Germ cells are not the primary factor for sexual fate determination in goldfish

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Abstract

The presence of germ cells in the early gonad is important for sexual fate determination and gonadal development in vertebrates. Recent studies in zebrafish and medaka have shown that a lack of germ cells in the early gonad induces sex reversal in favor of a male phenotype. However, it is uncertain whether the gonadal somatic cells or the germ cells are predominant in determining gonadal fate in other vertebrate. Here, we investigated the role of germ cells in gonadal differentiation in goldfish, a gonochoristic species that possesses an XX-XY genetic sex determination system. The primordial germ cells (PGCs) of the fish were eliminated during embryogenesis by injection of a morpholino oligonucleotide against the *dead end* gene. Fish without germ cells showed two types of gonadal morphology: one with an ovarian cavity; the other with seminiferous tubules. Next, we tested whether function could be restored to these empty gonads by transplantation of a single PGC into each embryo, and also determined the gonadal sex of the resulting germline chimeras. Transplantation of a single GFP-labeled PGC successfully produced a germline chimera in 42.7% of the embryos. Some of the adult germline chimeras had a developed gonad on one side that contained donor derived germ cells, while the contralateral gonad lacked any early germ cell stages. Female germline chimeras possessed a normal ovary and a germ-cell free ovary-like structure on the contralateral side; this structure was similar to those seen in female morphants. Male germline chimeras possessed a testis and a contralateral empty testis that contained some sperm in the tubular lumens. Analysis of *aromatase*, *foxl2* and *amh* expression in gonads of morphants and germline chimeras suggested that somatic transdifferentiation did not occur. The offspring of fertile germline chimeras all had the donor-derived phenotype, indicating that germline replacement had occurred and that

the transplanted PGC had rescued both female and male gonadal function. These findings suggest that the absence of germ cells did not affect the pathway for ovary or testis development and that phenotypic sex in goldfish is determined by somatic cells under genetic sex control rather than an interaction between the germ cells and somatic cells.

Keywords

Sex differentiation; Primordial germ cells; Gonadal fate determination; Transplantation; SPT; Teleost

INTRODUCTION

The interaction between germ cells and gonadal somatic cells is important for early gonadal formation and sex differentiation in vertebrates. Recent studies employing knockout or knockdown strategies to investigate the functions of genes involved in early gonadal differentiation have considerably increased our understanding of the contributions of germ cells and somatic cells to gonadal formation. Thus, mice that are null for *Dnd*1, *nanos*2, *nanos*3, *Figla*, *Nobox* or *Sohlh2*, which are expressed in the germ cells, show loss of primordial germ cells (PGCs) or, in females, loss of oocytes before or during early formation of the ovary and failure to form or maintain follicles (Youngren et al., 2005; Choi and Rajkovic, 2006; Choi et al., 2008). The loss of the germ cells did not affect testicular soma differentiation in *nanos*2 and *nanos*3 null mice or in *Ter* mutants; these mice showed normal testicular structure with seminiferous tubules (Tsuda et al., 2003; Youngren et al., 2005). Therefore, the presence of germ cells is more important for folliculogenesis rather than for primary sex determination or gonadal differentiation in mammals.

The contribution of the germ cells to gonadal formation is more dramatic in teleosts than mammals. Complete ablation of the germ cells was first reported in zebrafish (Weidinger et al., 2003). Knockdown of the *dead end* (*dnd)* gene during zebrafish embryogenesis causes abnormal migration of the PGCs and, consequently, induces germ cell deficiency in the fish. These germ cell deficient fish all develop as males with normal expression of the testicular genes, *amh*, *sox9a* and *11b-HSD*, which are involved in testicular differentiation and development in intact males (Siegfried and Nüsslein-Volhard, 2008). These reports indicate that absence of germ cells biases sex determination toward maleness in zebrafish. In medaka, a similar response occurs to

produce sex reversal in XX germ cell deficient fish (Kurokawa et al., 2007). Inhibition of *cxcr4* or *nanos,* genes involved in PGC migration or development, causes female-to-male sex reversal. The somatic cell lineages of XX individuals initially express *aromatase* and *foxl2*, which are involved in ovarian differentiation; however, the expression of these genes is not maintained. The cells eventually express *P45011b*, a gene specific to Leydig cells, in the same manner as seen in zebrafish. In medaka, the male-determining gene *DMY*, located on the Y chromosome, has a strong influence on phenotypic sex determination (Matsuda et al., 2002). Therefore, the absence of germ cells from the early gonad induces transdifferentiation of somatic cells to the male pattern regardless of the chromosomal sex determination system. However, this outcome is not consistently seen in other fish species. We recently reported that absence of germ cells in the loach gonad did not affect gonadal fate determination (Fujimoto et al., 2010). In this species, fish with induced germ cell depletion following knockdown of *dnd* developed either as females or males, indicating that the germ cells are not important for ovarian differentiation in the loach.

During embryogenesis, primordial germ cells (PGCs) have the potential to enter either spermatogenesis or oogenesis. In the mouse, the sex chromosome constitution of the PGCs does not influence the decision on whether the cells undergo spermatogenesis or oogenesis; most PGCs up to 11.5 dpc develop as prospermatogonia in a male urogenital ridge environment or as oocytes in a female urogenital ridge environment (12.5 dpc), regardless of the chromosomal sex of the PGCs (Adams and McLaren, 2002). In teleosts, however, it is uncertain whether the germ cells or gonadal somatic cells predominantly determine primary gonadal sex. Zebrafish germline chimeras that are produced by transplantation of a single PGC (derived from a somite

stage embryo) into blastula stage embryos, only developed as males; this suggests that chromosomal constitution has a weak effect on sexual fate determination (Saito et al., 2008). By contrast, the transplantation of zebrafish ovarian germ cells derived from adult ovaries into 2-week-old larvae of a danio interspecies hybrid (*Danio rerio* x *Pearl danio*), resulted in the appearance of male and female fish among these germline chimeras (Wong et al., 2011). This implies that ovarian germ cells can differentiate into either spermatogonia or oogonia. Overall, these reports strongly suggest that the germ cells themselves, or even differentiated germ cells (oogonia), are not sufficient for inducing ovarian differentiation in these species.

Goldfish possess an XX-XY sex determination system (Yamamoto and Kajishima, 1968). This species has a long history of use as a model for fish developmental biology because it is possible to control spawning, fertilization, and embryonic development by varying the water temperature and to manipulate both genetic and phenotypic sex by gynogenesis and temperature control (Yamaha et al., 1986; Yamaha et al., 1999; Goto-Kazeto et al., 2006). Previous reports indicate that goldfish PGCs, or their precursor cells, are located in the lower part of the blastoderm at the mid-blastula stage (Kazama-Wakabayashi et al., 1999; Otani et al., 2002). Transplantation experiments involving grafting blastomeres or blastoderm from this region into a host blastula showed that donor PGCs migrated towards the gonadal ridge in the chimeras (Yamaha et al., 2001; 2003). Although this transplantation approach allowed the introduction of a donor germline into the host, it was not feasible to prevent the simultaneous introduction of donor somatic cells. To eliminate contamination by donor somatic cells, a protocol for transplantation of a single PGC into each host embryo was developed. This technique is termed the single PGC transplantation (SPT)

method. Through use of this approach, germline chimeras carrying donor-derived gametes have been generated with relatively high efficiency in zebrafish (Saito et al., 2008). In combination with the complete ablation of host PGCs prior to donor PGC transplantation, the SPT method provides a powerful tool for investigating the contribution of germ cells to early gonad formation and to primary sex differentiation.

In this study, we used goldfish to determine whether germ cells or gonadal somatic cells determine primary sex differentiation. Additionally, we sought to test whether it is feasible to induce ovarian differentiation naturally by transplantation of a single PGC. First, we isolated the goldfish *dnd* gene, which plays a vital role in the migration and survival of PGCs. We then designed an anti-sense morpholino oligonucleotide against this gene to induce elimination of endogenous PGCs before they enter the gonadal ridge. Second, the gonadal morphology of germ cell depleted fish was examined histologically and the patterns of expression of the germ cell marker gene, *vasa*, and candidate sex-determining genes, *cyp19a1*, *foxl2* and *amh*, were determined to identify gonadal sex. Third, we tested the contribution of a single PGC to gonadal formation and sex differentiation following transplantation into a sterilized host embryo. Our results from the goldfish were different to those reported for zebrafish and medaka. Sexually dimorphic gonads were observed in germ cell-depleted goldfish as has been found in the loach. Furthermore, transplantation of a single donor PGC induced formation of both ovaries and testis, and did not bias the primary sex determination outcome.

MATERIALS AND METHODS

Ethics

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Hokkaido University and Field Science Center for Northern Biosphere, Hokkaido University.

Fish and tissue collection

Goldfish (*Carassius auratus*) were kept in the Nanae Fresh Water Laboratory, Hokkaido University. Adult albino goldfish were obtained from a commercial supplier. Artificial fertilization was performed as described by Yamaha et al. (2001). The dechorionation and culture conditions for the embryos were as described by Yamaha and Yamazaki (1993). We used published criteria for classification of embryonic developmental stages (Kajishima, 1960; Yamaha et al., 1999). Fertilized eggs and embryos were kept at 20˚C, and held at this temperature for 3 months to avoid temperature induced female-to-male sex reversal (Goto-Kazeto et al., 2006). Fry were fed *Artemia nauplii* twice per day, mature fish were fed artificial flakes once per day. Under laboratory conditions, male fish take approximately six months to reach puberty, while females take at least one year until they become reproductively competent. Morphants and germline chimeras were collected from 5 months to 2-years-old.

Morpholino injection for PGC depletion

The full sequence *dnd* cDNA was isolated by RACE (GenBank ID: JN578697). The deduced protein showed 79.5% identity with *dnd* of zebrafish (GenBank ID: AY225448) and 63.1% with the loach (GenBank ID: AB531494); an RNA binding

domain was present in this protein (Supplementary Fig. 1A). The 5'UTR sequence of the longer form was 66 nt, but one of the four 5' RACE clones was found to be shorter and to be missing 27 nt from a position 8 nt upstream of the start codon (Supplementary Fig. 1B). Identification of a target site for the morpholino oligonucleotide (MO) was determined according to manufacturer's protocol (GeneTools, LLC); the selected site corresponded to the sequence of the dominant form. Whole mount *in situ* hybridization showed that *dnd* mRNA localized in the cleavage furrows and in PGCs in goldfish embryos, with a similar distribution pattern as *vasa* mRNA expression (Supplementary Fig. 1C) (Otani et al., 2002). Endogenous PGCs were targeted by injecting the MO into the lower part of the blastodisc of goldfish embryos at the 1-4 cell stage. The injection volume was estimated under the microscope to approximate to 1/7th of the diameter of each egg. To determine the optimal dose for depletion of PGCs, different concentrations of morpholino (0, 50, 100 and 200 μM in 0.2M KCl) were compared by examination of the rate of abnormality and gonadal phenotype of the resultant fish (Supplementary Fig. 2). Ablation of PGCs was checked by co-injecting *dnd* MO and GFP-*nos*1 3'UTR mRNA, and the gonads of 9-month-old morphants were examined to confirm the success of the ablation treatment. As a result of the analyses, we chose a concentration of 50 μM for subsequent experiments.

