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Early Depletion of Primordial Germ Cells in Zebrafish Promotes Testis Formation

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SUMMARY

As complete absence of germ cells leads to sterile males in zebrafish, we explored the relationship between primordial germ cell (PGC) number and sexual development. Our results revealed dimorphic proliferation of PGCs in the early zebrafish larvae, marking the beginning of sexual differentiation. We applied morpholino-based gene knockdown and cell transplantation strategies to demonstrate that a threshold number of PGCs is required for the stability of ovarian fate. Using histology and transcriptomic analyses, we determined that zebrafish gonads are in a meiotic ovarian stage at 14 days postfertilization and identified signaling pathways supporting meiotic oocyte differentiation and eventual female fate. The development of PGC-depleted gonads appears to be restrained and delayed, suggesting that PGC number may directly regulate the variability and length of gonadal transformation and testicular differentiation in zebrafish. We propose that gonadal transformation may function as a developmental buffering mechanism to ensure the reproductive outcome.

INTRODUCTION

Primordial germ cells (PGCs) are the stem cells of the gametes, providing genome transmission to future generations (Lesch and Page, 2012). During development, PGCs undergo specification, migration, and proliferation. Reciprocal interactions between germ cells and somatic cells are important for gonadal differentiation (Kocer et al., 2009). However, little is known about the regulatory role of germ cells during sexual development.

In mammals, agametic male gonads develop into a normal testis cord, while loss of germ cells in ovaries at birth disrupts ovarian structures and folliculogenesis (Merchant-Larios and Centeno, 1981). In teleosts, the requirement of germ cells for gonadal development appears to be variable. Their absence leads to exclusive male development in medaka and zebrafish (Kurokawa et al., 2007; Siegfried and Nüsslein-Volhard, 2008; Slanchev et al., 2005), but not in goldfish or loach (Fujimoto et al., 2010; Goto et al., 2012).

Mammalian sex determination is regulated by antagonistic pathways, which direct the bipotential embryonic gonad toward ovarian or testicular fate (Warr and Greenfield, 2012). Moreover, evidence indicates that somatic sex needs to be reinforced throughout adulthood. In mice, loss of FOXL2 in mature ovary or DMRT1 in mature testis causes transdifferentiation of somatic cells (Matson et al., 2011; Uhlenhaut et al., 2009). In zebrafish, oocytes appear essential for the development of females in juveniles and for maintenance of the sexual phenotype in adults (Dranow et al., 2013).

The number of PGCs likely plays an important role in teleost sexual differentiation. For medaka and stickleback, females possess more germ cells than males due to their sexually dimorphic proliferation (Lewis et al., 2008; Saito et al., 2007). Transplantation of a single PGC into a germline-deficient zebrafish embryo generates males exclusively (Saito et al., 2008). *ziwi* mutants with reduced PGC numbers can develop as males or females; however, a greater reduction due to a hypomorphic allele in *trans* to a null allele gives rise to males (Houwing et al., 2007). These data argue that the absolute number of germ cells is important in determining the sexual phenotype of zebrafish.

Zebrafish are undifferentiated gonochorists since all individuals first initiate oogenesis via forming an immature ovary (Takahashi, 1974). In developing males, but not in

females, a gonad transformation arises from apoptosis-driven degeneration of oocytes (Uchida et al., 2002; Wang and Orban, 2007) about 23–35 days postfertilization (dpf) leading to subsequent testis development (Orban et al., 2009; Uchida et al., 2002). Molecular control of sex determination and gonad differentiation in zebrafish appears to be complex (Liew and Orban, 2014; Orban et al., 2009) and variable across domesticated strains versus wild populations (Liew et al., 2012; Wilson et al., 2014).

In this study, we analyze the relationship between the number of PGCs and sexual differentiation in zebrafish. By tracking changes in the PGC number during development, we demonstrate that a dimorphic proliferation of PGCs occurs in the early larvae, underlining the beginning of sexual differentiation. By creating zebrafish containing various numbers of PGCs, we show that a threshold number of PGCs is required for stabilizing the ovarian fate and that PGC number may directly regulate the progression of gonadal transformation.

