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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.

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

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(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* × *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, 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