Whole mount *in situ* hybridization (WISH) and histology

WISH was carried out essentially as described previously (Saito et al., 2004). Briefly, chemically dechorionated embryos were fixed overnight at 4˚C in freshly prepared 4% paraformaldehyde, then washed in PBS containing 0.1% Tween 20 (PBST), dehydrated through a methanol series, and stored in 100% methanol at -20˚C prior to further

processing. After rehydration, embryos at the somite stage were treated for 10 to 20 min with proteinase K (10 µg/ml in PBST). After prehybridization for 3 h, the embryos were hybridized overnight at 62˚C in hybridization buffer containing anti-sense or sense probe at 50 ng/ml. Embryos were then washed at high stringency and transferred to blocking buffer, PBST with 10% blocking reagent (Roche), for 3 h. Following preblocking, embryos were incubated overnight at 4˚C with an anti-digoxigenin-alkaline phosphate conjugate at a 1/8000 dilution in blocking buffer. The staining was carried out using NBT/BCIP and the preparations were postfixed in 4% paraformaldehyde.

Anti-sense and sense *dnd* probes (GenBank accession JN578697) for the goldfish were prepared using a digoxigenin (DIG) RNA labeling kit (Roche). A 500 bp fragment from the coding region was generated by PCR from *dnd* cDNA using gene-specific primers (forward primer, 5'-TACAGGTGCGCCATCACAGGTG-3'; reverse primer, 5'-GTCAGAGATCATTCGCAGCACCGTCAG-3'). The zebrafish anti-sense *vasa* probe is able to detect goldfish PGCs, as reported previously (Otani et al., 2002).

For histology, tissues were fixed in Bouin's solution overnight and stored in 70% ethanol until further processing. Paraffin sections (8 µm) were cut and stained with Hematoxylin and Eosin. Photographs were taken using a LEICA DM2500 microscope or a LEICA MZ16F stereomicroscope, each equipped with a CCD camera.

RT-PCR

Frozen tissues in RNAlater solution (Sigma-Aldrich, Japan) were cut into small pieces and total RNAs were extracted using TRIzol reagent (Invitrogen, Japan) according to the manufacturer's protocol. RNA from type I and II gonads of morphants and the

empty gonads of germline chimeras was precipitated by adding a high salt solution (1.2M NaCl, 0.8M sodium citrate) to the isopropanol. PrimeScript reverse transcriptase (Takara, Japan) was used for cDNA synthesis. Gene expression in the gonads of morphants and germline chimeras was examined using *cyp19a1* and *foxl2* as ovarian markers, *amh* as a testicular marker, *vasa* as a germ cell marker, and *b-actin* as an internal control. To date, *foxl2* and *amh* have not reported in goldfish; therefore, we isolated their partial cDNAs, and submitted the sequences to GenBank (*foxl2*, JN578698; *amh*, JN578699). Briefly, the deduced protein sequence of goldfish foxl2 showed 94.3% identity to that of the zebrafish, while AMH had 82.1% identity. RT-PCR was carried out using gene specific primers with the following conditions: 30 cycles of 94˚C for 30 s, optimal annealing temperature for 30 s and 72˚C for 30 s (Table 1).

Generation of germline chimeras

To eliminate the possible influence of donor derived somatic cells on gonadal sex differentiation in germline chimeras, a single PGC was transplanted into each host embryo as described by Saito et al. (2008). To test germline transmission to the next generation, we used the albino phenotype as donor and wild type as host. Briefly, GFP-labeled donor PGCs were generated by injecting GFP- nos1 3'UTR mRNA into albino phenotype embryos at the 1-4 somite stage, and the labeled PGCs at the 5- to 10-somite stage were used for transplantation into wild type blastulae. Development of the host PGCs was blocked by injection of a 50 μM solution of *dnd* MO. Chimeric embryos were identified and photographed using a LEICA AF 6000 fluorescence stereomicroscope equipped with a CCD camera. Embryos were cultured for two days in

a 96-well plate filled with Ringer's solution supplemented with antibiotics, then they were transferred to a 24-well plate. Germline chimeras were identified by the presence of donor PGCs in the host embryo at days 2 and 3 after transplantation.

Examination of the fertility and offspring of germline chimeras

To determine whether the gametes produced by the germline chimeras were functional, gametes of chimeric fish were used in a fertlization experiment with gametes of albino phenotype fish; this procedure was performed three times at 6- to 9- month intervals. Germline chimeras were given an HCG injection to induce spermiation or ovulation one day before the fertilization experiment (see Yamaha et al., 2001). Offspring were raised as described above and their phenotypes were recorded.

Results

dnd is expressed in PGCs and is required for the maintenance and migration of PGCs

In situ hybridization using a *dnd* anti-sense probe showed that *dnd* mRNA was located in PGCs during goldfish embryonic development, as has already been reported for zebrafish, medaka and loach (Weidinger et al., 2003; Liu et al., 2009; Fujimoto et al., 2010). The *dnd* mRNA signal was also detected in the cleavage planes of 4-cell stage embryos and in some single cells located in the marginal region of the lower part of the blastodisc at the blastula stage (Supplementary Fig. 1C(a, b)). At the somite stage, *dnd* mRNA was expressed in cells located along both sides of the embryonic body (Supplementary Fig. 1C(c-e)). The pattern of localization of *dnd* mRNA was the same as that of *vasa* mRNA, which is a germ cell marker gene in all vertebrates (Fig. 1A,B,E,F).

Injection of an MO against *dnd* into1- to 4-cell stage embryos caused depletion and mal-migration of PGCs. *In situ* hybridization analysis using a *vasa* anti-sense probe identified a few PGCs with a weak signal in a gonadal region of the embryonic body of morphants at the 5-somite stage (Fig. 1C and D). Most of the PGCs had disappeared from the morphants by day 2 (the late somite stage) although a few were still present in extra-gonadal regions, such as the tail (Fig 1G and H). This observation was confirmed by co-injection of *dnd* MO and GFP-*nos*1 3'UTR mRNA into 1- to 4-cell stage embryos. At day 3, the embryos contained GFP-labeled PGCs that were aligned along the axis of both sides of the embryonic body; however, almost no GFP-labeled PGCs were found in morphants (Fig. 2A,B and Table 2). Further

examination of PGC ablation in the mophants was carried out using a histological analysis of the trunk region on day 10. At this stage, PGCs had reached the dorsal peritoneum of the upper part of the body cavity. The PGCs were observed as single, relatively large cells that were occasionally associated with somatic cells. No PGC-like cells were found in morphants (n = 10) (Fig. 3).

The concentration of MO did not seem to affect the rate of abnormal embryos, although there were clear differences between different batches of embryos (Supplementary Fig. 2A). The most common deformity at day 2 was pericardiac edema and hematoma (Supplementary Fig. 2B). These abnormal embryos did not survive. Dose dependent sterility was observed in 9-month-old fish that had been injected with the MO in the concentration range 10 – 200 µM (Supplementary Fig. 2C). In the controls, all fish possessed either a well-developed ovary or testis on both sides (described as "large-bilateral" in Supplementary Fig. 2C). Half of the embryos injected with 10 µM MO possessed a "string-like" gonad on both sides due to the complete elimination of the germ cells. These string-like gonads could be sub-divided into two categories, type I and type II. Various types of ovarian and testicular structures were identified in the remainder of the embryos by external inspection: well-developed gonad on one side ("large-unilateral"); slender, but not "empty", gonad on one side or both sides ("small-unilateral" or "small-bilateral", respectively); or partially slender gonad on one side. Most of the embryos injected with 50, 100 or 200 µM MO possessed string-like gonads, with a single exception that received 100 µM; therefore, inhibition of *dnd* translation, and thus the elimination of the germ cells, was achieved between 50 and 200 µM MO.

Germ cell depletion does not alter gonadal fate determination

In 5-month-old fish, the ovary and testis were distinguishable by their external appearance (Fig. 4A and C). Ovaries had an ovarian cavity and lamellar structure with oocytes at the perinuclear stage (Fig. 4E and I); the testis was attached to the coelomic wall and had the full stages of germ cell development in each cyst (Fig. 4G and M). The gonads of morphants had two types of external appearance: flat and relatively transparent (type I); or, narrow and tube-like (type II) (Fig. 4B and D). Histological analysis of these gonads showed that type I was an "empty ovary" with a clear ovarian cavity, blood vessels and connective tissues (Fig. 4F). In the type I gonad, no ovarian lamellae had developed at this stage; however, mesh-like structures were observed in the central zone of type I gonads in 1-year-old female morphants (Supplementary Fig. 3). These mesh-like structures developed on the side of the gonads equivalent to the area of ovarian lamellae formation in control ovaries. Externally, the type I gonads in some female morphants were flat and transparent with two parallel lines in each gonad. The type II gonad was an "empty testis" with a tubular structure, blood vessels and efferent duct (Fig. 4H). In control testes, the seminiferous tubules contain cysts in which the germ cells undergo spermatogenesis synchronously, with spermatozoa in the tubular lumen (Fig. 4M). In type II gonads, the seminiferous tubules were lined with cells that were similar to Sertoli cells and contained an empty central tubular lumen (Fig. 4N). The tubular lumens became more prominent in 1-year-old male morphants (Supplementary Fig. 3).

Analysis of the expression of candidate sex-determining genes in the gonads of morphants and control fish gave results consistent with their external gonadal morphology. *vasa*, a gene expressed specifically in germ cells, was present in control

gonads but could not be detected in the gonads of morphants (Fig. 5). Expression of *foxl2* and *cyp19a1*, genes mainly expressed in ovarian follicles, was detected in the ovaries of control fish and in type I gonads. Three control testes were analyzed by RT-PCR and one was found to express *foxl2*; no expression was detected in any male morphant type II gonads. *amh* is known to be expressed in the testis at a higher level than in the ovary (Siegfried and Nüsslein-Volhard, 2008; Wang and Orban, 2007; Vizziano et al., 2008). We found *amh* expression in control ovaries and testes, and in morphant type I and II gonads; however, expression was relatively weak in the ovaries. The expression of *amh* showed that somatic cell differentiation of granulosa cells in the ovaries or Sertoli cells in the testes had occurred. Overall, the absence of germ cells did not affect the expression patterns of *foxl2*, *cyp19a1* or *amh*.

