*) 作为受体, 开展草鱼借腹生殖研究。利用 morpholino 敲降 *dnd* 基因, 获得缺乏生殖细胞的不育金鱼受体, 并将从 1.5 龄草鱼精巢中制备的供体细胞移植至出膜后 4 天金鱼仔鱼的腹腔。移植后 6 个月, 21 尾雄性金鱼受体中有 11 尾成功产生了具有正常功能的供体来源草鱼精子, 平均精液量为 754 μ L。此外, 与生殖细胞缺失后无法形成卵巢的不育斑马鱼受体不同, 不育金鱼受体的性腺体细胞微环境能够支持供体草鱼精原干细胞分化为卵原细胞, 并在移植后 10 个月进一步发育为卵黄生成后期的卵母细胞。上述结果表明, 金鱼是具有应用潜力的草鱼生殖干细胞移植受体, 可为供体草鱼精子的规模化、快速生产提供技术基础, 并为未来通过生殖调控获得供体草鱼卵子提供研究依据。

关键词: 草鱼; 生殖干细胞移植; 金鱼; 代孕繁殖; 精子