RESULTS

Dimorphic Proliferation of PGCs Occurred during Early Larval Stages in Zebrafish

To understand how the PGC count might be involved in sexual differentiation, we examined their numbers at different development stages using the *Tg(vasa:vasa-EGFP)* (i.e., *zf45Tg*) transgenic zebrafish line (Figure 1A). For clarity, the term "PGC" was used before 2 weeks of zebrafish development. First, we estimated the number of PGCs present in the gonadal region between 1 dpf and 8 dpf with the squash method. The number of PGCs ranged from 25 to 44 at 1 dpf, without obvious fluctuations in the PGC number during the first week of development (Figure 1B).

Next we used optical sectioning to precisely count PGC numbers at 7 and 14 dpf in WT larvae from two different families (FI and FII). The PGC count at 7 dpf appeared to follow a unimodal distribution (Figure 1C). At 14 dpf, the number of PGCs showed a bimodal distribution between two distinct populations (means = 40.3 ± 7.7 and 87.7 ± 12.1; Figure 1D), and the difference between the means was significant (Student's t test, $p < 0.05$). Morphologically, green fluorescent protein (GFP)-expressing gonadal regions exhibited a smaller and less dense morphology at 7 dpf (Figure 1E) and became larger as the number of GFP-expressing cells increased at 14 dpf. In 14 dpf individuals with a higher number of PGCs, gonadal regions were broader and denser due to the increased number of cells populating that region (Figures 1F and 1G). To determine whether this bimodal distribution in PGC number was due to a family-specific effect, we examined the distribution of PGC number by family. Intriguingly, 83.3% of individuals in FI, the family with a slightly female-biased offspring sex ratio (60%), exhibited gonadal regions with a higher number of PGCs, while nearly 60% of individuals in the male-biased FII (84%) contained a lower number of PGCs at 14 dpf (Figures 1H and 1I). Our data suggest that a dimorphic proliferation of PGCs occurs between 7 and 14 dpf and that progeny sex ratios within families may correlate with the divergent distribution of PGC numbers.

Depletion of PGCs in the Embryos Resulted in Masculinization of Zebrafish Gonads

To further investigate whether there was a relationship between early PGC count and sexual development, we compared unmanipulated *Tg(vasa:vasa-EGFP)* individuals with low PGC and high PGC counts and found no difference in their trunk-based expression at 22 dpf (Figure S1A available online) or adult sex ratio (Figure S1B). Next, we generated zebrafish morphants with depleted PGCs using two different methods. In the first approach, we microinjected a diluted morpholino oligonucleotide (MO) directed against the *dead end* gene (*dnd*) into *Tg(vasa:vasa-EGFP)* zebrafish embryos. Injected embryos were categorized based on the PGC number observed at 24–32 hours postfertilization (hpf) and then grown to adulthood together with uninjected embryos (control), and sexual phenotype was assessed.

Next, we used optical sectioning to count PGC numbers in *dnd* morphants at 7 and 14 dpf. In the majority of larvae from zero PGC and severely depleted PGC groups (PGC count <6 at 24–32 hpf), gonadal structures were absent (Figures 2A and 2B), and these individuals were excluded from further microscopic analysis. In the remaining PGC-depleted group (PGC count 6–9 at 24–32 hpf), the majority of the larvae at 7 and 14 dpf had loose aggregates of PGCs, but were lacking clear gonadal structures. Of the undepleted gonads (>20 PGCs at 24–32 hpf), more than 60% showed clear gonadal structures at 7 dpf.

Among larvae with visible PGCs, the average PGC number was 7.8 ± 4.8 in the PGC-depleted gonads (6–9) and 29.1 ± 9.4 in undepleted gonads (>20) at 7 dpf. At 14 dpf, the morphology of PGC-depleted gonads was similar to those in 7 dpf; however, all of the larvae with undepleted gonads (>20) exhibited distinct gonadal structures (Figure 2B). The average PGC number was 25.6 ± 16.8 in PGC-depleted gonads; in contrast, the PGC count in undepleted gonads displayed a bimodal distribution comparable to that observed in uninjected larvae (means = 47.4 ± 9.8 and 99.3 ± 20.9; Figure 2C, right). This suggested that morpholino-induced PGC depletion below a threshold effectively prevented subsequent PGC proliferation in most individuals and that the effect was maintained during later stages.