Donor PGC migration and germline chimeras

To examine whether function could be restored to the empty gonads of morphants, we created germline chimeras by transplanting a single PGC into each *dnd* MO treated host blastula. To aid identification of PGCs, they were labeled with GFP-*nos*1 3'UTR mRNA. Fluorescent PGCs were easy to distinguish after the late epiboly stage. In goldfish, PGCs are more widely distributed than in zebrafish embryos (Otani et al., 2002; Saito et al., 2006). In day 3 embryos, the labeled PGCs were mostly located on both sides of the trunk region between somites 6 and 13 (87.7% of the total PGCs counted in 9 embryos), when observed from the lateral side of the body (Fig. 6A). From a dorsal viewpoint, 94.3% of PGCs were observed in region B, below the notochord and somites (Fig. 6A). The remaining PGCs (5.8%) were located at the body surface in region A (Fig. 6A). The transplanted PGC migrated to the gonadal ridge of the host

embryos. Chimeras in which the donor PGC was located between somites 6 and 13 (from a lateral viewpoint) and below region B were classified as germline chimeras. In total, 42.7±1.0% (*n*= 28) of the chimeras had donor PGCs at the gonadal region (Table 3). Approximately half of the chimeras (50.0±2.9%) had a donor PGC located at various positions outside the gonadal region. In some of the host embryos (15/28 of germline chimeras, 53.6%), the transplanted PGC divided once after transplantation (Fig. 6B(a)). PGC division was observed in both day 1 and day 2 chimeras (Fig. 6B(b-c)). In some cases, donor PGCs that had originated from a single PGC did not migrate simultaneously, even when both PGCs were in the correct position on day 1 (Fig. 6B(a-c)). Some donor germ cells could still be detected by fluorescence microscopy up to 1.5 months after transplantation; these germ cells were located just under the swim bladder (Fig. 6B(d)). There were 28 germline chimeras at day 3; however, only 21 germline chimeras survived until the time of examination.

Gonadal development and sex of germline chimeras

Previous analyses have shown that various factors determine whether or not a donor-derived PGC contributes to gonadal development in a germline chimera: the relationship of the donor and host, *i.e.* inter- or intra-species; sterilization of the host; and, transplantation of the PGC with or without somatic cells (Saito et al., 2010). Even if the donor PGC settles at the gonadal region during embryogenesis, it may not contribute to gametogenesis. Over the course of the study, SPT germline chimeras were examined from 5 months to 2 years. Nine of the 21 germline chimeras possessed a fully developed ovary or testis on one side of the body (Fig. 7A-C, Fig. 8A-C, Table 4) but a poorly developed contralateral gonad (Fig. 7B,D and Fig. 8B,D). However the degree of

gonadal development varied among the fish (Table 5). A well-developed ovary or testis similar to that of a control was observed in 7 of the 9 germline chimeras (Fig. 7A,B, Fig. 8A,B). The remaining 2 chimeras showed partial development of the gonad in which the germ cells did not extend from the anterior to posterior ends. The other 12 germline chimeras lacked a developed gonad, but instead had an "empty" gonadal structure indicating either that the donor PGC was lost at some point during gametogenesis or that the PGC remained quiescent and failed to differentiate after transplantation. In zebrafish, single PGC transplantation induces formation of a functional gonad in SPT chimeras at a high rate, but these chimeras only develop as males (Saito et al., 2008). In this study, we show for the first time that single PGC transplantation can induce formation of a functional ovary in goldfish SPT chimeras. Examination of histological sections showed that the oocytes in the ovaries of these chimeras were at different stages of development, in a similar fashion to a control ovary (Fig. 7C). Moreover, the contralateral gonad in these chimeras was found to be free of germ cells and similar to the type I gonad of female morphants (Fig. 7D). In the male SPT chimeras, a well developed testis contained seminiferous tubules with the germ cells undergoing spermatogenesis synchronously in each cyst; the lumen of seminiferous tubules contained spermatozoa (Fig. 8C). In the contralateral gonad, the tubular lumen also contained spermatozoa despite the absence of obvious cysts lining the seminiferous lumen and a lack of other germ cell stages (Fig. 8D). It is possible that these spermatozoa had migrated from the other testis through the efferent duct.

The patterns of expression of candidate sex-determining genes in both gonads of the germline chimeras were examined by PCR. We found that the germ cell-free gonads of female and male chimeras showed similar patterns to those in morphants,

with the exception of *foxl2* expression in male chimeras (Fig. 5). None of the morphant type II gonads expressed *foxl2;* by contrast, however, some testes and contralateral gonads (type II) in the male chimeras expressed *foxl2* gene to a similar extent as control testes. Although there was histological evidence of spermatozoa in the type II gonads of male germline chimeras (see above), *vasa* expression was not detected in the RT-PCR analysis. This was expected since *vasa* is not expressed in spermatids and spermatozoa (Komiya et al., 1994; Kobayashi et al., 2000).

Complete replacement of germline and donor PGC derived offspring

To determine whether the gametes produced by the germline chimeras were functional, they were fertilized using gametes of albino fish; this process was carried out three times with 6- to 9-month intervals. Since the albino phenotype is determined by a recessive gene, then if host derived (wild type) gametes are present in the germline chimeras, the phenotype of the F_1 fish will be wild type. Three of the 9 chimeric fish, SPTC-1 (female), SPTC-7 (male) and SPTC-8 (male), produced gametes one day after HCG injection. The male chimeras, SPTC-7 and SPTC-8, were each mated to three albino females (Table 6). In each cross, the phenotype of the offspring was albino, i.e., the germlines of these chimeric fish had been completely replaced by the donor PGC (Fig. 9D). The female chimera (SPTC-1) was crossed on five occasions to different albino males. Although most of the offspring were albino, wild type progeny also appeared in three of the five crosses, at rates of 6.7%, 7.5% and 1.4% (Table 6). The wild type embryos were distinguishable by their pigmented eyes on day 3 (Fig. 9E). Egg sizes differ between wild type and albino fish, with the former being much larger. The wild type embryos hatched but they were abnormal (data not shown). In control

crosses between chimeric and wild type fish, all of the offspring were wild type (Fig. 9C). The remaining 6 chimeras, 2 females and 4 males, did not respond to HCG injections. Histological analysis of their gonads showed that germ cells were not present in 5 of the germline chimeras, similar to the situation in morphants. The sixth chimera, a female, possessed small numbers of vitellogenic and degenerating oocytes.

DISCUSSION

We have shown that germ cells are not essential for gonadal fate determination in goldfish. Depletion of host PGCs by knockdown of the *dnd* gene proved an effective approach since the host germline cells failed to migrate properly and disappeared before entering the gonadal ridge. As a consequence, early gonadal differentiation could be analyzed in the complete absence of germ cells. Our results demonstrate that determination of the developmental pathway towards an ovary or a testis occurs independently of the presence of germ cells. The present findings in the goldfish are consistent with previous results from the loach, turtles and mammals (Fujimoto et al., 2010; DiNapoli and Capel, 2007; Defalco and Capel, 2009). This was also confirmed by the experiments in which a single PGC was transplanted. In zebrafish, SPT chimeras only develop as males, and feminization by treatment with 17β-estradiol (E2) is needed to produce female SPT chimeras (Saito et al., 2008). Up till now, no spontaneous ovarian differentiation had been observed in teleost SPT chimeras. In this study, however, the gonads of goldfish SPT chimeras could follow either the female or male developmental pathways to produce functional eggs and sperms. This opens the possibility that the gonadal somatic cells may have the potential to induce differentiation of PGCs into either oogonia or spermatogonia in goldfish. One of the male SPT chimeras produced almost 100% female offspring (data not shown), suggesting that an XX donor PGC had been induced to differentiate towards spermatogenesis under the XY host somatic cell lineage. Further investigations will be necessary to provide conclusive proof of the validity of this interpretation.

Contribution of germ cells to gonadal fate determination in teleosts

Two models can be proposed from our increased understanding of the role of germ cells in gonadal sex differentiation in fish: first, PGC-dependent sex differentiation; and second, somatic cell-dependent sex differentiation. The first model applies to zebrafish and medaka. In these species, fish without germ cells in early embryogenesis develop as males by transdifferentiation of gonadal somatic cells (Weidinger et al., 2003; Kurokawa et al., 2007). Even if a single PGC is transplanted into these germ cell depleted embryos, they develop as males in zebrafish (Saito et al., 2008). In the hotei mutant of medaka, fish with an excessive number of germ cells compared to normal become female (Morinaga et al., 2007). These findings suggest that gonadal sex is determined by the balance of germ cells and gonadal somatic cells. This model was also proposed by Siegfried and Nüsslein-Volhard (2008). The second model applies in goldfish and loach. We could not verify chromosomal sex in the goldfish since sex-linked DNA markers are currently unavailable. However, goldfish phenotypic sex is determined genetically when specimens were raised under optimal breeding conditions (Goto-Kazeto et al., 2006). As shown in this study, PGC depletion did not influence sex differentiation, as both female and male morphants appeared in the normal sex ratio. Gonadal structures were similar in these morphants as in the empty gonads of germline chimeras in both sexes. In the loach, secondary sex characteristics, such as differences in the size and shape of the pectoral fin have been identified in morphants (Fujimoto et al., 2010). Overall, these reports suggest that no transdifferentiation of gonadal somatic cells occurs in fish without germ cells, thus the gonadal somatic cells are the determinant for gonadal fate determination in these species.

Germ cell depletion by *dnd* gene knock down

Analysis of the localization of *dnd* mRNA in developing goldfish embryos by WISH showed that the gene was expressed in PGCs, as has also been reported for other species (Weidinger et al., 2003; Liu et al., 2009; Fujimoto et al., 2010). Knockdown of the *dnd* gene by a morpholino oligonucleotide caused a decrease in the number of PGCs at early somite stages and almost complete loss of PGCs by late somite stages. From an examination of the external appearance of gonads in the morphants, we concluded that treating the embryos with a 50-200 μM concentration of the morpholino was sufficient to induce complete loss of PGCs in goldfish. This dose range is similar to that used in other fish species, although slight adjustments are needed depending on the size of the eggs. The occurrence of abnormalities in the morphants varied between egg batches rather than the amount of morpholino injected. Embryonic abnormalities may be caused by damage resulting either from manipulating the eggs or as a side effect of the morpholino, which may explain why the rate of anomalies varies between egg batches.

Donor PGC migration and the rate of formation of germline chimeras

GFP-labeled PGCs were identified and their migration and settlement patterns tracked. In goldfish, ectopic migration by PGCs was observed more frequently than in zebrafish (Saito et al., 2006). However, most labeled PGCs were located in the trunk region between somites 6 and 13, as viewed from a lateral perspective. These PGCs were observed in the vicinity of the notochord and somites when viewed from a dorsal perspective. We found that 42.7% of transplanted PGCs were in this area. However, not all donor PGCs that settled in gonadal regions contributed to subsequent gametogenesis. Only approximately half of the germline chimeras showed partial or fully developed gonads on one side of the body in goldfish. This result suggests that only donor PGCs

that settled in a specific area involved in proliferation and differentiation of germ cells could rescue gametogenesis in the gonad.

Donor derived-gametes in germline chimeras

In this study, nine germline chimeras were used in the mating analysis. Three of the nine (one female and two male) were shown to be fertile after HCG induction of gametes. Since the donor gametes encoded the recessive albino phenotype, then if host gametes were completely replaced by donor derived germ cells, all of the progeny should show the albino phenotype. The two male chimeras only produced albino offspring, whereas the female chimera produced both albino and wild type offspring. The proportion of wild type offspring from female chimeras varied from 0 to 7.5% among batches. This may be have been due to any remaining host PGCs failing to proliferate or differentiate following exposure to the morpholino, in contrast to the donor PGC. The occasional host PGC may have settled in the host gonad and have contributed to gametogenesis. The size difference between host and donor derived eggs is of interest. Egg size is determined by the amount of egg yolk that accumulates in the eggs during vitellogenesis; therefore, all of the eggs in a single batch are generally similarly sized in goldfish. The difference in egg sizes observed here between host and donor suggest that genetic factors also play some role in egg size determination. A somewhat similar finding has been reported in triploid crucian carp-diploid goldfish chimeric fish, which produce eggs of different sizes in the same batch (Yamaha et al., 2001).

Gene expression in the gonads of germline chimeras

Expression of the germ cell marker gene, *vasa*, and the candidate sex determining genes, *foxl2*, *cyp19a1* and *amh*, was screened in the gonads of the germline chimeras. *foxl2* and *cyp19a1* are mainly expressed in the granulosa cells, while *amh* is expressed in both granulosa and Sertoli cells. We detected expression of these genes not only in the ovary and testis but also in type I and type II gonads, suggesting that somatic cells could differentiate and develop in the germ cell free gonads in the same manner as in intact gonads. Therefore, differentiation and development of gonadal somatic cells appeared to be largely independent of the germ cells, as has been reported in zebrafish and loach (Siegfried and Nüsslein-Volhard, 2008, Fujimoto et al., 2010). In this study, *foxl2* expression was also found in the testes of some control and male chimeric fish. *foxl2* is known act as a transcription factor to regulate expression of the aromatase gene, and is important for ovarian differentiation and development. However, the expression of *foxl2* has also been reported in the adult testis of various species of fish including tilapia and southern catfish (Wang et al., 2004; 2007; Liu et al, 2007). These reports suggest that *foxl2* might be a general regulator of steroidogenesis in gonads (Wang et al., 2007). Further investigation will be needed to fully elucidate the role of *foxl2* in the testis. Currently, marker genes for testicular somatic cells, such as *sox*9 and *cyp11b*, are not available in the goldfish; therefore, conclusive evidence for differentiation of testicular somatic cells could not be obtained in morphant type II gonads. However, the morphological similarity of gonads in morphants and germline chimeras suggested that neither transdifferentiation nor masculinization occurs in morphant gonads.

CONCLUSIONS

Gonadal fate determination in teleosts presumably occurs as a consequence of the balance between germ cells and gonadal somatic cells during sex differentiation. We have shown that gonadal fate is determined by the gonadal somatic cells in goldfish. However, we have not determined whether the gonadal somatic cells have the potential to reverse the germ cell genetic sex. Unlike the model fish species medaka and zebrafish, there is less information available from the goldfish on genome sequences information. In particular, we lack sex-linked DNA markers and have yet to identify sex-related chromosomal differences. Sex determination and differentiation are variable processes among fish species; thus, an understanding of the mechanism of sex determination in any single species requires a wide range of infromation. In this respect, we believe that the goldfish offers more advantages than disadvantages for investigating interactions between germ cells and somatic cells during sex differentiation in fish.

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FIGURE LEGENDS

Fig. 1 Distribution of *vasa*-positive cells in goldfish embryos injected with a *dnd* morpholino oligonucleotide. Dorsal (A, C, E, and G) and lateral views (B, D, F and H) of early- and late-somite stage embryos, respectively. Treated embryos received the *dnd* morpholino injection after fertilization (C, D, G and H). Black arrows indicate *vasa*-positive cells at the normal position and white arrows indicate cells in an ectopic position. Scale bar, 100 μm.

Fig. 2 Ablation of PGCs in goldfish embryos treated with a *dnd* morpholino oligonucleotide. Lateral view captured using a fluorescence microscope of a day 3 embryo (left) and higher magnification image of the boxed area (right). GFP-labeled PGCs were distributed in the gonadal region of the control embryo that received an injection of GFP-*nos1* 3'UTR mRNA (A). No GFP-labeled PGCs were observed in the *dnd* morphant embryo that received co-injection of the *dnd* morpholino oligonucleotide and GFP-*nos1* 3'UTR mRNA.

Fig. 3 Histological analysis of *dnd* morphant. Transverse section of the trunk region of day 10 fish received *dnd* morpholino oligo injection at 1- to 4-cell stage embryo. In the control fish, PGCs had reached the dorsal peritoneum of the upper part of the body cavity and were observed as single, relatively large cells (black arrow). No PGC-like cells were found in morphants(n=10). g, gut; m, mesonephros; c, coelomic cavity.

Fig. 4 Morphological differences between gonads of PGC-depleted goldfish. External appearance (A-D) and histological observations of gonads (E-N) in fish at 5-months-old. The gonads of PGC-depleted goldfish, which received a *dnd* morpholino injection after fertilization, showed two categories of external appearance: type I, which is flat and relatively transparent (B); and type II, which is narrow and tube-like (D). Section through a type I gonad that formed an ovarian cavity through connection to the peritoneal wall (F and K) as seen in the control ovary (E and I). White arrows indicate the position where the gonads attach to the peritoneal wall to form an ovarian cavity. Unlike the control ovary, the type I gonad did not contain germ cells, either oogonia or oocyte, on the interior side (J and L). The control testis has seminiferous tubules (area outlined by the black line) that contain different stages of germ cells in the cysts, and tubular lumens that are filled with spermatozoa (G and M). A section through a type II gonad showing a lack of germ cells in the seminiferous tubules and efferent duct (H and N). pn, peri-nuclear stage; oc, ovarian cavity; bv, blood vessel; sz, spermatozoa; ef, efferent duct.

Fig. 5 RT-PCR analysis of *vasa*, *foxl2*, *cyp19a1* and *amh* in the gonads of PGC-depleted and germline chimeras. *vasa* is expressed in the ovary and testis of control fish and germline chimeras, but not in the gonads of fish which received a *dnd* morpholino injection after fertilization (type I and type II gonads of *dnd* MO and germline chimeras). *amh* is expressed in all the gonads tested: ovary, testis, type I and type II gonads. *foxl2* and *cyp19a1* are expressed in the ovary and type I gonad, but not in the testis or type II gonad, although there was exceptionally weak expression of *foxl2* in some testes and type II gonad in control male and male germline chimera.

Fig. 6 Schematic illustration of the distribution of PGCs in a day 3 goldfish embryo (A) and the localization of a transplanted donor PGC in the host goldfish (B). (A) The lateral view shows the gonadal region where PGCs settled at a rate of 87.7%. The dorsal view shows the area where PGCs settled at a rate of 94.3%, with a rate of 5.8% ectopic migration to the body surface. (B) Germline chimeras produced by transplantation of a single PGC: day 1 (a), dorsal and lateral views at day 3 (b and c) and at 3 weeks (d). The GFP-labeled donor PGC has divided once on day 1, with one PGC migrating and settling in the gonadal region (arrow), while the other is migrating ectopically (arrowhead). GFP fluorescence in the donor germ cell gradually weakened, but was still detectable at 3 weeks (d).

Fig. 7 Gonadal morphology of female germline chimeras in goldfish. External appearance (A) and histological sections through the gonads (B-D) in 1-year-old germline chimeras. Transverse sections of the trunk region showed a well developed ovary on the right and a poorly developed contralateral gonad on the left (B). Transverse sections of the ovary show an ovarian cavity and oocytes at different stages of development (C). Higher magnification of the poorly developed contralateral gonad indicated by the arrowhead in (B). The structure of this gonad was similar to the type I gonads of female morphants (see Fig. 4F). oc, ovarian cavity; pn, peri-nuclear stage; v, vitellogenic stage.

Fig. 8 Gonadal morphology of male germline chimeras in goldfish. External appearance (A) and histological sections through the gonads (B-D) of 1-year-old

germline chimeras. Transverse sections of a developed testis on the right side of the body and a poorly developed contralateral gonad on the left (B). Transverse sections of a testis showing cysts with germ cells at different stages of development and a tubular lumen full of spermatozoa (C). Numerous spermatozoa are present in the tubular lumen in the left gonad but no other germ cell stages are present suggesting that these spermatozoa originated from the right testis by migration through the efferent duct (D). sz, spermatozoa; sc, spermatocyte; bv, blood vesicle.

Fig. 9 Germline chimeras generated from a wild phenotype host (A) and an albino phenotype donor (B). Crossing germline chimeras with wild type fish generated an F1 generation with wild type phenotype (C). Crossing germline chimeras with albino fish produces an F1 generation with an albino phenotype (D). The female germline chimeras had both albino eggs and a low rate of wild type eggs (E). Wild type eggs (arrowhead) are larger than albino phenotype eggs (arrows).

Supplementary Figure 1 Goldfish *dead end* (*dnd*) is expressed in primordial germ cells. (A). Diagram of the structure of the goldfish *dnd* mRNA showing a 375 amino acid ORF (grey box) and an RNA binding domain (meshwork box). (B). The 5'UTR of *dnd* mRNA has two different lengths. Start codon sequences are in bold, and the target sequences of the morpholino oligonucleotide are underlined. (C). Whole-mount *in situ* hybridization of embryos with *dnd* anti-sense RNA probe showing signals (arrows) in the cleavage furrows of the 4-cell embryos (C-a), in the lower part of the blastodisc at blastula stage (C-b), and aligned along the embryonic body at the somite stage (C-c,d,e).

Supplementary Figure 2 Knock-down of *dnd end (dnd)* by injection of a morpholino oligonucleotide into goldfish embryos. (A). Rate of abnormal embryos after injection of different concentration of the morpholino oligonucleotide. (B). Control and abnormal embryos. Control embryo at day 3 (a), embryo showing pericardiac edema (b) and embryo showing hematoma (c). (C). Gonadal development in 9-month-old goldfish after treatment with different concentrations of morpholino oligos at the embryonic stage.

Supplementary Figure 3 Gonadal morphology of adult *dead end* (*dnd*) morphant goldfish.The fish were injected with the morpholino oligonucleotide as 1- to 4-cell stage embryos. External appearance and histological sections of a 1-year-old female (upper images) and a 1-year-old male *dnd* morphant (lower images). The section of the type I gonad shows a mesh-like structure in the area where the ovarian lamella develops in a normal ovary. Arrows indicate where the gonad is attached to the peritoneal wall. The section of the type II gonad shows seminiferous tubules that are lined with cells interpreted as Sertoli cells and that contain an empty central tubular lumen.

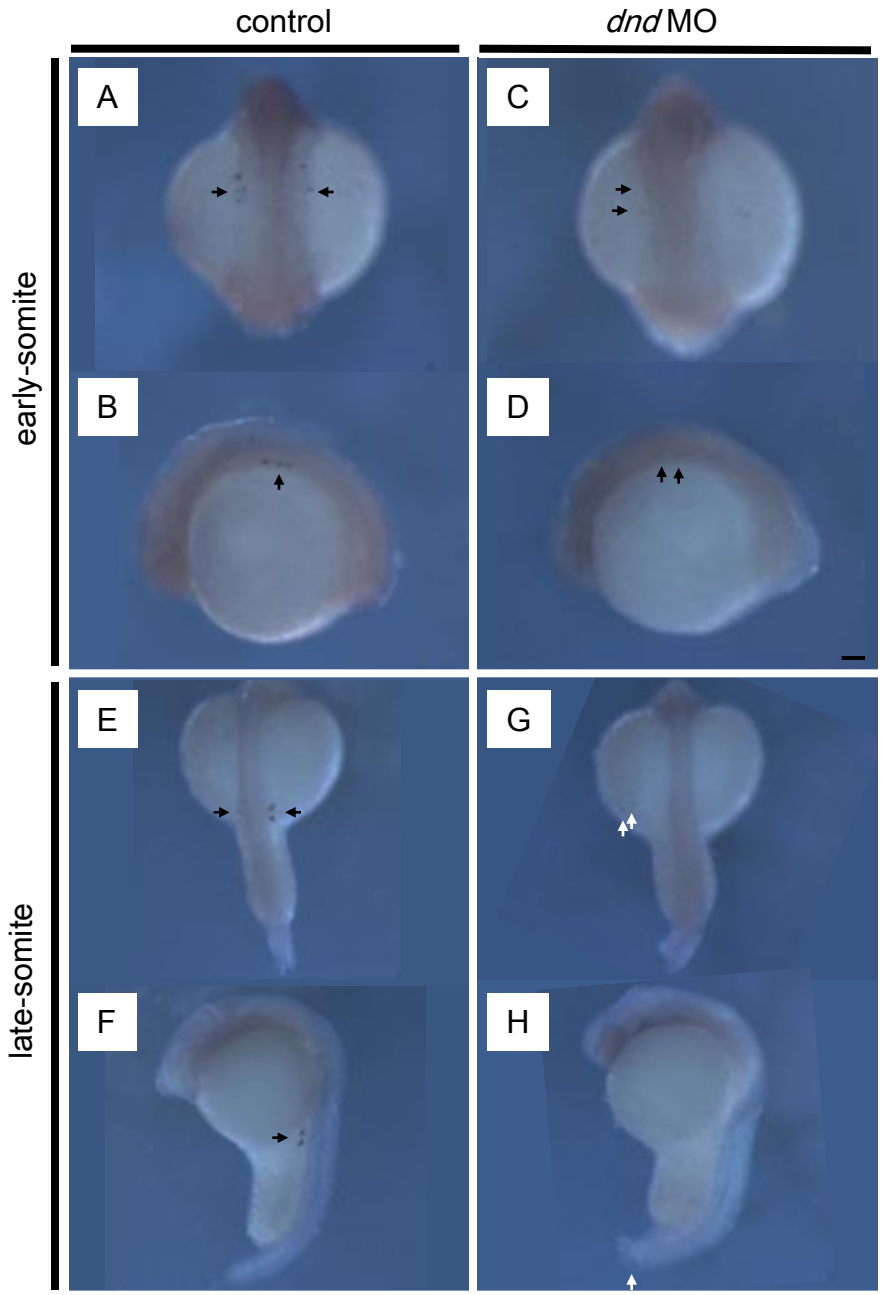


Fig 1

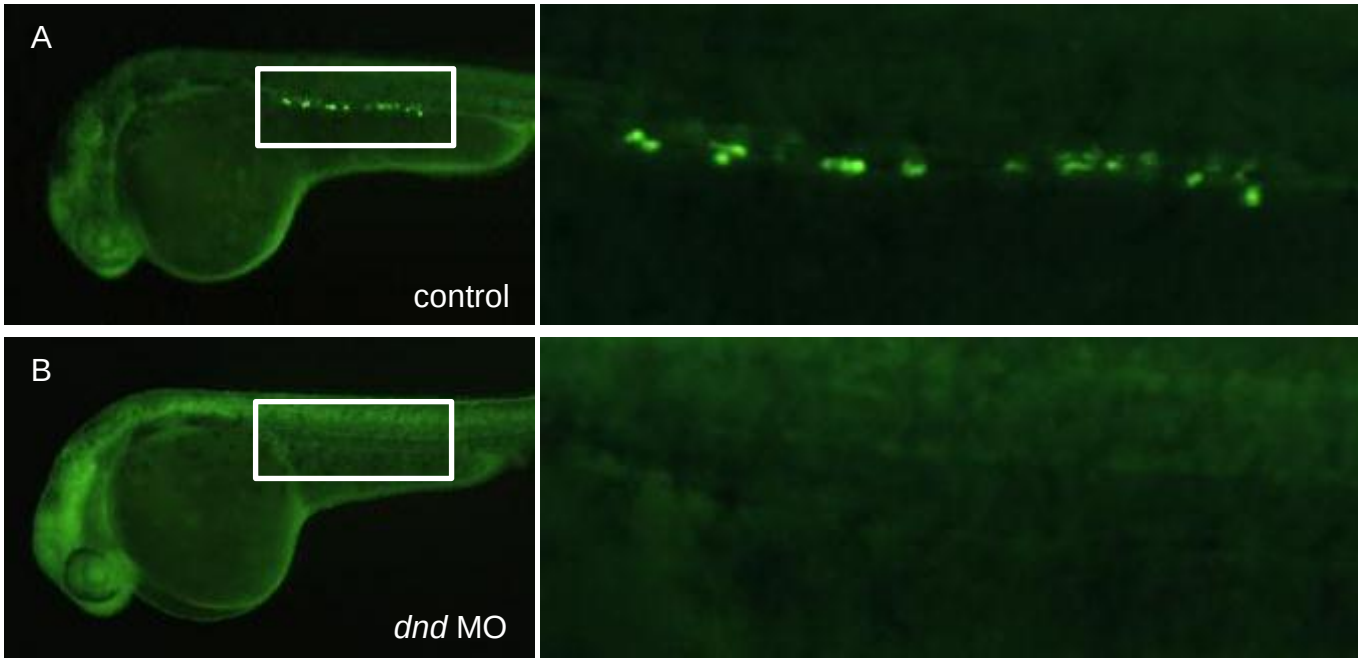
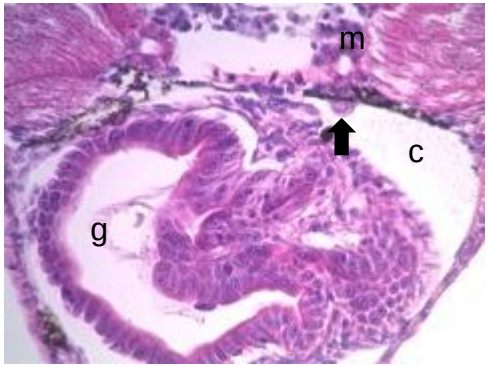


Fig 2

control



dnd MO

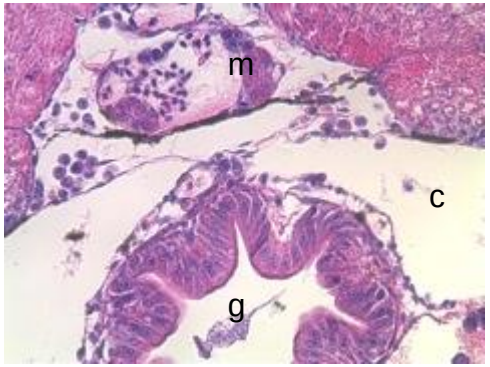
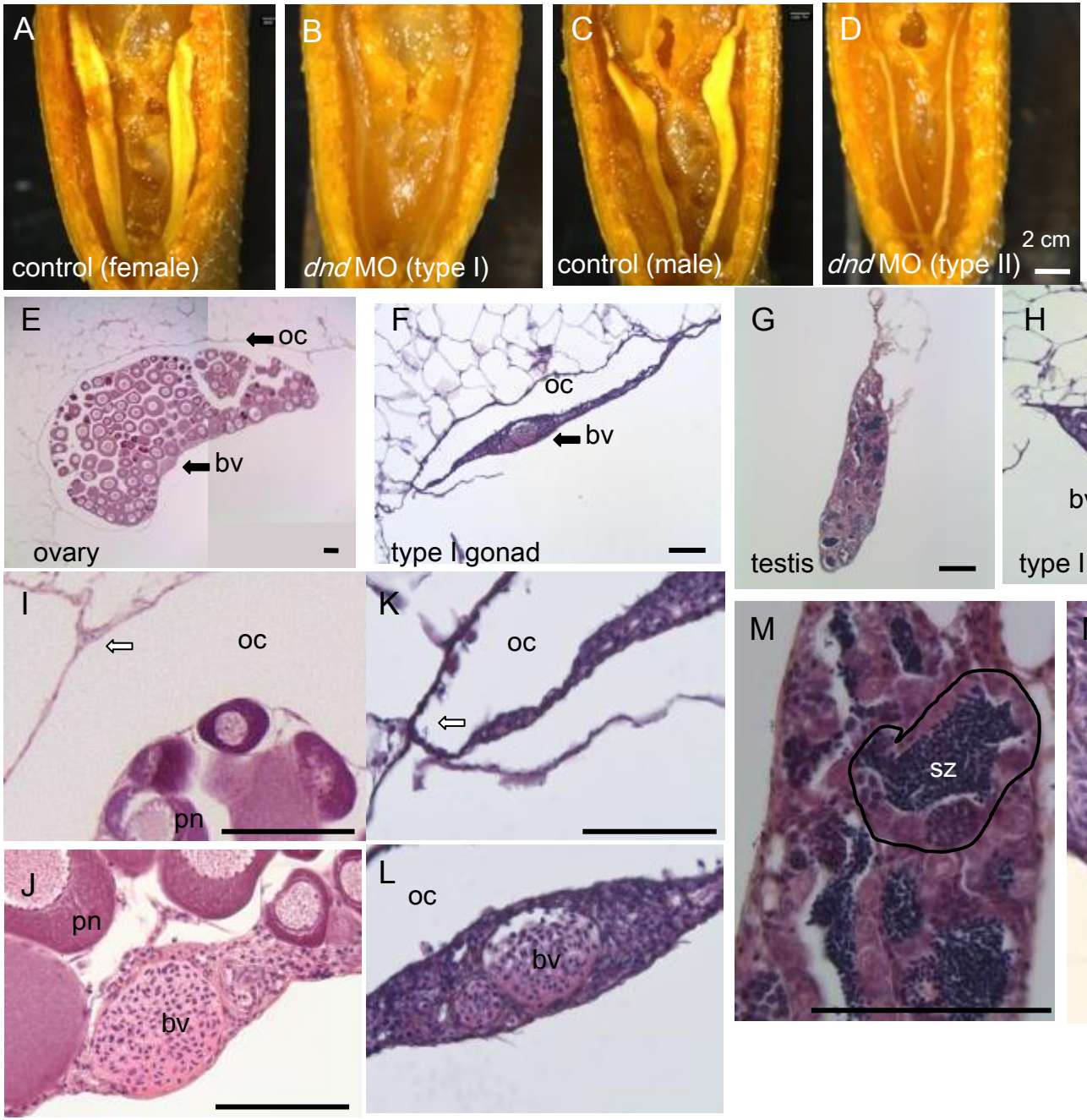


Fig 3


A
control (female)
B
dnd MO (type I)
C
control (male)
D
dnd MO (type II)
2 cm
E
oc
bv
ovary
F
oc
bv
type I gonad
G
testis
H
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type II gonad
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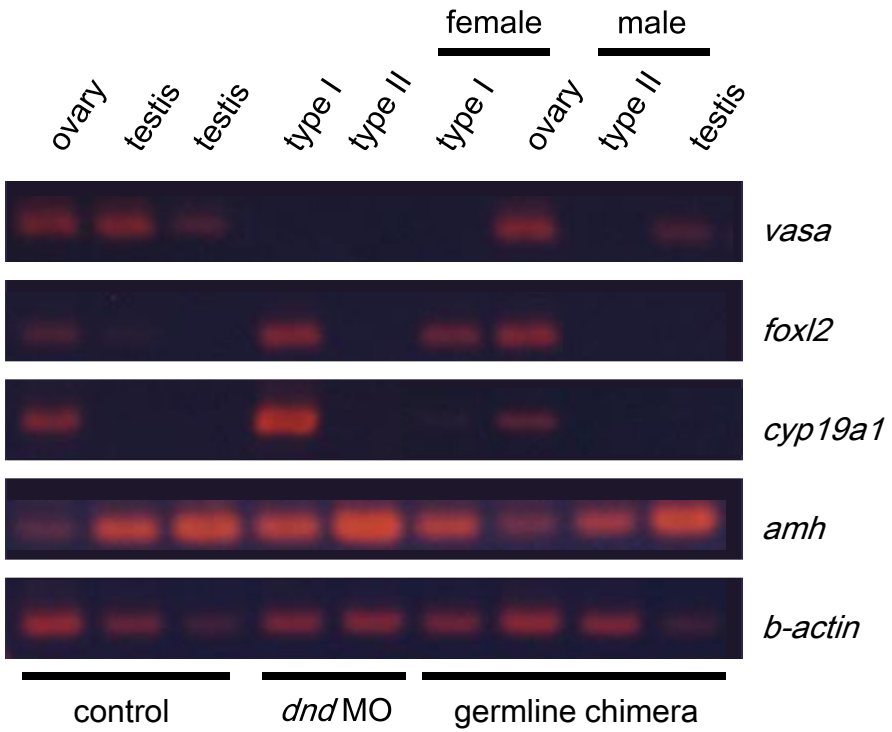
Fig. 4


female
male
ovary
testis
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type I
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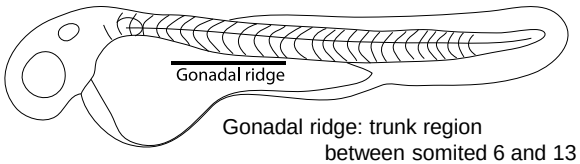
type I
ovary
type II
testis
vasa
foxl2
cyp19a1
amh
b-actin
control
dnd MO
germline chimera

Fig 5

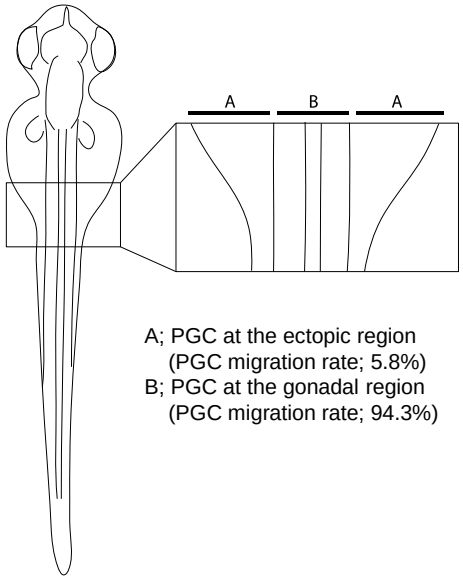
A

Lateral view of Day 3 embryo



Gonadal ridge: trunk region
between somited 6 and 13
(PGC migration rate; 87.7%)

Dorsal view of Day 3 embryo



A; PGC at the ectopic region
(PGC migration rate; 5.8%)
B; PGC at the gonadal region
(PGC migration rate; 94.3%)

B

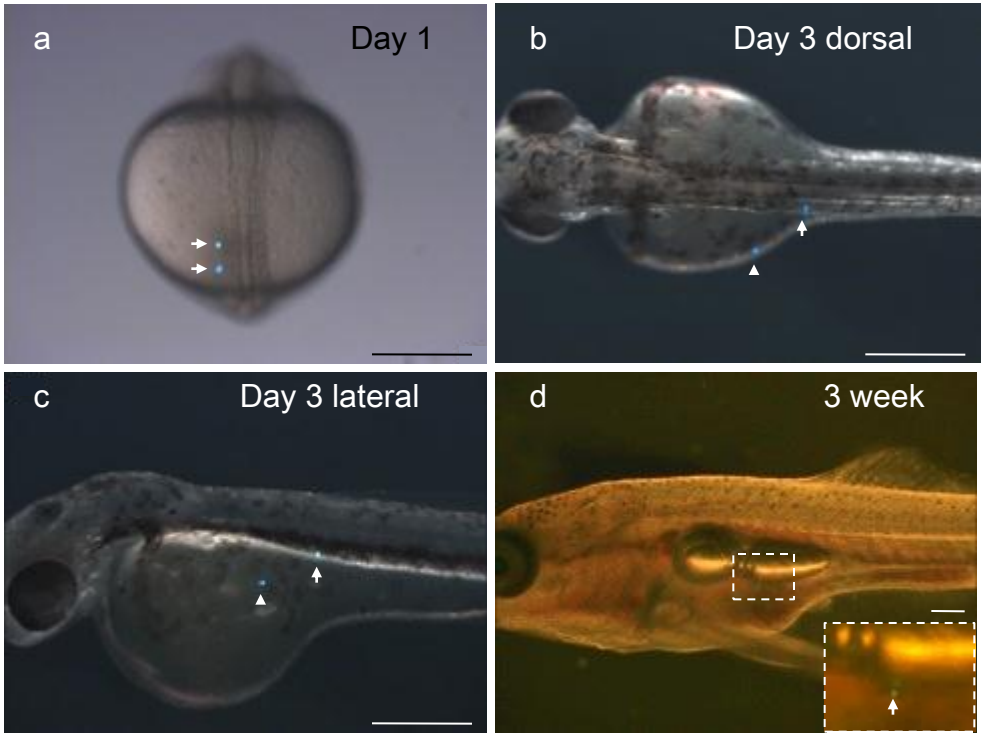


Fig 6

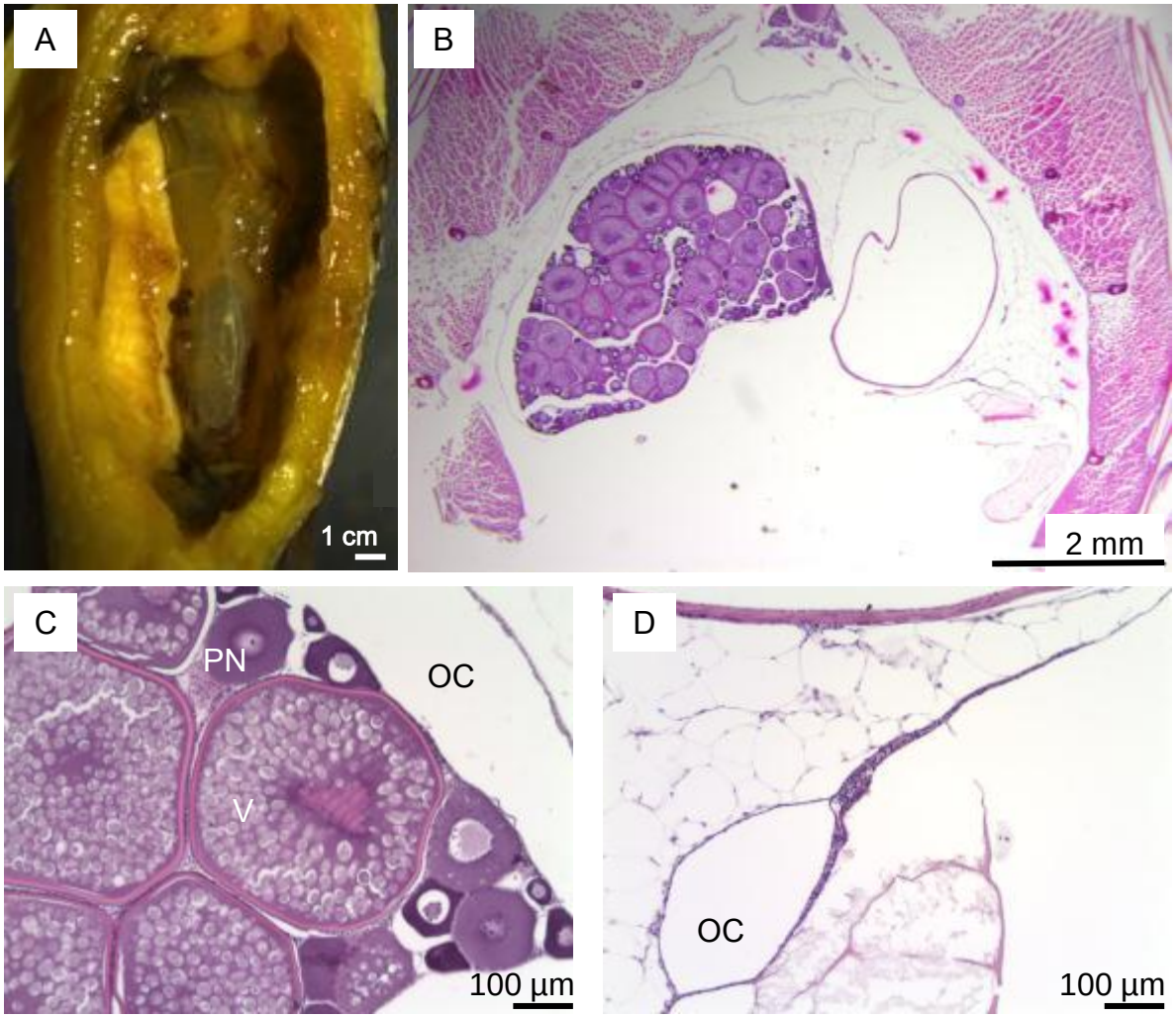


Fig 7

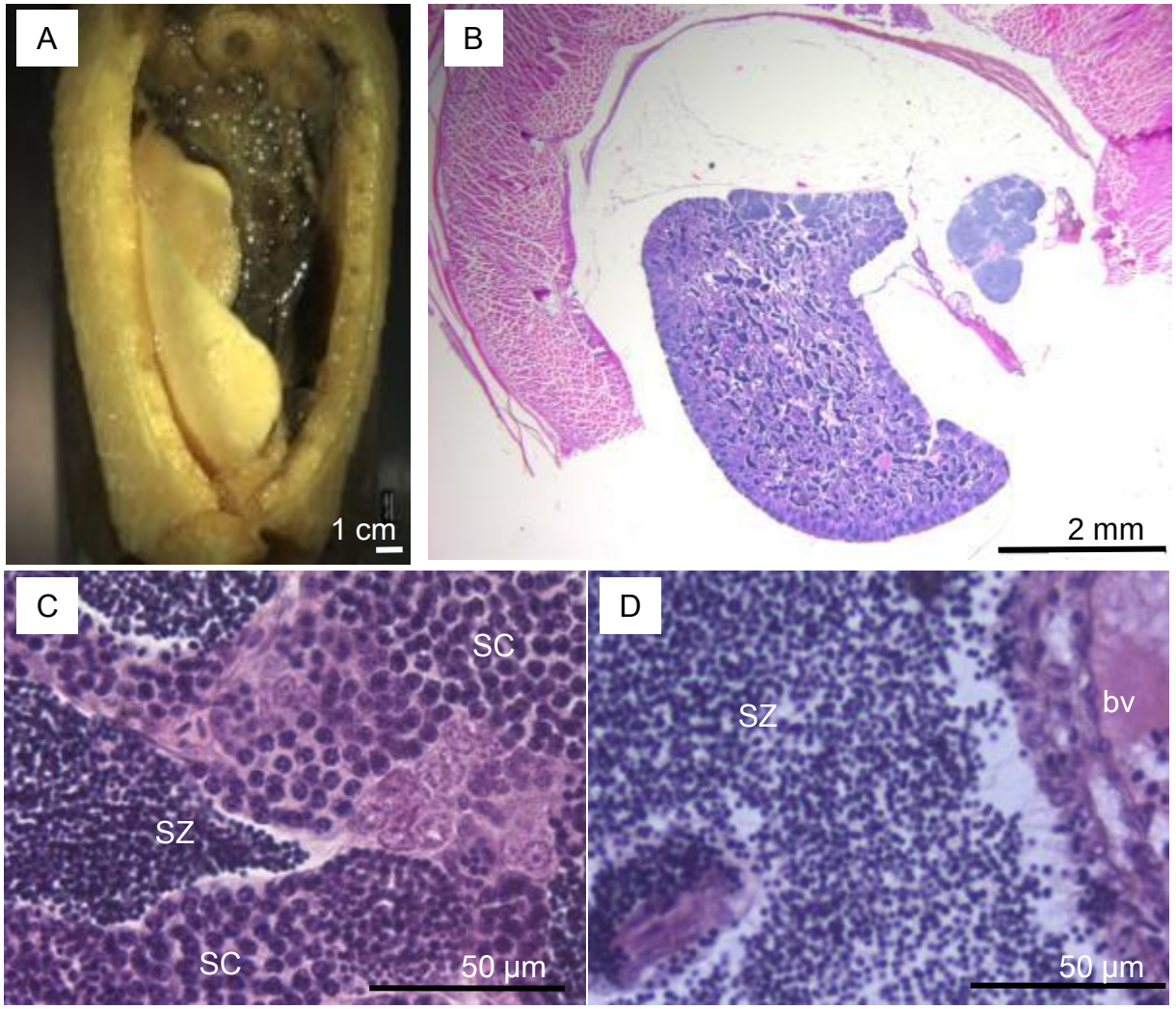


Fig 8

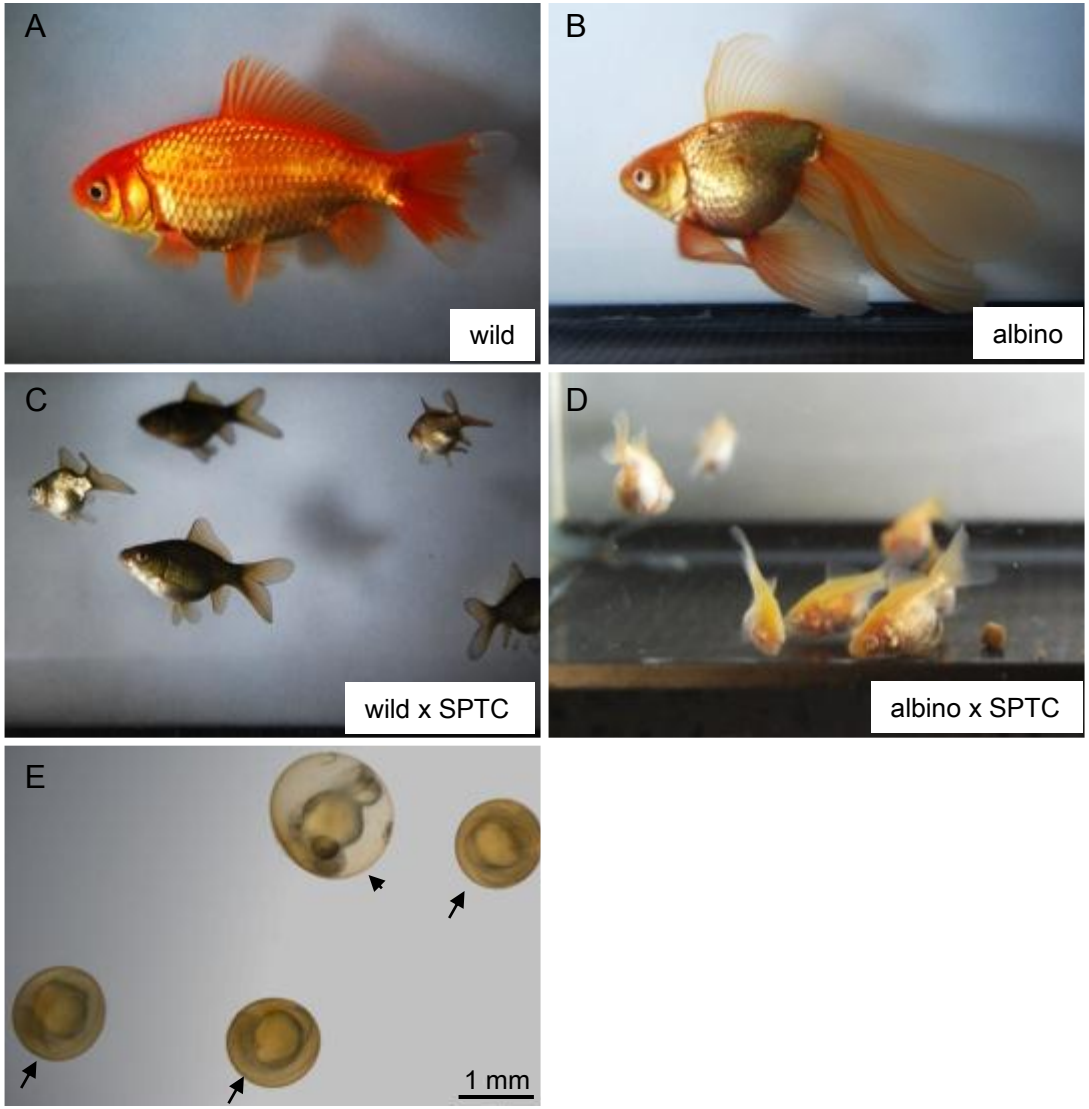
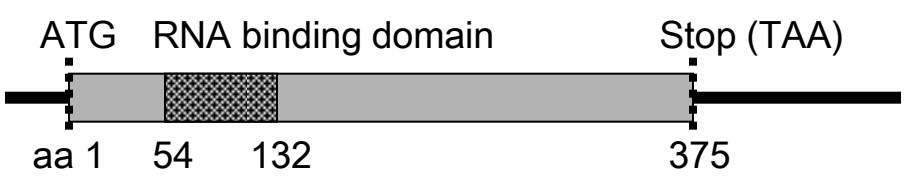


Fig 9

Supplementary Figure 1

A

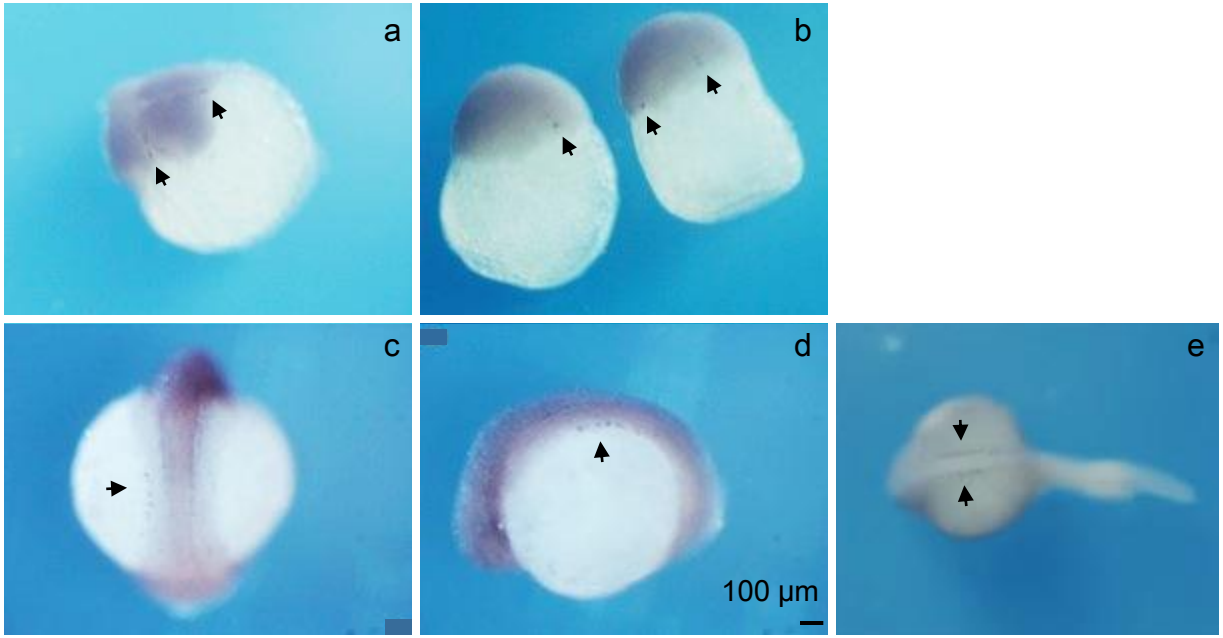


B

Short form 5'AAGCAGTGGTATCAACGCAGAGTACGCGGGG---------
Long form 5'AAGCAGTGGTATCAACGCAGAGTACGCGGGGGATTTACAG

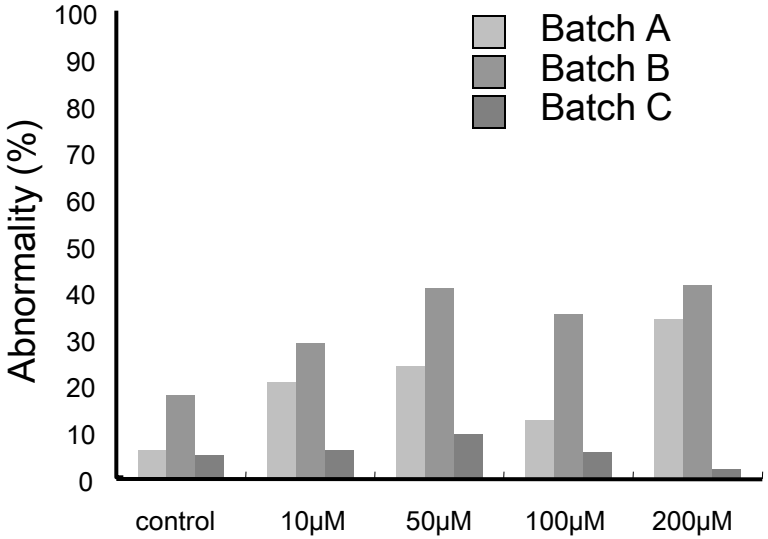
----------ACAGCGGC**ATG**GAGGGACAGCA3'
GTGTGCCATCACAGGTGGACAGCGGC**ATG**GAGGGACAGCA3'

C

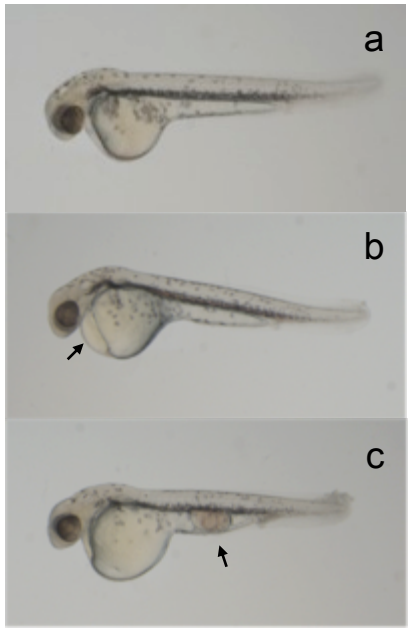


Supplementary Figure 2

A



B

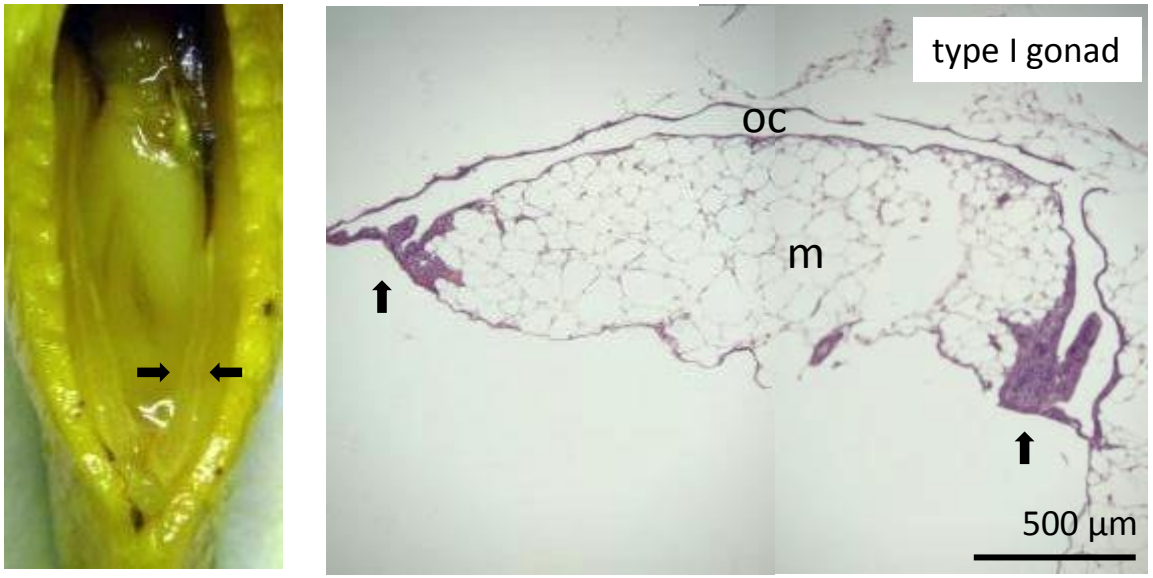


C

Morpholino concentration (μM)	% fish possessed				Characterization of developed ovary or testis
	Developed			Undeveloped (string-like)	
	ovary		testis		
0	51.7	(15/29)	48.3 (14/29)	0.0 (0/29)	Large-bilateral
10	15.8	(3/19)	31.6 (6/19)	52.6 (10/19)	Large-unilateral, small-unilateral, small-bilateral, small-bilateral (partial)
50	0.0	(0/19)	0.0 (0/19)	100.0 (19/19)	-
100	0.0	(0/14)	7.1 (1/14)	92.8 (13/14)	Small-bilateral (partial)
200	0.0	(0/14)	0.0 (0/14)	100.0 (14/14)	-

Supplementary Figure 3

dnd morphant (1-year-old female)



dnd morphant (1-year-old male)

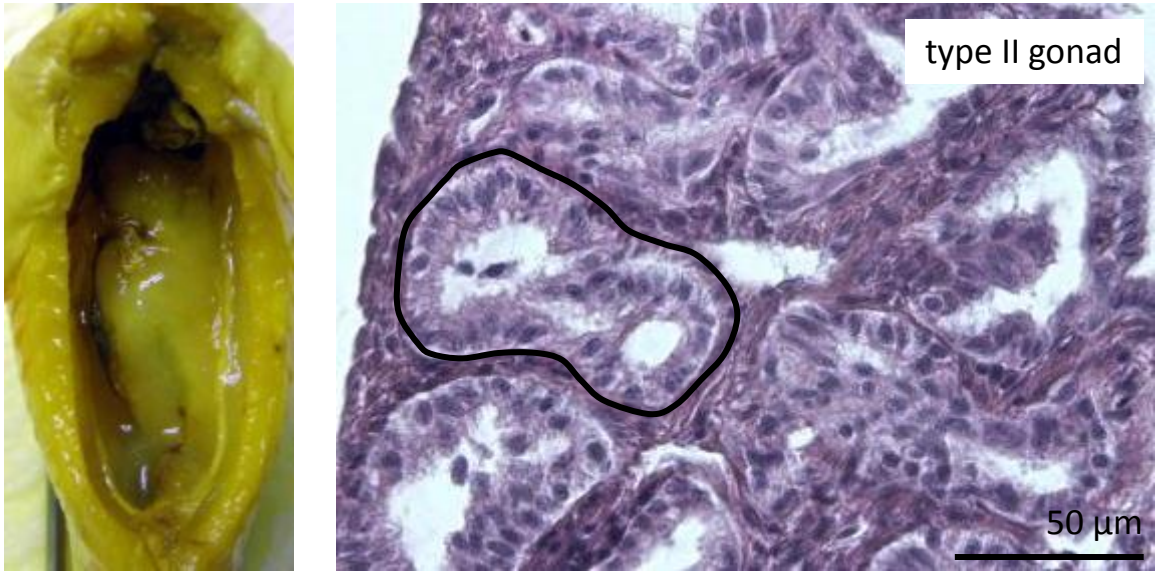


Table 1 Gene specific primers used for RT-PCR.

Gene	Forward primers (5'→ 3')	Reverse Primers (5'→ 3')	Annealing Tm
cyp19a1	TACGGAGACATTGTGCGGGTTTGGA	CCTGCAACTCCTGAGCGTCT	60 ˚C
foxl2	TGGACACTACCTCAAGCTCA	TGAACGGTCTCTTCATGCGT	60 ˚C
amh	ACTCAGTACCTGGGAGGTGG	GCAGAGAGAGCGATTGACGG	63 ˚C
vasa	TGATGGCTGATGGTGTGGCT	TTCAGGGTAGGTGGCGCTGA	60 ˚C
b-actin	ACGGTATTGTGACCAACTGG	CTCGGTCAGGATCTTCATCA	57 ˚C

Table 2 The number of GFP-labeled PGC in a gonadal region of *dnd* morphant embryos at day 3 in goldfish.

Morpholino concentration (μM)	No. of embryos	No. of embryos without GFP-labeled PGC at gonadal region	No. of GFP-labeled PGC at	
			gonadal region	ectopic region
0	28	0	16.5 ± 9.2	3.0 ± 3.7
50	23	20	0.1 ± 0.3	0.1± 0.5
100	18	17	0.1 ± 0.2	0.3 ±1.0

Table 3. Location of transplanted goldfish PGCs following transplantation into sterilized blastulae.

Group no.	Experimental group**	Total no. of embryos	Number of normal embryos (%)	No. of embryos with PGC at‡		
				None (%)	Gonadal region (%)	Ectopic (%)
1	Chimeras	39	35 (89.7)	1 (2.9)	15 (42.9)	19 (54.3)
	MO	43	41 (95.4)	-	-	-
2	Chimeras	23	22 (95.7)	2 (9.1)	9 (40.9)	11 (50.0)
	MO	37	35 (94.6)	-	-	-
3	Chimeras	13	9 (69.2)	1 (11.1)	4 (44.4)	4 (44.4)
	MO	40	30 (75.0)	-	-	-
Total*	Chimeras	75	66 (84.9±8.0)	4 (7.7±2.5)	28 (42.7±1.0)	34 (50.0±2.9)
	MO	120	106 (88.3±6.7)	-		

*Standard error is indicated.
**Development of host PGCs was blocked by injection of a *dead end* (*dnd*) antisense morpholino oligonucleotide (MO).
‡ -, not determined.

Table 4 Gonadal phenotype of germline chimeras generated by single PGC transplantation in goldfish.

Group	Total no. of fish	Gonadal phenotype			
		ovary	testis	Type I (empty ovary)	Type II (empty testis)
control	38	22	16	-	-
dnd MO	35	-	-	17	18
SPTC*	21	4**	5**	9	3

* SPTC; germline chimera generated by Single PGC Transplantation (SPT).

** These germline chimeras possessed a poorly developed contralateral gonad of type I and type II in female and male, respectively.

Table 5 Characterization and external appearance of gonads in germline chimeras in goldfish.

SPTC*	Characterization of gonad	Analysis
No. 1, 2	Developed ovary, unilateral	No. 1 Mating No. 2 Histology
No. 3, 4	Developed partial ovary, unilateral	No. 3, 4 Histology
No. 5, 6, 7, 8, 9	Developed testis, unilateral	No. 5, 6 Mating No. 7, 8, 9 Histology

* SPTC; germline chimera generated by Single PGC Transplantation (SPT).

Table 6 Phenotype of F1 population produced from germline chimeras in goldfish.

Mating pair	No. of F1 fish		% albino phenotype
	Wild	Albino	
Albino (F1) x Albino (M1)	0	39	100.0 %
SPTC-7 (M)	0	71	100.0 %
SPTC-8 (M)	0	64	100.0 %
Wild (M1)	74	0	0.0 %
Albino (F2) x Albino (M1)	0	56	100.0 %
SPTC-7 (M)	0	11	100.0 %
SPTC-8 (M)	0	47	100.0 %
Wild (M1)	68	0	0.0 %
Albino (F3) x Albino (M1)	0	35	100.0 %
SPTC-7 (M)	0	24	100.0 %
SPTC-8 (M)	0	18	100.0 %
Wild (M1)	26	0	0.0 %
SPTC*-1 (F) x Albino (M1)	2	28	93.3 %
Albino (M2)	0	86	100.0 %
Albino (M3)	5	62	92.5 %
Albino (M4)	1	70	98.6 %
Albino (M5)	0	21	100.0 %
Wild (M1)	48	0	0.0 %

* SPTC; germline chimera generated by Single PGC Transplantation (SPT).